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Diapause induces remodelling of the fatty acid composition of membrane and storage lipids in overwintering larvae of *Ostrinia nubilalis*, Hubn. (Lepidoptera: Crambidae)

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Abstract

Seasonal changes in the FA composition of triacylglycerols and phospholipids prepared from the whole body of non-diapausing and diapausing fifth instar larvae of *Ostrinia nubilalis*, Hubn. (Lepidoptera: Crambidae) were determined to evaluate the role of these lipids in diapause. Substantial changes in the FA composition of triacylglycerols and phospholipids were triggered by diapause development. This led to a significant increase in the overall FA unsaturation (UFAs/SFAs ratio), attributable to an increase in the relative proportion of MUFAs and the concomitant decrease in PUFAs and SFAs. In triacylglycerols, the significant changes in FAs composition is the result of an increase in the relative proportions of MUFAs, palmitoleic acid (16:1n-7) and oleic acid (18:1n-9), and a concomitant reduction in composition of SFAs and PUFAs, mainly palmitic acid (16:0) and linoleic acid (18:2n-6), respectively. Changes in the composition of phospholipids were more subtle with FAs contributing to the overall increase of FA unsaturation. Differential scanning calorimetry (DSC) analysis revealed that the melt transition temperatures of total lipids prepared from whole larvae, primarily attributable to the triacylglycerol component, were significantly lower during the time course of diapause compared with non-diapause. These observations were correlated to the FA composition of triacylglycerols, most likely enabling them to remain functional during colder winter conditions. We conclude that *O. nubilalis* undergoes remodelling of FA profiles of both energy storage triacylglycerols and membrane

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phospholipids as an element of its overwintering physiology which may improve the ability to cold harden during diapause.

Key words: Diapause, Cold hardiness, Fatty acid composition, Triacylglycerols, Phospholipids, Melt transition temperatures.

Abbreviations: HVA, homeoviscous adaptation; DSC, differential scanning calorimetry; FA, fatty acid; SFA, saturated fatty acid; UFA, unsaturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; UFAs/SFAs, ratio of unsaturated/saturated FAs; FAME, Fatty acid methyl ester;

Introduction

Diapause is a genetically programmed developmental response into which insects enter to survive unfavourable environmental conditions. It is an environmentally induced dormancy that can occur during any stage of development and is both specific to, and consistent within a species (Denlinger, 1991; Bale and Hayward, 2009). Winter diapause in many insects of temperate and polar zones is closely related to their cold hardiness (Denlinger, 1991). Metabolism decreases during diapause to enable survival over non-feeding periods during cold winters (Bennet et al., 1997; Watanabe and Tanaka, 2000) and is associated with a range of biochemical and physiological changes (Denlinger, 1991; Košťál and Šimek, 1995; Hochachka and Somero, 2002; Denlinger and Lee, 2010). Beside the biosynthesis of low molecular weight organic solutes, which function as cryo/anhydroprotectants and play a role in the preservation of the structural and functional integrity of membranes, adjustments of FA composition of membrane and storage lipids is another important physiological mechanism underlying phenotypic responses to low temperature (Sinesky, 1974; Cossins and Raynard, 1987; Hazel, 1989; Hazel, 1995). FAs, as the main constituents of membrane and storage lipids, have a major influence on cold hardiness.

Analysis of the FA compositions of insects would suggest that these profiles are not fixed characteristics, but are the end products of adaptive biochemical and physiological processes, changing seasonally to serve the vital physiological needs of each species (Sinesky, 1974; Spike et al., 1991; Hazel, 1995; Hochachka and Somero, 2002). Insects preserve the fluidity of lipids and lower their liquid-crystal to gel phase transition temperatures during overwintering mainly by increasing the level of unsaturation of their FAs (Sinesky, 1974;

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Ohtsu et al., 1993; Hazel, 1989; Hazel, 1995; Hochachka and Somero, 2002; Haubert et al., 2008; Van Dooremalen and Ellers, 2010; Koštál, 2010; Goto and Katagiri, 2011). A relationship between increased FA unsaturation (UFAs/SFAs ratio), and diapause initiation has been observed in many insect species (Kuthiala and Chippendale, 1989; Ohtsu et al., 1993; Bennet et al., 1997; Buckner and Hagen, 2003; Bashan and Cakmak, 2005; Michaud and Denlinger, 2006; Koštál, 2010; Vukašinović et al., 2013). In most poikilotherms, the strong effect of temperature on the biophysical properties of lipids is compensated by a process known as homeoviscous adaptation (HVA) (Sinensky, 1974; Hazel, 1995; Hochachka and Somero, 2002). One of the predicted ways to achieve HVA is that membrane FAs become more unsaturated, together with changes in phospholipid headgroups and cholesterol content, during a cold response. HVA compensate the negative effect of membrane solidification which can lead to reduced efficiency of proteins and enzymes associated with the membrane at low temperature and alter its permeability (Sinensky, 1974; Hazel, 1989; Hazel, 1995; Hochachka and Somero, 2002; Koštál, 2010).

The European corn borer, *Ostrinia nubilalis*, is one of the most important Lepidopteran pests of grain, particularly maize (*Zea mays*, L.), that cause substantial economic loss in temperate regions of Eurasia and North America. It overwinters as a diapausing fifth instar larva, usually inside the corn stalks left on harvested fields. Overwintering larva of *O. nubilalis* require a period of cold acclimation during photoperiod-induced diapause in order to gradually develop cold hardiness. Diapausing larvae are freeze tolerant, lose water during dormancy and accumulate substantial amounts of cryo/anhydroprotectants such as glycerol and trehalose in their haemolymph (Grubor-Lajsic et al., 1991, 1992; Kojić et al., 2010). The pentose phosphate pathway, which is intensive during early and mid diapause, serves as a source of NADPH that plays a vital role in the antioxidative defence system and the intensive biosynthetic pathways of protective molecules (Stanić et al, 2004; Jovanović-Galović et al, 2004; Jovanović-Galović et al, 2007).

Given that there are few studies on lipid rearrangements in freeze tolerant insect species and that diapausing larvae of *O. nubilalis* are freeze tolerant, we analyzed the triacylglycerol and phospholipid FA composition of total lipids extracted from the whole bodies of non-diapausing and diapausing fifth instar larvae of *O. nubilalis*. The main purpose of this study was to provide a detailed analysis of seasonal changes of storage and membrane FA composition, as well as storage lipid fluidity in overwintering larvae of *O. nubilalis*. This data will supplement our previous results on the composition and biophysical properties of lipids during diapause (Vukašinović et al., 2013) and further extend our knowledge

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concerning the overwintering physiology and cold tolerance in this species. In addition to detailed biochemical characterization of the FA composition of triacylglycerols and phospholipids, we also measured the associated biophysical changes in the form of melt transition temperatures which are related to lipid phase behaviour and fluidity.

Material and methods

Insect collection

Non-diapausing and diapausing fifth instar larvae of *O. nubilalis* were sampled from corn stalks from the fields in the region of Vojvodina (Serbia) between August 2010 and May 2011. Actively feeding, non-diapausing larvae of *O. nubilalis* (larvae that would continue direct development to pupae that season) were collected during late summer (August 2010, ND, non-diapause). Diapausing larvae were collected during autumn (October 2010, ED, early diapause), winter (December 2010; January and February 2011, MD, mid-diapause), and early spring (April and May 2011, TD, termination of diapause). This sampling regime was chosen to include all of the phases of diapause. Maize stalks containing diapausing larvae were left in corn stalks, on harvested fields, until each sampling date. Three pools, of three larvae per pool, were prepared for each experimental group.

Lipid extraction

Fresh weight of the larvae pool was measured immediately after sampling and stored at -80°C until analysis. Total lipids were extracted by homogenizing (T10 Basic Ultra-Turrax Homogenizer, IKA, Germany) whole bodies of three larvae per sample in ice-cold chloroform:methanol (2:1, v/v) according to a modified method of Folch et al. (1957). The homogenates were filtered through Whatman N^{o}1 filter paper, pre-washed prior to filtration. After the addition of KCl (0.88%, w/v), samples were thoroughly vortexed and centrifuged for 5 minutes at 1500 rpm to achieve phase separation. The upper methanol/water layer was discarded, while the lower chloroform phase containing the total lipid extract was dried under a stream of nitrogen using an N-EVAP system (Organomation). Total lipid extracts were resuspended in chloroform (10 mg ml^{-1} final concentration) and stored at -20°C until analysis.

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Triacylglycerol and phospholipid FA composition analysis

Triacylgylcerol and phospholipid fractions prepared from total lipid extracts were separated by thin-layer chromatography (TLC) using 20 x 20 cm silica gel plates with hexane:diethyl ether:glacial acetic acid (80:20:2, v/v/v). Lipid fractions were visualized under an ultraviolet light (254 nm), after spraying plates with 2`,7`-dichlorofluorescein dissolved in methanol (0.1%, w/v), and identified by comparison with commercial standards. Triacylgylcerol and phospholipid fractions were scraped from the plates into reaction vials and trans-methylated with 1% sulphuric acid in methanol in sealed vials at 50°C for 16 hrs (Christie, 1982). After trans-methylation, mili-Q water and hexane:diethyl ether (1:1, v/v), in the ratio of 1:1 v/v, was added to each vial, which was vortexed and centrifuged at 1500 rpm for 2 min. From the resultant two-phase mixture, the upper organic phase containing the FAMEs was removed and the extraction was repeated by adding hexane:diethyl ether (1:1, v/v). After the re-extraction, the upper organic phase containing the FAMEs was removed again. 2 ml $NaHCO_3$ (2%, w/v) was added to the combined upper organic phase which was then vortexed and centrifuged at 1500 rpm for 2 min. The lower layer was discarded while the upper phase containing the FAMEs was evaporated under nitrogen. The unpurified FAMEs from triacylglycerol and phospholipid fractions were re-dissolved in 50-100 µL of hexan and applied to the base of the 20 x 20 cm silica gel TLC plate using a Hamilton micro syringe. The TLC plate was developed in a hexane:diethyl ether:glacial acetic acid (90:10:1, v/v/v) solvent system. The FAMEs were visualized under an ultraviolet light (254 nm) after spraying plates with 2`,7`-dichlorofluorescein dissolved in methanol (0.1%, w/v). Their position was then marked using a pencil and scraped from the plates into reaction vials. Hexane:diethyl ether (1:1, v/v) and $NaHCO_3$ (2%, w/v), in the ratio of 2:1 v/v, was added to each vial, which were then vortexed and centrifuged at 1500 rpm for 2 min. The upper layer was then removed to a second clean vial. An additional quantity of hexane:diethyl ether (1:1, v/v) was added to the lower phase in the first vial, which was vortexed and centrifuged at 1500 rpm for 2 min. The collected upper layer containing the purified FAMEs was finally evaporated under a stream of nitrogen. The purified FAMEs from triacylglycerol and phospholipid fractions were re-dissolved and analyzed using a TRACE 2000, Thermo Electron, Gas Chromatograph (Thermo Scientific, UK) equipped with a Restek Stabilwax column (0.32 mm i.d. × 30 m). Hydrogen was used as the carrier gas (Pond et al., 2008).

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Differential scanning calorimetry (DSC) analysis

Differential Scanning Calorimetry (DCS 820, Mettler Toledo) was used to examine thermal changes in total lipids prepared from the whole bodies of non-diapausing and diapausing fifth instar larvae of *O. nubilalis*. Some of the purified triaclyglycerols were also analyzed to determine if total lipid and triacylglycerol samples were sufficiently different to warrant similar treatment of all samples. Total lipids were extracted from whole bodies of non-diapausing (ND) and diapausing larvae (D) of *O. nubilalis*. Total lipid extracts were placed in 40 µL aluminium pans and dried under a stream of nitrogen (approximately 1.5 mg of dried extract) before being sealed and analyzed using the following DSC temperature programme. Samples were initially cooled to -60°C, held isothermally for 1 min at this temperature then warmed to 40°C at 4°C min^{-1}. The melt endotherms on thermograms were evaluated using STARe software (version 6). For each endothermic event (melt) the peak, onset, end set and enthalpy change during the melt were measured. The peak of the melt endotherm was taken as the temperature at which the difference between the DSC curve and the interpolated baseline is at a maximum; the onset temperature the temperature at which melting starts and the end set, the temperature at which melting finishes. The area under the melt endotherms represent the enthalpy change during melting (mJ) which is the amount of heat energy taken up by the sample during melting. This gives an indication of the amount of total lipid from the whole body which undergoes a phase transition (solid-to-liquid).

Statistical analysis

All results were expressed as mean ± standard error (SE). The peak areas were converted to percentages of total content of FAs within the same chromatogram (relative proportions) for each FA detected and the logarithmic transformations of these values were analyzed using one-way analyses of variance (one-way ANOVA) followed by Tukey's HSD post hoc test with a level of significance at least $p<0.05$ between groups (Hinkle et al., 1994) using STATISTICA software, version 12 (StatSoft Inc., Tulsa, OK, USA).

To summarize overall change in FA unsaturation separately for storage and membrane lipids, the ratio of unsaturated to saturated fatty acids (UFAs/SFAs ratio) was calculated as the ratio of the total proportion of all unsaturated fatty acids (UFAs=(MUFAs+PUFAs)) over the total proportion of all saturated fatty acids (SFAs).

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Results

The FA composition of triacylglycerols and phospholipids

The FA composition of triacylglycerols and phospholipids prepared from the whole bodies of non-diapausing and diapausing larvae of *O. nubilalis* were analyzed to determine the seasonal changes in their overall FA unsaturation. In total seven FAs were identified: palmitic acid (16:0), palmitoleic acid (16:1n-7), stearic (18:0), oleic acid (18:1n-9), vaccenic (18:1n-7), linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) (Fig. 1A, B). The relative proportion of most of these determined FAs significantly differed in both triacylglycerols and phospholipids between non-diapausing and diapausing larvae as well as during the time course of diapause (Fig. 1A, B). In Fig. 2A, B results are summarized by considering structural classes of FAs: saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs).

In the FA composition of triacylglycerols, the major structural class of FAs was MUFAs (Fig. 2A), with palmitoleic acid (16:1n-7) being the dominant FA in both non-diapausing and diapausing larvae (Fig. 1A). The proportion of MUFAs significantly increased from 56.35% in non-diapause (ND) to 82.14% in early diapause (ED) (Fig. 2A). Furthermore, MUFAs did not change significantly during the time course of diapause, except in the termination period (TD), when it decreased from 86.39% to 78.29% (Fig. 2A). However, while the proportion of the second most abundant structural class of FAs, SFAs, decreased significantly from 28.57% in non-diapause (ND) to 11.56% in early diapause (ED), they did not significantly change during diapause development, but significantly increased during termination of diapause (TD) from 11.27% to 17.07%. The least abundant class of triacylglycerol FAs were PUFAs (Fig. 2A). The relative abundance of PUFAs significantly decreased from 15.08% in non-diapause (ND) to 6.30% in early diapause (ED), while it increased from 1.77% in mid diapause (MD) to 4.64% in termination of diapause (TD). This change in PUFAs during the time course of diapause is the result of changes in the proportion of linoleic acid (18:2n-6) (Fig. 1A), since the proportion of α- linolenic acid (18:3n-3) remained constant (Fig. 1A).

Overall changes of FA unsaturation in triacylglycerols were expressed using the ratio of unsaturated and saturated fatty acids (UFAs/SFAs ratio). The UFAs/SFAs ratio significantly differed between the two distinct physiological states of non-diapause and diapause as well as during the development of diapause (Fig. 2C). The UFAs/SFAs ratio increased 3.05 times

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during transition from non-diapause state (ND) to early diapause (ED), remained at a high level until mid diapause (MD) and then decreased 1.62 times during the termination of diapause (TD). This trend in changes of the UFAs/SFAs ratio in triacylglycerols is mainly the result of the prominent increase in the relative proportions of dominant MUFAs, palmitoleic (16:1n-7) and oleic acid (18:1n-9), and a concomitant decrease in dominant SFAs and PUFAs, palmitic acid (16:0) and linoleic acid (18:2n-6), respectively (Fig. 1A).

Unlike in the FA composition of triacylglycerols, identified FAs were more evenly distributed in the pool of phospholipids (Fig. 1B). PUFAs were the major structural class in non-diapausing larvae, while MUFAs were dominant in diapausing larvae (Fig. 2B). The proportion of PUFAs significantly decreased during transition from non-diapausing to early diapause (ED), from 47.40% to 30.60%, respectively (Fig. 2B). The relative proportion of PUFAs continued decreasing to 23.15% until mid-diapause (MD) and then increased to 31.42% during the termination of diapause (TD). This trend was mainly a result of a change in the proportion of the dominant PUFA, linoleic acid (18:2n-6), since the proportion of α-linolenic acid (18:3n-3) did not significantly change during diapause (Fig. 1B). The proportion of MUFAs significantly increased from 27.45% in non-diapausing larvae to 52.96% in early diapausing larvae (ED), reached a maximum of 59.81% in mid diapause (MD) and then significantly decreased to 45.69% during termination of diapause (TD) (Fig. 2B). This trend was a result of the change in the proportion of oleic acid (18:1n-9); the dominant MUFA in the phospholipids FA pool in both the non-diapausing and diapausing states (Fig. 1B). SFAs were the least abundant structural class in the FA composition of phospholipids (Fig. 2B). The relative proportion of SFAs decreased significantly from 25.15% in non-diapause to 16.44% in early diapause (ED) and remained stable until the termination of diapause (TD) when it increased to 22.89% (Fig. 2B). Such a trend is a result of changes in the proportion of stearic acid (18:0), since the proportion of palmitic acid (16:0) did not significantly change during the time course of diapause (Fig. 1B).

To summarize, the overall change in phospholipids FA unsaturation as well as the ratio of unsaturated to saturated fatty acids (UFAs/SFAs) was calculated and proved to be significantly different between non-diapause and diapause (Fig. 2D). The UFAs/SFAs ratio showed a similar trend in both triacylglycerols and phospholipids (Fig. 2C), but with a lower overall change in phospholipids (Fig., 2B), which is the consequence of a more even distribution of analyzed FAs in this fraction (Fig. 1B).

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Thermal changes in total lipids

Differential Scanning Calorimetry (DSC) was used to examine how the seasonal change in overall FA unsaturation affected the lipid melting temperature (Fig. 3). The whole body total lipids (TLs) *vs*. triacylglycerols fraction (TAGs) DSC thermal curves showed a high similarity and indicated that enthalpy changes were predominately attributable to TAGs with other lipid classes contributing little to the melt of the complex mixture of total lipids (Fig. S1, supplement).

The DSC thermal analysis of the whole body lipids of diapausing larvae detected two clearly defined melt endotherms: endotherm 1 and endotherm 2 (Fig. 3), while in non-diapausing larvae endotherm 1 was very different in shape being much broader and being made up of two shallow melt peaks (I and Ia) with endotherm 2 still present (Fig. 3). The melt peaks of both endotherms shifted significantly toward the left between both non-diapausing and diapausing larvae (Fig. 3). Detailed data is presented in Table 1. The peaks of melt endotherm 1(I and Ia), were detected at -18.3°C and -6.1°C, respectively, while the peak of melt endotherm 2 was 12.0°C for the total lipids from whole non-diapausing larvae (ND). The two melt peaks for all stages of diapausing larvae were similar with peak 1 varying only within the range -18.2 to -19.3°C while peak 2 was similar for early and mid-diapause but increased towards termination from 3.0 to 7.8°C (Fig. 3; Table 1). In summary, the differences between samples of storage lipids taken at different seasonal times indicate that the composition of storage lipid mixtures changes seasonally. A decrease in melting temperatures during the transition from non-diapause (ND) to diapause (ED) was accompained by a change in enthalpy (ΔH) of the endothermic event reflecting the quantitative change in lipid composition. During the time course of diapause, significant change occured at the termination of diapause (TD) when the melting (phase-transition) temperatures increased (Fig. 3; Table 1).

Discussion

The FA composition of lipids has been thoroughly analyzed in many insects to understand the dynamic and complex physiological state of diapause (Spike et al., 1991; Ohtsu et al., 1993; Koštál et al., 2003; Basham and Cakmak, 2005; Michaud and Denlinger, 2006; Khani et al., 2007; Koštál, 2010; Rozsypal et al., 2014). The FA composition of triacylglycerols and phospholipids extracted from the whole bodies of non-diapausing and

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diapausing larvae of *O. nubilalis*, included C16 and C18 UFAs and SFAs; palmitic acid (16:0), palmitoleic acid (16:1n-7), stearic (18:0), oleic acid (18:1n-9), vaccenic (18:1n-7), linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3), which is in accordance with previous studies on Lepidopteran species (Fast, 1970; Thompson 1973; Atapour et al., 2007; Khani et al., 2007). Generally, Lepidoptera exhibit typical animal FA profiles which are characterized mainly by low proportions of palmitoleic acid (16:1n-7) and high proportions of linoleic acid (18:2n-6) and/or α-linolenic acid (18:3n-3) (Fast, 1970). In this study on *O. nubilalis*, palmitoleic acid (16:1n-7) comprise a prominent proportion of the total FAs in triacylglycerol and phospholipid FA pools, which is unusual for a Lepidopteran species but more characteristic of Dipteran species (Fast, 1970; Thompson 1973; Buckner et al., 2004). Many authors have found that changes in the FA composition of triacylglycerols and phospholipids are strongly associated with a physiological state of diapause during which many insects develop cold hardiness (Bennet et al., 1997; Kostal and Simek, 1998; Koštál et al., 2003; Buckner et al., 2004; Bashan and Cakmak, 2005; Michaud and Denlinger, 2006; Rozsypal et al., 2014). In *O. nubilalis*, changes in the FA composition of triacylglycerols and phospholipids led to a significant increase in the overall FA unsaturation (UFAs/SFAs ratio) during transition and time course of diapause, which significantly decreased during the termination period. Such a trend in this freeze tolerant Lepidopteran insect species is mainly a consequence of an increase in MUFAs and the concomitant decrease in PUFAs and SFAs.

In triacylglycerols, the significant changes in FAs composition is the result of an increase in the relative proportions of MUFAs, palmitoleic acid (16:1n-7) and oleic acid (18:1n-9), and a contaminant reduction in composition of SFAs and PUFAs, mainly palmitic acid (16:0) and linoleic acid (18:2n-6), respectively. Changes in the composition of phospholipids were more subtle with FAs contributing to the overall increase of FA unsaturation. In both analyzed pools, the proportion of palmitoleic acid (C16:1n-7) increased at the expense of palmitic acid (16:0), while the proportion of oleic acid (C18:1n-9) increased at the expense of linoleic acid (C18:2n-6) during transition to diapause.

The increase described above in the relative proportion of MUFAs at the expense of PUFAs and SFAs, triggered by diapause development, differs significantly from the trend in the majority of insect species studied (reviewed by Koštál, 2010), but are in accordance with the findings of Ohtsu *et al*. (1998). Ohtsu *et al*. (1998) found that such changes in FA composition enable *Drosophila* species to retain broad temperature tolerance, which is necessary for survival in temperate habitats. Results in this study confirm that MUFAs play a key role in the maintenance of the functionality of lipids during overwintering of freeze

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tolerant larvae of *O. nubilalis*, since PUFAs are significantly reduced during diapause. There are several reasons why MUFAs have an advantage over PUFAs in insects of temperate regions since MUFAs maintain the wide temperature range of fluidity of lipids, which is important for species exposed to extreme environmental temperature fluctuations (Ohtsu et al., 1998; Michaud and Denlinger, 2006). Also, MUFAs, due to the central position of the double bond in FA, maximize the lateral displacement of the end of the chain and contribute to overall membrane disorder (Barton and Gunston, 1975). Thus, high levels of *cis* oleic acid (18:1n-9) in the membrane phosphoslipids of *O. nubilalis*, maintain fluidity of the membrane and provide a suitable environment for the correct function of sensitive membrane associated proteins and enzymes (Cossin and Raynard, 1987; Michaud and Denlinger, 2006). The fluidity of storage lipids (triacylglycerols) is particularly important in freeze tolerant insects overwintering in the non-feeding state of diapause. The prominent proportion of MUFAs, especially palmitoleic acid (C16:1n-7) shown in this study, may help to maintain this fluidity. Other studies have also shown that MUFAs play an important role in cold hardiness development in freeze tolerant Lepidopteran (*Chilio supressalis*, Atapour et al., 2007) and Dipteran species (*Eurosta solidaginis*, Bennet et al., 1997). In these studies, palmitoleic acid (16:1n-7) dominated in the FA composition of whole body lipids of diapausing larvae, while oleic acid (C18:1n-9) was dominant in both, triacylglycerol and phospholipid FA compositions of the fat body, respectivelly. On another hand, some studies on freeze tolerant Dipteran (*Chymomyza costata*, Koštál et al, 2003) and Lepidopteran species (*Cydia pomonella*, Rozsypal et al, 2014) have shown that PUFAs play a role in the maintenance of the functionality of lipids overwintering as the relative proportion of PUFAs increase at the expense of MUFAs and/or SFAs. Because of the above, conclusions about the existence of a unique FA composition in freeze tolerant Lepidopteran and Dipteran species cannot be made.

The seasonal increase in overall FA unsaturation of storage and membrane lipids is considered to be an adaptation to optimize phase behaviour that is crucial for controlling membrane fluidity and storage utilization during low winter temperatures (Sinesky, 1974; Hazel, 1989; Hazel, 1995; Hazel and Williams, 1990; Ohtsu et al., 1993; Kostal and Simek, 1998; Hochachka and Somero, 2002; Koštál et al., 2003; Michaud and Denlinger, 2006; Overgaard et al., 2008; Van Dooremalen and Ellers, 2010; Rozsypal et al., 2014). Namely, increased FA unsaturation of membrane phospholipids contributes to the maintenance of the vital static order and dynamic properties of membranes whereas triacylglycerols maintain the fluidity of storage lipids facilitating accessibility of enzymes to energy lipid reserves during periods of low winter temperatures. This is important as solid phase triacylglycerols are not as readily

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metabolized by lipases as those in a liquid phase (Sinesky, 1974; Ohtsu et al., 1993; Hazel, 1987; Hazel, 1995; Joanisse and Storey, 1996; Bennet et al., 1997; Kostal and Simek, 1998; Buckner et al., 2004; Atapour et al., 2007; Van Dooremalen et al., 2011; Rozsypal et al., 2014).

This study suggests that compositional remodelling of lipids in *O. nubilalis* is a function of photoperiod-induced diapause rather than a direct response to low temperature during diapause, since the major changes in FAs composition in both triacylglycerols and phospholipids occur early in diapause (ED), when larvae of *O. nubilalis* have yet to be exposed to subzero temperatures.

In *O. nubilalis*, DSC thermogrames of whole body total lipids *vs*. the triacylglycerol fraction of total lipids are very similar and indicate that enthalpy changes associated with the melt of total lipids are primarily attributable to triacylglycerols. Thermal analysis revealed that the melt transition temperatures of *O. nubilalis* larvae triacylglycerols were significantly lower during all phases of diapause compared with non-diapause, with a significant increase during the termination period. The observed changes in both enthalpy and melt transition temperatures of total lipids occur in parallel with the seasonal changes in the FA unsaturation (UFAs/SFAs ratio). This means that the range of temperatures over which storage lipids remain fluid significantly shifts towards lower temperatures during transition to diapause and maintain during time course of diapause confirming the hypothesis that storage lipid, must remain fluid to be accessible as an energy substrate for metabolism during overwintering (Ohtsu et al., 1993; Joanisse and Storey, 1996; Ohtsu et al., 1998). These results are in agreement with our previous findings that the melt transition temperatures of total lipids extracted from the fat body tissue, which is the primary site of triacylglycerols storage in *O. nubilalis* larvae, are lower during diapause (Vukašinović et al., 2013). Recent work has established that the neutral lipid of overwintering freeze tolerant larvae of *Eurosta solidaginis* is dominated by acetylated triacylglycerols rather than as previously thought, the energy-dense long-chain triacylglycerols (Marshall et al., 2014), generally used by animals. We found no evidence of acetylated triacylglycerols in *O. nubilalis* which would have been visible on the HPTLC plates if present.

The FA composition of storage lipids in ectotherms is expected to change with temperature in a similar manner to the membrane phospholipids, so the maintenance of lipid fluidity is also expected to be adaptive (Hochachka and Somero, 2002; Haubert et al., 2008; Van Dooremalen and Ellers, 2010). Our conclusion that FA unsaturation of the energy storage

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triacylglycerols increases during acquisition of cold tolerance is in accordance with other authors (Bennet et al., 1997; Ohtsu et al., 1993; Kostal and Simek, 1998; Atapour et al., 2007; Van Dooremalen and Ellers, 2010), and agrees with the suggestion that the theory of HVA should be extent to body lipids in general, even though it was formulated on the thermal response of membrane lipids (Sinesky, 1974). Our findings suggest that photoperiodical induction of diapause in *O. nubilalis* imposes FA remodelling, which in turn leads to the adaptation of membrane and storage lipids and occurs prior to the occurrence of environmental low temperatures. This is because the overall FA unsaturation increased early in diapause (ED) before larvae were exposed to subzero temperatures. Unsaturation of FA (mainly due to MUFAs) is maintained at a high level during the time course of diapause and decreases during the termination period (TD). In the termination period, the opposite FA re-modelling processes occurs resulting in a state of quiescence; a state between non-diapause and diapause (Koštál, 2006).

We conclude that *O. nubilalis* undergoes remodeling of FA profiles of both membrane phospholipids and energy storage triacylglycerols as an element of its overwintering physiology which is likely to be an important factor controlling cold tolerance during diapause.

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Table 1. Enthalpy of melting and melt transition temperatures (peak, onset and end set) of total lipids extracted from the whole bodies of non-diapausing (ND) and diapausing larvae of *O. nubilalis* through different months during diapause; ED- early diapause (late October), MD- mid diapause (MD_D- December and MD_F- February), TD- termination of diapause (TD_A- April and TD_M- May). Melt endotherms (1and 2) indicate phase transitions (solid to liquid). The peaks (I, Ia and II) of melt endotherms (1 and 2) indicates the temperature at which the difference between the DSC curve and the interpolated baseline is at a maximum.

Fig. 1A, B The FA composition of triacylglycerols (A) and phospholipids (B) prepared from the whole bodies of non-diapausing (ND) and diapausing (ED early diapause, MD mid diapause, TD termination of diapause) fifth instar larvae of *O. nubilalis*. The FAs are palmitic acid (16:0), palmitoleic acid (C16:1n-7), stearic acid (18:0), oleic acid (18:1n-9), vaccenic acid (18:1n-7), linoleic acid (18:2n-6), and α-linolenic acid (18:3n-3). Bars represent mean ± standard error, n = 3 groups, 3 larvae per group. Different letters (a, b, c) above the bar indicate statistically significant differences in fatty acid proportion between groups (ND, ED, MD and TD) determined by Tukey's HSD post hoc test, while bars followed by the same letter above are not significantly different in FA proportion between groups. Level of significance between groups for that fatty acid: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Fig. 2A, B, C, D Summarised results of the FA composition of triacylglycerols (A) and phospholipids (B) prepared from the whole body of non-diapausing (ND) and diapausing (ED early diapause, MD mid diapause, TD termination of diapause) fifth instar larvae of *O. nubilalis* showing the three major structural classes of FAs: saturates (SFAs), monounsaturates (MUFAs) and polyunsaturates (PUFAs) of total lipids, and changes in overall FA unsaturation (UFAs/SFAs ratio) of triacylglycerols (C) as well as phospholipids (D). SFAs is the sum of all saturated fatty acids, MUFAs is the sum of all monounsaturated fatty acids, PUFAs is the sum of all polyunsaturated fatty acids, UFAs/SFAs ratio is the ratio between the unsaturated fatty acids (UFAs=MUFAs+PUFAs) and the SFAs. Bars represent mean ± standard error, n = 3 groups, 3 larvae per group. Different letters above the bars (a, b, c, d) indicate statistically significant differences between groups (ND, ED, MD and TD) determined by Tukey's HSD post hoc-test, while bars followed by the same letter indicate that there was no significant difference between groups. Level of significance between groups of structural classes of FAs (SFAs, MUFAs and PUFAs) between examined group: *** = $p < 0.001$, ** = $p < 0.01$, *= $p < 0.05$.

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Fig. 3 DSC heating thermograms of total lipids extracted from the whole bodies of non-diapausing (ND) and diapausing larvae of *O. nubilalis* at different stages of diapause; ED- early diapause (late October), MD- mid diapause (MD_D- December and MD_F- February), TD- termination of diapause (TD_A- April and TD_M- May). Highlighted areas under melt endotherms (melt endotherm 1 and 2) indicate solid-liquid phase transitions. The peaks of melt endotherm 1 (I, and Ia) and the peak of melt endotherm 2 (II) presented in Table 1, indicates the temperature at which the difference between the DSC curve and the interpolated baseline is at a maximum.

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Table 1. Enthalpy of melting and melt transition temperatures (peak, onset and end set) of total lipids extracted from the whole bodies of non-diapausing (ND) and diapausing larvae of *O. nubilalis* through different months during diapause; ED- early diapause (late October), MD- mid diapause (MD_D- December and MD_F- February), TD- termination of diapause (TD_A- April and TD_M- May).

	Enthalpy of melting [mJ]			Peak of melt [°C]			Onset [°C]			End set [°C]		
	I	Ia	II	I	Ia	II	I	Ia	II	I	Ia	II
ND	-24.1	-12.0	-86.0	-18.3	-6.1	12.0	-23.2	-9.8	0.4	-11.7	-5.8	13.7
ED	-84.4	/	-46.8	-19.2	/	3.0	-28.0	/	-10.6	-14.6	/	5.6
MD_D	-69.9	/	-38.9	-19.3	/	3.1	-26.1	/	-10.6	-14.2	/	5.6
MD_F	-66.2	/	-39.1	-19.0	/	3.6	-25.2	/	-9.8	-14.5	/	6.3
TD_A	-51.3	/	-57.9	-18.5	/	7.3	-25.8	/	-7.9	-11.8	/	9.1
TD_M	-46.5	/	-60.3	-18.2	/	7.8	-27.9	/	-7.5	-11.3	/	9.9

Note: The temperature at the peak of melt indicates the temperature at which the difference between the DSC curve and the interpolated baseline is at a maximum (Fig. 3). The melt endotherm is determined as well by the onset temperature (temperature at which melting starts) and the end set temperature (temperature at which melting finishes).

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Fig. 1A, B

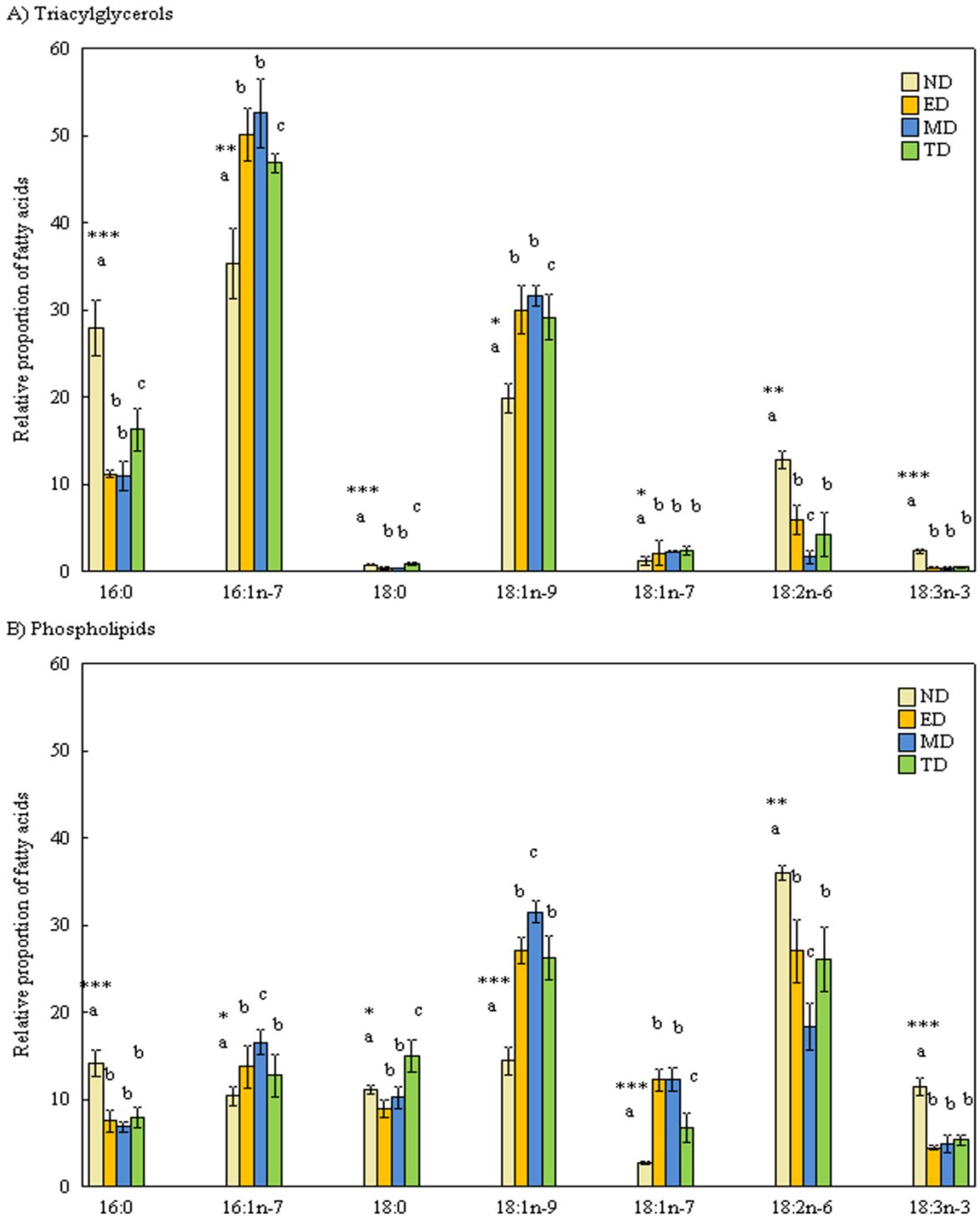

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Fig. 2A, B, C, D

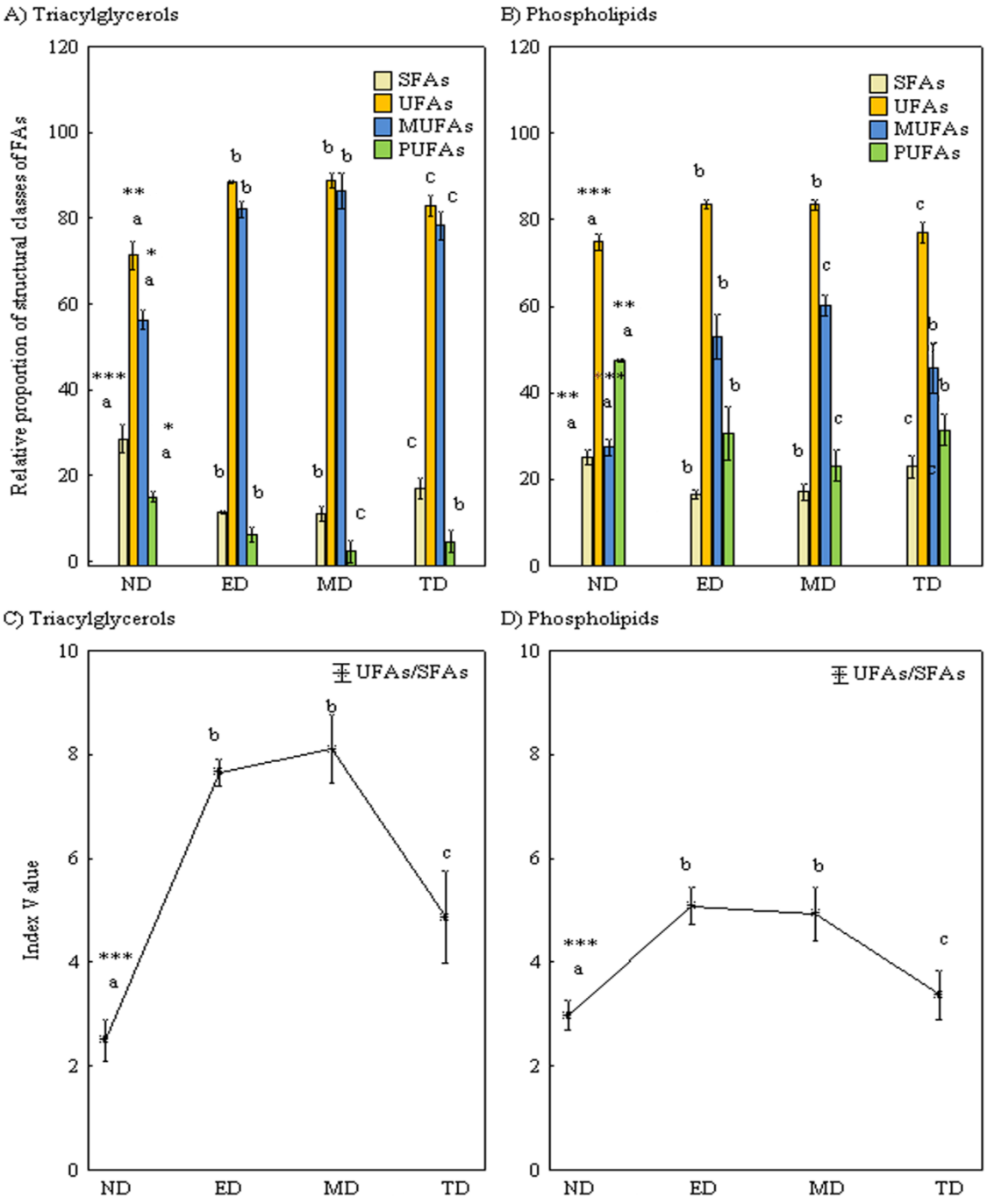

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Fig. 3