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Temperature adaptation of lipids in diapausing *Ostrinia nubilalis*: an experimental study to distinguish environmental versus endogenous controls

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Abstract

Larvae of the European corn borer (*Ostrinia nubilalis* Hubn.) were cold acclimated during different phases of diapause to determine if changes in the fatty acid composition lipids occur as part of a programmed diapause strategy, or as a response to low temperatures during winter. Cold acclimation of fifth instar larvae of *O. nubilalis* during diapause had modest effects further on the readjustments in fatty acid composition of triacylglycerols and phospholipids. Overall FA unsaturation (UFAs/SFAs ratio) was stable, with the exception of the triacylglycerols fraction after exposure to -3°C and -10°C in mid-diapause (MD) when it significantly increased. Differential Scanning Calorimetry (DSC) was used to examine phase transitions of total body lipid of cold acclimated larvae in diapasue. Thermal analysis indicated that

changes in the melt transition temperatures of whole body total lipids were subtle, but consistent with the modest changes in the level of FA unsaturation observed. We conclude that lipid rearrangements are a function of the endogenous “diapause program” rather than a direct effect of low temperatures, which proved to have limited impact on lipid changes in diapausing larvae of *O. nubilalis*.

Key words Cold acclimation; Fatty acid composition; Insect; Melt transition temperatures; Phospholipid; Triacylglycerol;

Abbreviations 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, ratio of unsaturated/saturated FAs; FAME, fatty acid methyl ester; GPEtn/GPChol ratio, ratio of glycerophosphoethanolamine/glycerophosphocholine.

Introduction

Some insects of temperate and colder climate zones survive harsh environmental conditions by overwintering in a state of diapause. In many of these insect species diapause is closely connected to cold hardiness and enhances cold stress tolerance (Denlinger, 1991; Denlinger, 2002; Bale and Hayward, 2010). Even though the level of metabolism is suppressed during dormancy, diapause is not a static state but rather a genetically controlled dynamic physiological process with different successive phases (Bennet et al., 1997; Koštál, 2006). Many biochemical and physiological changes that occur during the onset of diapause prepare the organism for forthcoming overwintering challenges. It is important to emphasise that these physiological mechanisms are initiated exclusively as a result of entering diapause and not as a direct response to low temperature. However, these physiological mechanisms may be further enhanced by subsequent cold exposure (Bale and Hayward, 2010). Specifically, diapause induces the synthesis and accumulation of cold-protective metabolites (e.g. sugars and sugar alcohols) and associated chaperones, a cessation of the cell cycle and changes in the storage and membrane lipid compositions (Tamariello and Denlinger, 1998; Hodková et al., 1999; Koštal and Šimek, 2000; Michaud and Denlinger, 2006; Tomčala et al., 2006; Rinehart et al., 2007; MacRae, 2010; Vukašinović et al., 2013, 2015).

In some poikilotherms, including insects, restructuring of the lipid composition of cellular membranes is closely related to changing ambient temperature (Cossins and Sinensky, 1984; Hazel, 1989, 1995; Koštal, 2010; Purać et al., 2011). The adaptive restructuring of membrane phospholipids involves an increase of the overall FA unsaturation

(UFAs/SFAs ratio) in conjunction with the modifications of phospholipid headgroups, as increase of the GPEth/GPchol ratio. Additional changes considering membrane cold adaptation involve an increase of the cholesterol content, the placement of unsaturated fatty acids on the *sn*2 instead *sn*1 position of the glycerol backbone of the molecule since unsaturated FA at *sn*-2 position has a greater effect on membrane fluidity than at the *sn*-1 position and an increase in the proportion of 16-carbon rather than 18-carbon fatty acids in the plasma membrane, to compensate for the rigidifying effect of sub-zero temperature during winter (Sinensky, 1974; Cossin, 1994; Hazel, 1995; Hochachka and Somero, 2002; Kostal et al., 2003; Michaud and Denlinger, 2006). Maintaining an optimal phase and fluidity of lipid deposits and membrane lipids at low body temperatures ensures the availability of energy stores during winter for enzymatic breakdown and maintains functionality and/or structural integrity (Cossins, 1994; Marshall et al., 2014).

The European corn borer, (*Ostrinia nubilalis*, Hubn.) is a widespread agricultural pest in temperate regions of Euroasia, North America and North Africa. This moth normally survives harsh winter conditions inside corn stalk debris, remaining on the soil surface after the crop has been harvested. It overwinters as a fifth instar diapausing larvae, which is freeze-tolerant with a considerable supercooling capacity of between -20.5 and -25 °C (Grubor-Lajšić et al., 1991). Metabolism of diapausing fifth instar larvae is suppressed and diverted towards: 1) The synthesis and accumulation of cold- protective metabolites e.g. glycerol and trehalose which are the main cryo and anhydroprotectors (Grubor-Lajsic et al., 1991, 1992; Kojić et al., 2010) and closely connected with regulation of water content during overwintering (Kojić et al., 2010), 2) Readjustment of lipid (storage and membrane) FA composition (Vukašinović et al., 2013; 2015), 3) The expression of stress-related genes (Popović et al., 2015) and 4) Re-arrangements of the antioxidative defense system (Stanic et al, 2004; Jovanović-Galović et al, 2004, 2007; Kojić et al, 2009). Since some of the overwintering cold stress-tolerance mechanisms in diapausing larvae of *O. nubilalis* reach their full potential only after exposure to low temperatures (Grubor-Lajsic et al., 1992; Stanić et al., 2004; Kojić et al., 2010), it is considered that exposure to low temperatures and cold-acclimation are essential for the development of cold hardiness during photoperiod-induced diapause.

Previous studies on larvae of *O. nubilalis* have shown that major compositional remodeling of lipids and changes of their biophysical properties were initiated by the diapause program and preceded the occurrence of low temperatures in fields (Vukašinović et al., 2013, 2015). Higher overall FA unsaturation (UFAs/SFAs ratio) in diapause was the result of concomitant increase in content of MUFAs and decrease in contents of PUFAs and SFAs. Such changes in the FA composition significantly contributed to the depression of melt transition temperatures during the time course of diapause, which was recorded using DSC (Vukašinović et al., 2015).

In this study the FA composition of triacylglycerols and phospholipids of cold acclimated fifth instar larvae of *O. nubilalis* during different phases of diapause were analyzed in order to clarify for the first time in this species, whether the changes in the FA compositions of lipids in overwintering larvae occur as a part of the programmed diapause (endogenous cues) and/or as a direct effect of exposure to low temperatures.

Material and methods

Insect collection

Fifth instar diapausing larvae of *O. nubilalis* were collected from corn stalk residues left on the soil surface of a harvested field in the vicinity of Novi Sad (AP Vojvodina, Serbia) throughout the diapausing period: autumn (October 2010, early diapause, ED), winter (January 2011, mid-diapause, MD) and early spring (March 2011, termination of diapause, TD). This sampling strategy included all phases of diapause and was consistent with earlier studies (Vukašinović et al., 2013; 2015). The range of ambient air temperatures in the weeks prior to larvae collection were: October 10°C to 5°C, January -5°C to -0.5°C, and March -14°C to 5.5°C.

Laboratory cold acclimation

Immediately after collection larvae were stored under controlled conditions (5°C) for 10 days. A sample was then taken for analysis and the remainder gradually cooled (1°C/ day) to the first experimental acclimation temperature (-3°C). At this point half were held at -3°C for a further 10 days while the remaining larvae were cooled at the same rate to -10°C. The latter sample was then held at -10°C for 10 days. All samples were prepared for analysis after the 10 day acclimation period. This treatment was repeated for each field sample (Fig. 1).

Extraction of total lipids

Three pools, of three larvae per pool, were prepared for each experimental group. Total lipids were extracted from whole bodies (3 larvae per pool) by homogenizing (T10 Basic Ultra-Turrax Homogenizer, IKA, Germany) them in ice-cold chloroform:methanol (2:1, v/v) according to a modified method of Folch et al. (1957). The homogenates of the whole body pool were filtered through Whatman N°1 filter paper, pre-washed prior to filtration with chloroform. After the addition of ¼ volume of KCl (0.88%, w/v) to the filtrate, samples were thoroughly vortexed and centrifuged

for 5 min 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 from larval whole bodies were resuspended in chloroform (10 mg ml^{-1} final concentration) and stored at -20°C until analysis.

Larval whole body triacylglycerol and phospholipid FA composition analysis

Triacylglycerol and phospholipid fractions prepared from total lipids extracted from whole bodies of fifth instar diapausing larvae of *O. nubilalis* 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). The analyzed lipid fraction was 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 developed plates into reaction vials and trans-methylated with 1% sulfuric acid in methanol in sealed vials at 50°C for 16 h (Christie, 1982). After trans-methylation, milli-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. Two ml of $NaHCO_3$ (2% w/v) was added to the combined upper organic phase which was then vortexed and centrifuged at 0.2G 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 hexane and applied to the base of a 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 was then vortexed and centrifuged at 0.2G 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 0.2G 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 TRACE 2000, Thermo Electron, Gas Chromatograph (Thermo Scientific, UK) equipped with a Restek Stabilwax column (0.32 mm i.d. x 30 m). Hydrogen was used as the carrier gas (Pond et al., 2008).

Differential Scanning Calorimetry analysis

A Differential Scanning Calorimeter (DSC 820, Mettler Toledo) was used to examine thermal changes in total lipids. Aliquots of dissolved total lipids extracted from whole bodies of fifth instar larvae of *O. nubilalis* were added to a 40 µl aluminium DSC sample pan and dried under a stream of nitrogen until a dry sample of about 1.5mg had been accumulated. A lid was then sealed onto the pan and the sample analysed in the DSC using the following temperature program. Samples were initially cooled to -60°C at 4°C min^{-1}, held isothermally for 1 min at this temperature then warmed to 40°C at 4°C min^{-1}. The thermograms were evaluated using STARe software (version 6).

Analysis was only conducted using whole body total lipids (TLs) and not the triacylglycerols fraction (TAGs) as in a previous study we determined that they resulted in very similar thermograms. We determined that melt changes were predominately attributable to TAGs with other lipid classes contributing little to the melt of the complex mixture of total lipids (Vukašinovic et al., 2015).

For each endothermic event (melt) the peak, onset and end set 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 endotherm represents the amount of heat energy [mJ] consumed during phase change, melting.

Statistical analysis

All results are 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 (Stat Soft Inc., Tulsa, OK, USA).

To summarize data, the overall change in FA unsaturation in the triacylglycerols and phospholipids, 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).

Results

FA composition of triacylglycerol and phospholipid fractions of cold acclimated diapausing larvae of O. nubilalis

First of all, the overall FA unsaturation in triacylglycerols and phospholipids of *O. nubilalis* during diapause (ED, MD, TD), regarding larvae stored under controlled conditions (5°C), were determined (Fig. 2A, B, C, D). The overall FA unsaturation (UFAs/SFAs ratio) did not change during time course of diapause until the termination period (TD), when it decreased (Fig. 2B, D), significantly in phospholipids (Fig. 2D), primarily as a consequence of a significant decrease in the relative proportion of MUFAs and a concomitant increase in the proportion of SFAs and PUFAs (Fig.2C).

Seven FAs with 16 and 18 carbons were determined in triacylglycerol and phospholipid fractions: palmitic (16:0), palmitoleic (16:1n-7), stearic (18:0), oleic (18:1n-9), vaccenic (18:1n-7), linoleic (18:2n-6) and α-linolenic acid (18:3n-3) (Fig. 3A, Fig. 4A). In triacylglycerol, palmitic (16:0), palmitoleic (16:1n-7) and oleic acid (18:1n-9) dominated and comprise more than 90% of the total FA content (Fig. 3A). FAs in phospholipids were more evenly represented with in general a higher abundance of PUFAs, especially linoleic acid (18:2n-6) (Fig. 4A).

In the case of triacylglycerols, the major structural class of FAs was MUFAs (Fig. 3B), with palmitoleic (16:1n-7) and oleic acid (18:1n-9) being the dominant FAs (Fig. 3A). The proportion of MUFAs did not change significantly in response to cold treatment during the time course of diapause (Fig. 3B). The relative abundance of SFAs changed only in mid diapause (MD), when significantly decreased after exposure to -3°C and -10°C (Fig. 3B), as a result of a significant decrease in the relative proportion of palmitic acid (16:0) (Fig. 3A). The least abundant class of triacylglycerol FAs, PUFAs, changed substantially only during early diapause (ED) after the cold acclimation to -3°C, as a result of a significant increase in the proportion of linoleic acid (18:2n-6) (Fig. 3A). To summarize, cold acclimation did not induce significant changes in the overall FA unsaturation (UFAs/SFAs ratio) of storage lipids during the time course of diapause, except in the mid-diapause (MD) after acclimation to -3°C and -10°C, which induced a significant increase in the UFAs/SFAs ratio (Fig. 3C).

The FA composition of phospholipids did not change significantly in response to cold acclimation during any phase of diapause (Fig. 4B, C), even though the temperature of exposure during different phases of diapause induced significant changes in the relative proportions of selected FAs (Fig. 4A). Namely, in early diapause (ED) cold acclimation to -3°C induced a significant increase in the relative proportion of vaccenic (18:1n-7) and linoleic acid (18:2n-6), while acclimation to -10°C a decrease in the relative proportion of α-linolenic acid (18:3n-3) (Fig. 4A). During mid diapause (MD) cold acclimation to -10°C induced a significant increase in the relative proportion of vaccenic (18:1n-7) and linoleic acid (18:2n-6) and a significant decrease in the relative proportion of stearic acid (18:0) (Fig. 4A). Cold acclimation during the termination of diapause (TD) did not induce any significant changes in the relative proportion of FA composition of phospholipids (Fig. 4A). Considering the overall FA unsaturation (UFAs/SFAs ratio), cold acclimation during different phases of diapause did not induce substantial changes in the FA composition of phospholipids (Fig. 4C). The UFAs/SFAs ratio showed a similar trend in both triacylglycerols and phospholipids (Fig. 3C, Fig. 4C), but with a lower overall FA unsaturation in phospholipids (Fig. 4C), which is the consequence of a more even distribution of FAs in this fraction (Fig. 4A).

Thermal changes in whole body total lipids of cold acclimated diapausing larvae of O. nubilalis

Differential Scanning Calorimetry (DSC) was used to examine the solid-liquid phase transition properties of total lipids extracted from the whole bodies of cold acclimated diapausing larvae of *O. nubilalis* focusing on the lipid melt transition temperatures (Fig. 5; Table 1).

Thermal analysis of total lipids detected two clearly defined melt endotherms: endotherm 1 and endotherm 2 (Fig. 5). The melt peaks were similar for cold acclimated samples during different diapause phases; the peak of endotherm 1 varied only within the range -18.09 to -19.61°C while the peak of endotherm 2 within the range 1.64 to 6.54°C (Fig. 5; Table 1).

Discussion

Lipid rearrangements occur in both non-dormant and dormant states in insects after exposure to low temperatures. However, in those insects in which a dormant state such as diapause is closely tied to cold hardiness, changes in the membrane lipid composition are not primarily a result of response to low temperatures, but are initiated with a photoperiodically induced diapause program and later intensified in response to cold (Kostal and Simek, 1998; Hodkova et al., 1999; Michaud and Denlinger, 2006; Bale and Hayward, 2010; Rozsypal et al., 2014). Thus, in cold

tolerant insects it is important to clarify whether lipid rearrangements are exclusively governed by a diapause program, by cold exposure, or a combination of both.

In our previous studies on field collected larvae of *O. nubilalis*, we demonstrated that the compositional remodeling of lipids is accompanied by changes in their biophysical properties and is a function of photoperiodically induced diapause program, rather than a result of exposure to low temperatures of diapausing larvae. Specifically, major changes in FA composition occur early in diapause, well ahead of occurrence of low temperatures during winter. A significant increase in the overall FA unsaturation (UFAs/SFAs ratio) resulted from an increase in the relative proportion of MUFAs, primarily palmitoleic (C16:1n-7) and oleic acids (C18:1n-9), and a concomitant decrease in PUFAs and SFAs content, α-linoleic (C18:2n-6) and palmitic acid (C16:0), respectively. Thermal analyses (DSC) of whole body total lipids have shown significantly lower melt transition temperatures during the time course of diapause, in comparison to non-diapause state (Vukašinović et al., 2015).

The aim of this study was to evaluate whether larvae of *O. nubilalis* are capable of responding to changes in environmental temperature during diapause or if the ability to acclimate is determined endogenously in diapause well ahead the occurrence of low winter temperature. Since *O. nubilalis* is a freeze-tolerant insect species with a high supercooling capacity in which diapause and cold hardiness are closely related, two different acclimation temperatures were chosen: -3°C, as a potentially critical temperature in initiating adaptation to low temperatures and -10°C as a typical low mid winter temperature.

Results compared to changes in the control group (5°C) showed that trend in the UFAs/SFAs ratio during diapause is similar to the results published in our previous studies, where the increase in the overall unsaturation (UFAs/SFAs ratio) occurs during transition to diapause and remains stable until the termination period, when it significantly decreases (Vukašinović et al., 2015). In the current study, the UFAs/SFAs ratio was high until the termination period (TD) when it decreased, significantly in phospholipids, mainly as a consequence of a decrease in dominant MUFAs and a concomitant increase in SFAs and PUFAs. In addition, the results of this study showed as well that cold acclimation of fifth instar *O. nubilalis* larvae during the state of diapause had modest effects further on the readjustments in FA composition of triacylglycerols and phospholipids. In this case the overall FA unsaturation (UFAs/SFAs ratio) was without significant change, except for the triacylglycerol fraction after the exposure to -3°C and -10°C in mid diapause (MD), where UFAs/SFAs ratio significantly increased as a result of a decrease in the relative proportion of SFAs, concretely palmitic acid (C16:0). Also, thermal analysis (DSC) of whole body total lipids revealed that cold acclimation of larvae during different phases of diapause slightly reduce the melt transition temperatures, which according to our previous findings significantly decreased during the transition to diapause, compared to the non-

diapause state (Vukašinović et al., 2015). The essential compositional adjustments of lipids (FAs rearrangements) are accompanied by changes in the biophysical properties of total lipids and is a reflection of the diapause program in larvae of *O. nubilalis*. This provides the ability to survive low winter temperatures without additional adaptations to increase fluidity of lipids during overwintering.

Lipids play a key role in protection against cold stress and are involved in the initial preparations for diapause which occur prior to the onset of winter conditions. Lipids also critically influence both the progression of diapause and its stress-tolerance phenotype as represent the front line protection against cold stress, representing the interface between the insect cells and their environment (Michaud and Denlinger, 2006; Bale and Hayward, 2010). It has been previously well documented that in some insect species winter diapause induces changes in the FA composition of triacylglycerol and phospholipid leading to a higher overall FA unsaturation (UFAs/SFAs ratio) (Bennet et al., 1997; Kostal and Simek, 1998; Hodkova et al., 1999; Michaud and Denlinger, 2006; Rozsypal et al., 2014). The results of this study are in accordance with these findings, i.e. cold acclimation resulted in only modest further changes in FA composition in *O. nubilalis* having changed significantly during transition to diapause (Vukašinović et al., 2015).

Since *O. nubilalis* is an atypical freeze-tolerant insect species that depresses its' supercooling point (SCP) to well-below the SCP of other freeze-tolerant species, it is highly likely that changes in the composition of lipids initiated early in diapause. These changes are mostly associated with MUFAs in both membrane and storage lipids and enhance thermal tolerance of this species in environments with wide fluctuations in winter temperatures. According to Michaud and Denlinger (2006) a dominance of MUFAs in membranes is energetically more favourable than synthesis of PUFAs since it allows a wide window of fluidity without a need for continuous readjustment of FAs in response to or in preparation for low-temperatures. In membrane lipids, the fluidity is maintained constitutively at a high level, probably to avoid the unregulated transition to the gel phase during cold spells. Based on the results of this study and considering our previous studies on lipids in *O. nubilalis* (Vukašinović et al., 2013, 2015), we conclude that lipid rearrangements are a function of the endogenous "diapause program" rather than a direct effect of low temperatures, which proved to have limited impact on lipid changes in diapausing larvae of *O. nubilalis*.

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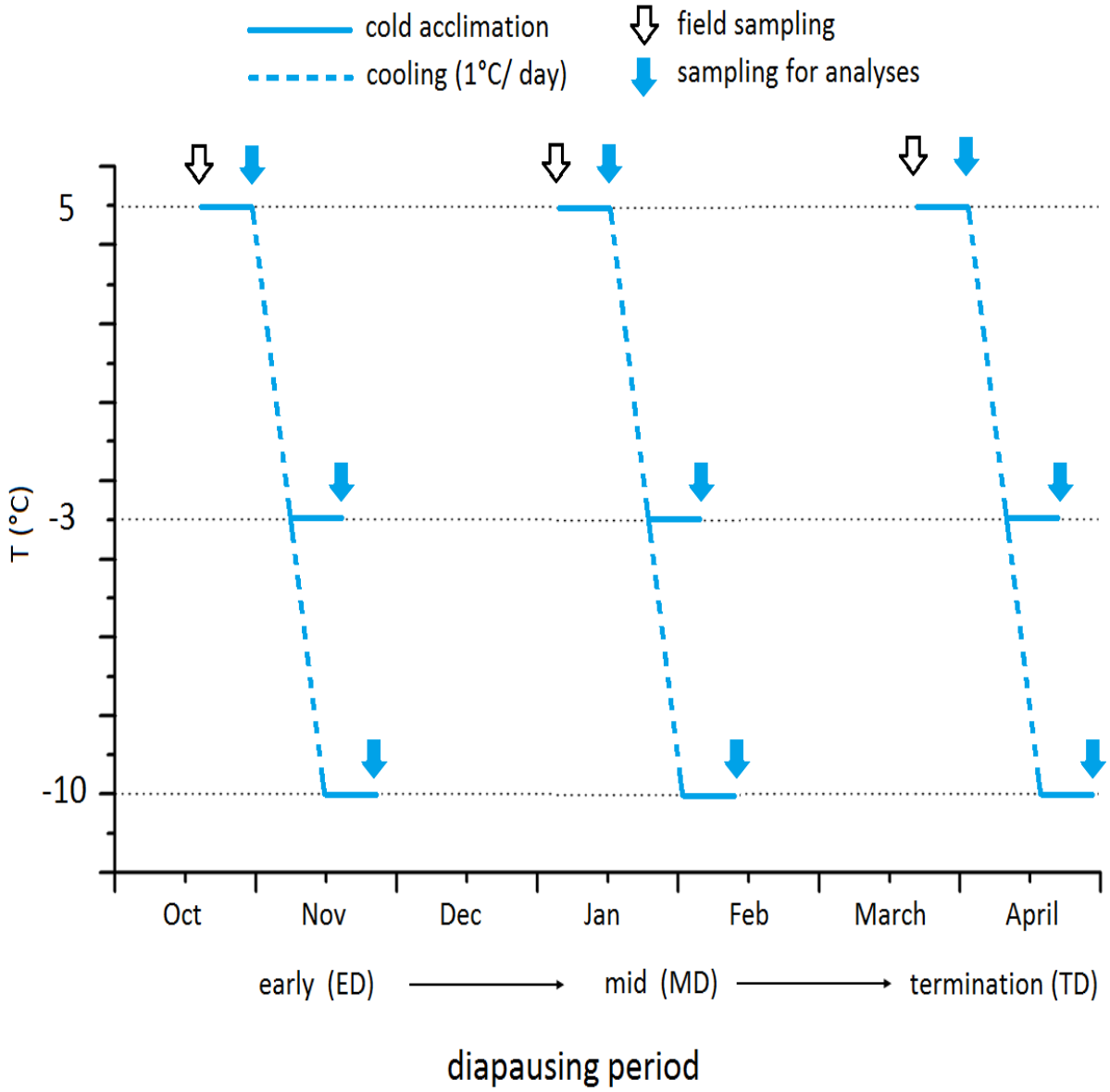

Fig. 1 Schematic illustration of experiment (explanation given in the text). ED – early diapause, MD – mid-diapause, TD – termination of diapause.

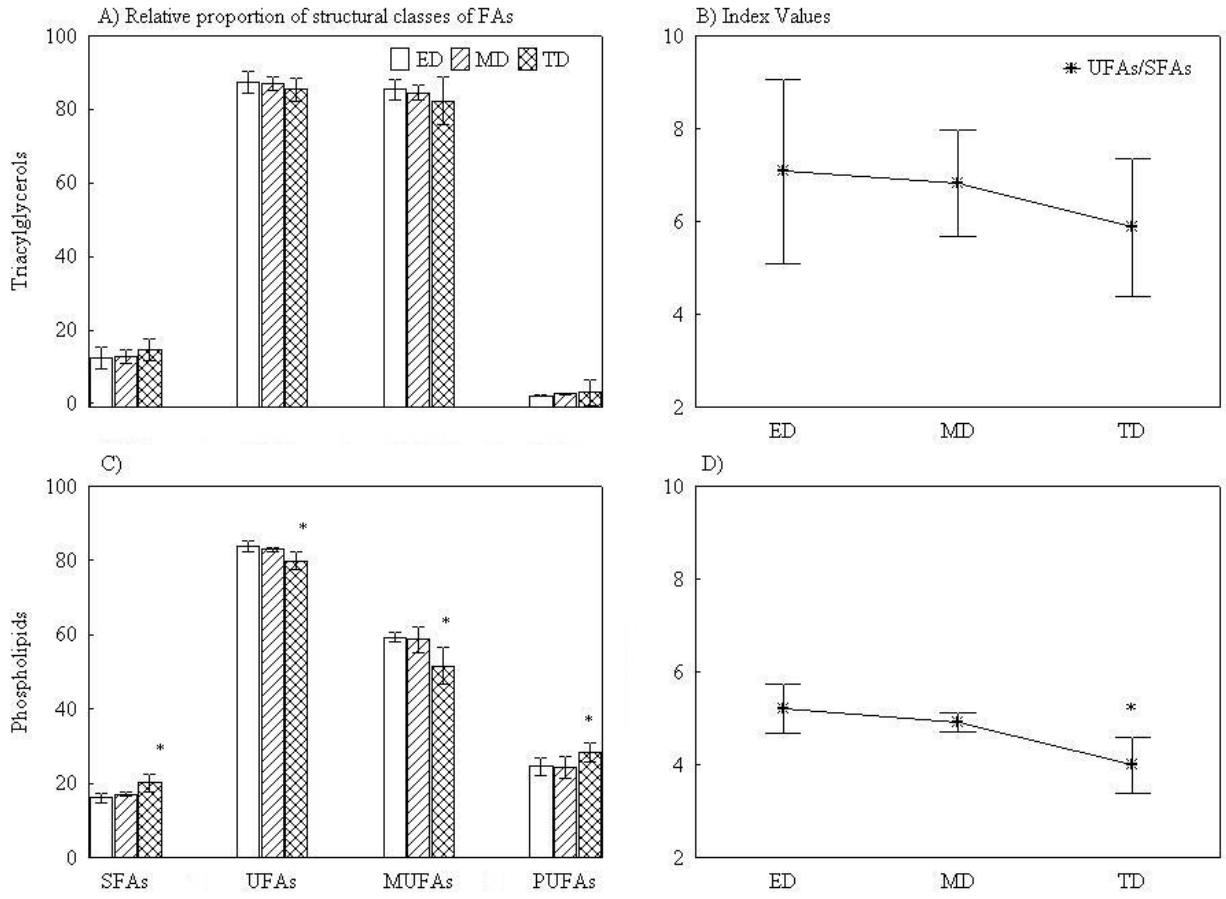

Fig. 2A, B, C, D The fatty acid composition of triacylglycerols and phospholipids of cold acclimated diapausing larvae of *O. nubilalis* to 5°C during early diapause (ED), mid-diapause (MD) and termination of diapause (TD) presenting the results considering major structural classes of FAs: saturates (SFAs), unsaturates (UFAs), monounsaturates (MUFAs) and polyunsaturates (PUFAs) (A and C), and changes in overall FA unsaturation (UFAs/SFAs ratio) (B and D). Bars represent mean ± standard error, n = 3 groups, 3 larvae per group. Asterisk above the bars indicate the level of statistically significant differences in the relative proportion of structural classes determined by Tukey's HSD post hoc-test in control group (5°C) during the time course of diapause (ED, MD, TD): *= $p < 0.05$.

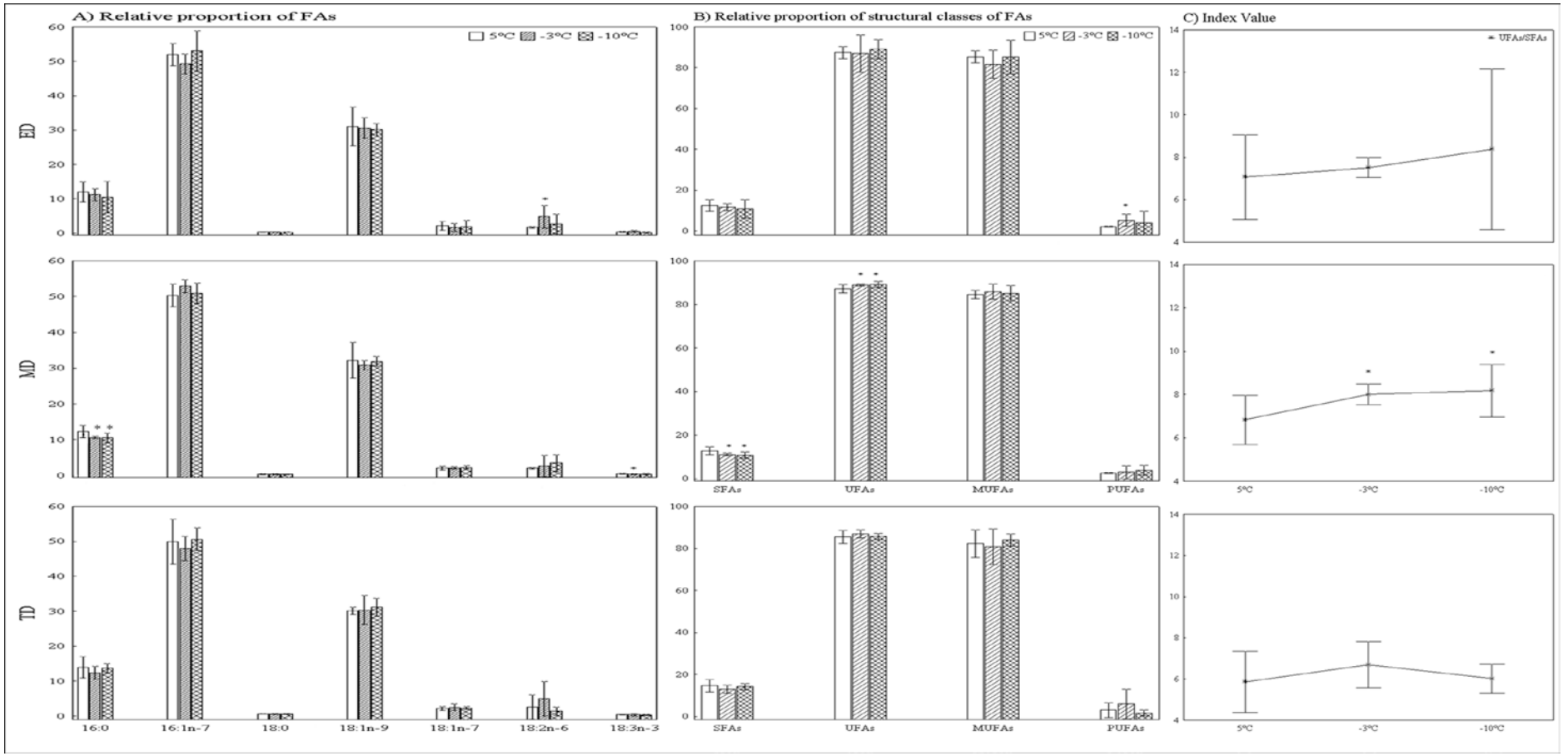

Fig. 3A, B, C The fatty acid composition of triacylglycerols of cold acclimated diapausing larvae of *O. nubilalis* to 5°C, -3°C and -10°C (A), during early diapause (ED), mid-diapause (MD) and termination of diapause (TD) presenting the results considering major structural classes of FAs: saturates (SFAs), unsaturates (UFAs), monounsaturates (MUFAs) and polyunsaturates (PUFAs) (B), and changes in overall FA unsaturation (UFAs/SFAs ratio) in triacylglycerols (C). Bars represent mean $\pm$ standard error, n = 3 groups, 3 larvae per group. Asterisk above the bars indicate the level of statistically significant differences determined by Tukey's HSD post hoc-test compared to control group (5°C) during the time course of diapause (ED, MD, TD): *= $p < 0.05$.

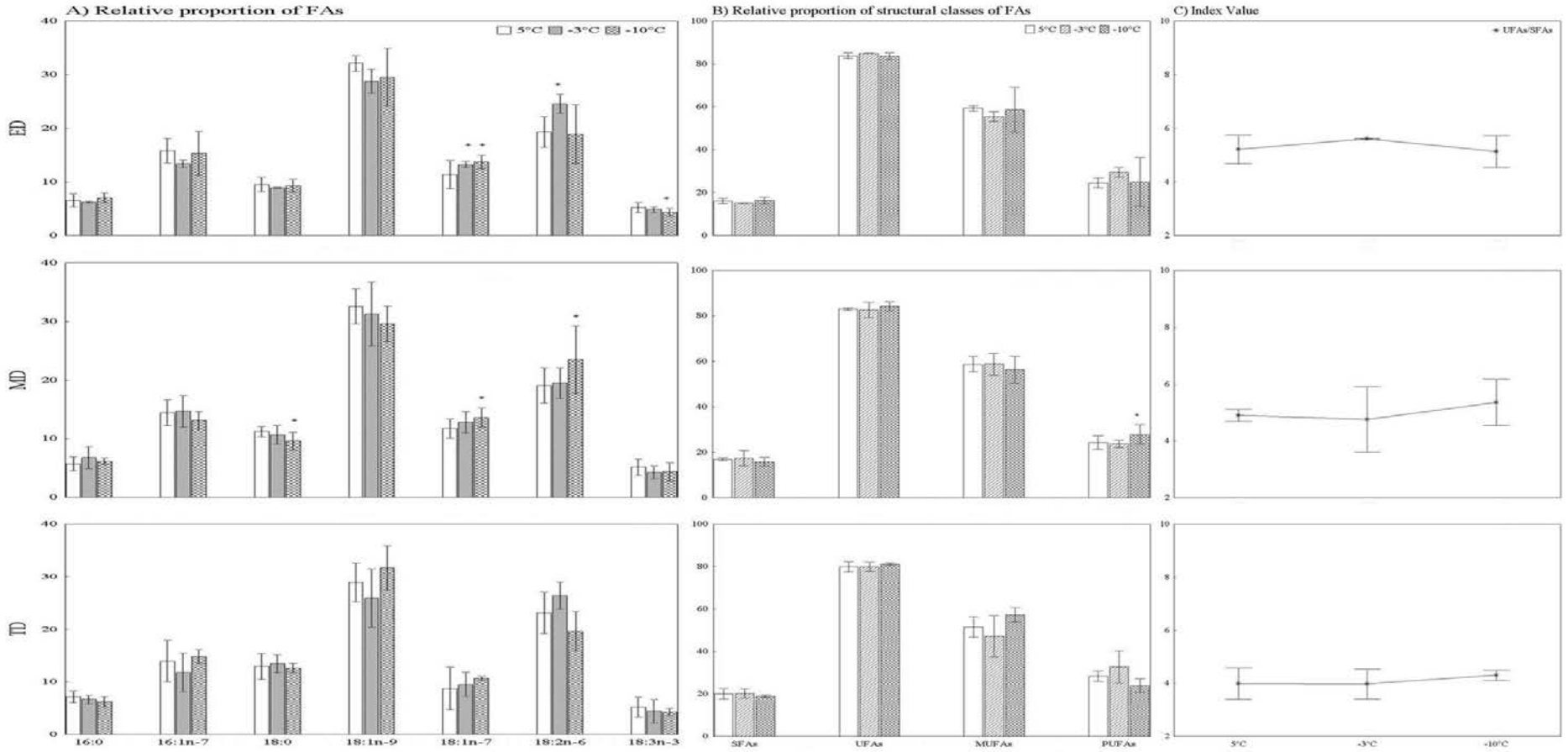

Fig. 4A, B, C The fatty acid composition of phospholipids of cold acclimated diapausing larvae of *O. nubilalis* to 5°C, -3°C and -10°C (A), during early diapause (ED), mid-diapause (MD) and termination of diapause (TD) presenting the results considering major structural classes of FAs: saturates (SFAs), unsaturates (UFAs), monounsaturates (MUFAs) and polyunsaturates (PUFAs) (B), and changes in overall FA unsaturation (UFAs/SFAs ratio) in phospholipids (C). Bars represent mean $\pm$ standard error, n = 3 groups, 3 larvae per group. Asterisk above the bars indicate the level of statistically significant differences determined by Tukey's HSD post hoc-test compared to control group (5°C) during the time course of diapause (ED, MD, TD): *= $p < 0.05$.

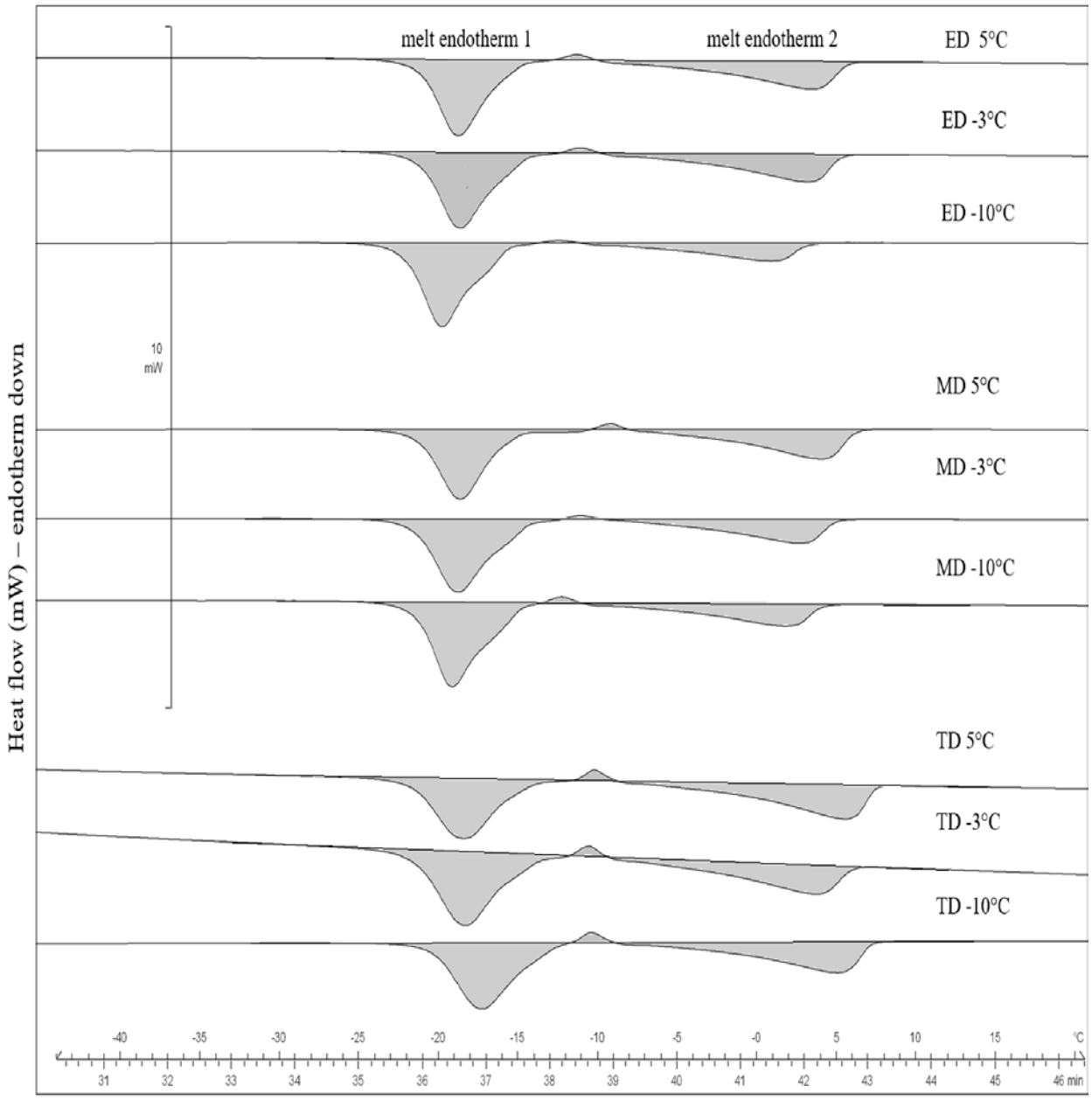

Fig. 5 DSC thermograms of total lipids of cold acclimated diapausing larvae of *O. nubilalis* to 5°C, -3°C and -10°C, during different phases of diapause; ED- early diapause, MD- mid diapause and TD- termination of diapause. Melt endotherm 1 and 2 indicate solid-liquid phase transitions. The peak of melt endotherm 1 and the peak of melt endotherm 2 presented in Table 1, indicates the temperature at which the difference between the DSC curve and the interpolated baseline is at a maximum. Since the total lipids comprise complex mixtures of molecules with different level of unsaturation, different chain lengh, melting is a multistep process.

	Peak of melt [°C]		Onset [°C]		End set [°C]	
ED	**1**	**2**	**1**	**2**	**1**	**2**
5°C	-18.92	3.91	-26.19	-9.49	-13.82	6.42
-3°C	-19.33	3.19	-26.02	-10.08	-13.11	5.97
-10°C	-19.61	1.64	-25.35	-9.77	-15.65	4.35
MD						
5°C	-18.65	4.70	-26.93	-8.91	-11.83	6.74
-3°C	-18.92	3.63	-24.29	-9.16	-14.78	5.92
-10°C	-19.01	2.96	-25.01	-9.13	-13.16	5.72
TD						
5°C	-18.26	5.98	-25.69	-7.07	-12.26	7.55
-3°C	-19.08	4.83	-26.21	-9.05	-11.84	7.22
-10°C	-17.46	5.21	-25.16	-11.68	-9.2	8.14

Note: The temperature at peak of melt indicates the temperature at which the difference between the DSC curve and the interpolated baseline is at maximum (Fig. 5). The melt endotherm (indicate phase transitions from solid to liquid) 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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