Metabolomic Analysis of Diapausing and Non-diapausing Larvae of the European Corn Borer *Ostrinia nubilalis* (Hbn.) (Lepidoptera: Crambidae)

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**Abstract**

In this study, an 1H-NMR-based metabolomic approach was used to investigate the biochemical mechanisms of diapause and cold hardiness in diapausing larvae of the European corn borer *Ostrinia nubilalis*. Metabolomic patterns in polar hemolymph extracts from non-diapausing and diapausing larvae of *O. nubilalis* were compared. Analysis indicated 13 metabolites: 7 amino acids, glycerol, acetate, citrate, succinate, lactate and putrescine. Results show that diapausing larvae display different metabolomic patterns compared to active non-diapausing larvae, with predominant metabolites identified as glycerol, proline and alanine. In specific diapausing larvae initially kept at 5 °C then gradually chilled to −3 °C and −16 °C, alanine, glycerol and acetate were predominant metabolites. 1H-NMR spectroscopy provides new insight into the metabolomic patterns associated with cold resistance and diapause in *O. nubilalis* larvae, suggesting distinct metabolomes function in actively developing and diapausing larvae.

**Keywords:** Cold hardiness, NMR spectroscopy, amino acids, polyamines, glycerol, intermediary metabolites

1. Introduction

Organisms, such as insects have evolved various mechanisms to adapt to adverse environmental conditions. As part of a general survival strategy, many insects have the ability to suppress basal metabolic rates and enter a hypometabolic or dormant state known as diapause. Besides insects, diapause is common in invertebrates such as rotifers, nematodes, earthworms, crustaceans and terrestrial gastropods.1

Diapause is a genetically determined, obligatory or facultative developmental arrest that can occur at the embryonic, larval, pupal, or adult stage of a given species. It is a dynamic process consisting of several distinct phases each characterized by a particular set of biochemical and physiological changes.1 Several comprehensive reviews have been published on the environmental,2,3 hormonal and molecular regulation of diapause.4–7 During diapause, overall metabolism is depressed, energy production and consumption processes are adjusted, and gene expression and biochemical pathways are diverted toward synthesis of protective molecules.

Many insects of temperate regions develop cold hardiness during winter diapause.8–10 Several eco-physiological and biochemical studies have been conducted to improve our understanding of the cellular mechanisms of cold hardening process during the hypometabolic state.11,12 The main function of metabolism during periods of cold accli-
mation is biosynthesis of low molecular weight organic solutes such as polyols and sugars that depress the body’s supercooling point (cryoprotectants), while stabilizing proteins and cellular membranes.\textsuperscript{13,14} Story and Story summarize recent research on the biochemical adaptations associated with insect cold hardness, including: regulation of cryoprotectant biosynthesis, mechanisms of metabolic rate depression, the role of aquaporins, and cell preservation strategies (chaperones, antioxidant defence systems, metal binding proteins and mitochondrial suppression).\textsuperscript{15}

Most studies on the molecular basis of insect cold hardness conducted to date have been based on the level of the genome, transcriptome and proteome.\textsuperscript{15–18} Despite significant progress in this area, many aspects of the metabolic adaptations underlying diapause and cold hardness remain undiscovered, especially in non-model organisms. We propose that a metabolomic approach could provide insight into the metabolite composition underlying adaptations in response to unfavourable environmental conditions, such as low temperature. Since comprehensive analysis of the metabolites present in an organism or cell is crucial to understanding cellular processes and function, reports describing metabolomic approaches for studying insect biochemistry have been gradually increasing over the past several years.\textsuperscript{19–25} Metabolomics as a part of systems biology can reveal the unique fingerprint of low molecular weight metabolites within a biological sample that are characteristic for a specific physiological state.\textsuperscript{26,27}

In the present study, we analyzed metabolomic profiles in diapausing larvae of the European corn borer \textit{Ostrinia nubilalis} (Lepidoptera: Crambidae), a maize pest species of Eurasia and North America. \textit{O. nubilalis} overwinters as a diapausing 5\textsuperscript{th} instar larvae mostly in corn stalks. Cold hardness in this species is tightly linked with diapause. During diapause, larvae gradually accumulate glycerol and, to less extent, trehalose in hemolymph and become cold tolerant with a supercooling point of approximately \(20\ ^\circ\text{C}\). To investigate the role of altered overall metabolic activity in cold hardening larvae of \textit{O. nubilalis}, in the present study we compare the metabolomic patterns of diapause and non-diapause larvae using high-resolution \(1\text{H}\) nuclear magnetic resonance (NMR) spectroscopy with multivariate analysis. In addition, we have also examined the metabolomic responses in diapausing larvae maintained at \(5\ ^\circ\text{C}\) and subsequently exposed to \(-3\ ^\circ\text{C}\) and \(-16\ ^\circ\text{C}\) for two weeks. To our knowledge, this is the first report describing application of NMR spectroscopy to provide insight into mechanisms of cold resistance and diapause in \textit{Ostrinia nubilalis}.

### 2. Experimental

#### 2.1. Materials and Methods

Specimens of non-diapausing larvae of \textit{Ostrinia nubilalis} were collected in July, while diapausing larvae were collected in October from maize plants from fields in Vojvodina Province, Serbia. Diapausing larvae were kept in a laboratory at \(5\ ^\circ\text{C}\) and \(70\%\) relative humidity for a week and then divided into two groups. The first was kept at \(5\ ^\circ\text{C}\) for two weeks and the second was divided into two subgroups by gradual exposure to temperatures of \(-3\ ^\circ\text{C}\) and \(-16\ ^\circ\text{C}\) (cooling rate \(-3\ ^\circ\text{C}\) per day). Larvae were kept at these temperatures for two weeks. Following this treatment, survival was \(100\%\) and samples were collected. Analysis included five replicate pools. For each pool \(150\ \mu\text{l}\) of hemolymph was sampled and merged from 10–12 animals by cutting the prolegs of larvae and squeezing hemolymph into 1.5 ml microcentrifuge tubes containing a few crystals of phenylthiourea to prevent melanisation. After removal of haemocytes by centrifugation at 12 000 \(\text{g}\) for 5 min at \(4\ ^\circ\text{C}\), the supernatant was lyophilized overnight.

#### 2.2. NMR Sample Preparation

Extraction of metabolites from the hemolymph of \textit{O. nubilalis} larvae was performed using a modified Bligh-Dyer procedure,\textsuperscript{29} all lyophilizates were resuspended in a 500\(\mu\text{l}\) mixture of methanol, chloroform and water at a final ratio of 2.0:2.0:1.8. Samples were vortexed for 60\(\text{s}\) and centrifuged at 10 000 \(\text{g}\) for 10\(\text{min}\) at \(4\ ^\circ\text{C}\) and then polar extracts (upper layer) were removed and lyophilized. Dried polar extracts were resuspended in 90\% \(\text{H}_2\text{O}/10\% \text{D}_2\text{O}\) (GOSS Scientific Instruments Ltd, Essex UK) prepared as 100\(\text{mM}\) phosphate buffer (pH 7.0), containing 0.5\(\text{mM}\) sodium 3-(trimethylsilyl)propionate-2,2,3,3-d\textsubscript{4} (TMSP, Cambridge Isotope Laboratories) as an internal reference.

#### 2.2.1. NMR Experiments and Data Processing

A 500 MHz Bruker spectrometer equipped with a cryogenically cooled probe was used for one-dimensional (1D) \(1\text{H}\) and two-dimensional (2D) \(1\text{H}-\text{J-resolved}\) NMR spectra (\(J\)-\text{RES})\textsuperscript{30} NMR data acquisition. In both cases, water resonance was suppressed using excitation sculpting.\textsuperscript{31} 1D spectra were acquired using a 90\(^\circ\) pulse, with 5 kHz spectral width, a relaxation delay of 3\(\text{s}\) and 128 transients. For quantitative analysis, 1D spectra were also acquired using a 30\(^\circ\) pulse and a relaxation delay of 15\(\text{s}\). 2D \(J\)-RES spectra were collected using a double spin echo sequence with 16 transients per increment and 32 increments. Strong coupling artefacts were suppressed by phase cycling.\textsuperscript{32} Before Fourier transformation, 2D \(J\)-RES spectra were multiplied by a combined sine-bell/exponential window function in the direct dimension and by a sine bell function in the incremented dimension.\textsuperscript{33} Skyline projections were calculated and spectra were aligned. Selected signals arising from residual solvents, and TMSP were excluded. Spectra were normalized according to probabilistic quotient normalization factors and binned at 0.005 ppm (4 data points). The generalized-log transformation was applied before conducting multivariate statistical analysis.\textsuperscript{34}
Spectra of the polar fraction were recorded with a simple 1D pulse sequence using a 60° flip angle, 512 transients and 8 steady state scans, a relaxation delay of 3 s, a spectra width of 7002 Hz and 32,768 data points. Data were processed by multiplying free induction decays by an exponential line broadening function of 0.5 Hz before Fourier transformation.

NMR data was processed using NMRLab in the MATLAB (The MathWorks, Inc., Natick, MA) programming environment. Multivariate statistical analysis of the projected J-RES NMR spectroscopy data was carried out using PLS toolbox 4.1 (Version 4.1; Eigenvector Research, Manson, WA). NMR resonances of metabolites were assigned using the Chenomx NMR Suite (version 5.0; Chenomx Inc., Edmonton, Canada).

3. Results and Discussion

In recent years, the number of metabolomic studies of insect biochemistry and diapause has been gradually increasing. The present study describes application of NMR-based metabolomics to characterise molecular and biochemical changes underlying diapause in the European corn borer *Ostrinia nubilalis*. We compared metabolomic profiles in polar hemolymph extracts of diapausing and non-diapausing larvae of *O. nubilalis* using high-resolution 1H-NMR spectroscopy, and show that diapause, as a special state of physiological dormancy, has a different metabolomic fingerprint compared to active non-diapausing phase. Furthermore, different metabolic profiles was measured in diapausing larvae maintained at 5 °C vs. those gradually chilled to –3 °C and –16 °C and kept for two weeks. In total, 13 metabolites were identified (Fig. 1) including: 7 amino acids, glycerol, lactate, putrescine, acetate, citrate and succinate.

Principal component analysis (PCA) was conducted on 1H-NMR spectra of metabolites extracted from 4 groups of *O. nubilalis* larvae: 1 non-diapausing and 3 diapausing (5 °C, –3 °C and –16 °C). The resulting PCA score plot (PC1 vs. PC2) is shown in Fig. 2. The first two principal components capture 93.9% of the variance in the data. This PCA plot shows excellent separation between...
non-diapausing and diapausing states in the first principal component, which explains the largest percentage (89.5%) of the data.

Among polar fraction metabolites extracted from the hemolymph of diapausing larvae, clear separation was observed by PCA analysis between larvae incubated at 5 °C and those kept at –3 °C and –16 °C (Fig. 4). The first two principal components shown on this plot explain 68.9% of the variation.

The most relevant discriminatory metabolites between diapausing groups kept at 5 °C (negative loadings) versus those incubated at –3 °C and –16 °C (positive loadings) are shown in Fig. 5. A section of the loadings plot (0.8–4.3 ppm) for the first principal component is presented. As can be seen, the most dominant metabolites observed in the hemolymph polar fraction from diapausing larvae were glycerol, and to a lesser extent the amino acids proline and alanine. The major metabolites observed in hemolymph extract from non-diapausing larvae were the amino acids serine and glutamine, as well as several amino acids with aliphatic side chains: valine, leucine and isoleucine. In addition, levels of citrate, acetate, succinate, putrescine and lactate were found to be significantly increased in the non-diapausing group.

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Glycerol. Moreover, alanine is a less toxic end product of the effectiveness of alanine is roughly the same as that of pyruvate; Pro, proline; Gln, glutamine; Citr, citrate; Ser, serine.

**Fig. 5.** Section of the loadings plot (0.8–4.3 ppm) for the first principal component. Signals representing the most relevant metabolites which discriminate diapausing *Ostrinia nubilalis* larvae exposed to 5 °C (negative loadings) from those exposed to –3 °C and –16 °C (positive loadings) are shown (Val, valine; Ile, isoleucine; Leu, leucine; Lac, lactate; Ala, alanine; Putr, putrescine; Ace, acetate; Pro, proline; Gln, glutamine; Citr, citrate; Ser, serine).

Protective in high concentrations (Fig. 3). Furthermore, the present study indicates that free amino acids alanine and proline are higher in diapausing larvae than in non-diapausing larvae (Fig. 3). Glycerol is the most common cryoprotectant in insects, which, together with other similarly acting metabolic compounds, is associated with a colligative depression of the freezing point, and protects cells by stabilizing membranes and macromolecules. The correlation between increased concentrations of alanine and cold tolerance has been reported for a large number of insect species. In support of this, the colligative effectiveness of alanine is roughly the same as that of glycerol. Moreover, alanine is a less toxic end product of pyruvate catabolism than lactate, which may be important during diapause when the aerobic portion of cellular respiration is slowed.

The increased level of proline observed in diapausing larvae is likely a consequence of accumulated alanine that is transformed to proline during diapause, as described in diapausing eggs of the silkworm *Bombyx mori*. Proline itself is a natural cryoprotectant expressed by numerous organisms under low-temperature stress: primarily in plants, but in some bacteria, invertebrates, protists, and algae as well. Besides alanine and proline, our NMR spectra suggest considerable involvement of other free amino acids (serine, glutamine, valine, leucine, isoleucine) in the main metabolic pathways of non-diapausing vs. diapausing larvae (Fig. 3).

Insect hemolymph is a dynamic and highly complex mixture of small molecular mass compounds like polyols, sugars, free amino acids, inorganic salts and variety of metabolic intermediates. Its composition varies considerably during development and stress exposure. Non-dia- pausing larvae were collected in the fifth instar, a developmental stage that is characterized by completion of food intake and preparation for pupariation. Thus, the increased content of free amino acids in the hemolymph in this period is probably due to their future use as building blocks for biosynthesis of new proteins or other nitrogen-containing compounds during metamorphosis. The increased levels of lactate, acetate and succinate (Fig. 3) observed in this study is consistent with high lipid and carbohydrate catabolism that match the cellular energy demands of actively developing larvae.

We observed clear separation in the PCA plot for metabolites from diapausing larvae reared at 5 °C vs. those kept at –3 °C and –16 °C (Fig. 4), indicating different metabolic profiles and suggesting that low temperatures have a significant influence on the larval metabolome. Since *O. nubilalis* larvae develop cold hardiness during diapause it is very difficult to distinguish the effects of thermal adaptation from the effects of diapause. The most dominant metabolite observed in hemolymph during diapause at subzero temperatures was alanine (Fig. 5). Alanine is, together with lactate, a well known end-product of anaerobic metabolism in many terrestrial insects; and could be produced from pyruvate in a one step transamination reaction. However, as reactions are readily reversible, another possibility is that up-regulation of alanine could be part of the intermediary metabolism of glycerol biosynthesis.

Along with alanine, glycerol and acetate were elevated in diapausing larvae kept at subzero temperatures (Fig. 5). Both glycerol and acetate could also accumulate during the anaerobic metabolism phase which is probably predominant at low temperatures. In general, the end products of anaerobic metabolism are mostly unknown for many insect species, including *O. nubilalis*. For example, *Drosophila melanogaster* produces lactate, alanine and acetate, but in other species a wide array of other products have been identified, including: sorbitol, succinate, glycerol, α-glycerol-3-phosphate, pyruvic acid, and fatty acids. Acetate could be produced from mitochondrial acetyl-CoA in the absence of oxygen or via alternative pathways.

Discriminatory metabolites in hemolymph which separate diapausing larvae exposed to 5 °C from those exposed to subzero temperatures were similar to those obtained when comparing non-diapausing and diapausing larvae, although concentrations were different (Fig. 5). The absence of succinate could be consistent with slowed aerobic respiration during diapause. Interestingly, the polyamine putrescine was found to discriminate non-diapausing from diapausing larvae, and diapausing larvae kept at 5 °C from diapausing larvae kept at subzero temperatures. This is a novel finding for this species and suggests a need for further research. Elevations of putrescine levels has been associated with stress in plants, earthworms, nema-
todes, leeches and planarians.\textsuperscript{22,44,45} In animals, the conversion of arginine to putrescine occurs via ornithine via arginase and ornithine decarboxylase. Since, arginine and ornithine are part of the urea cycle, whose presence in insects has not been confirmed, the origin of putrescine in hemolymph of \textit{O. nubilalis} remains unclear. The primary excretory product of the catabolism of nitrogen compounds, nucleic acids and proteins in insects is uric acid and urea is present only in trace amounts. Our results do not suggest changes in the concentration of urea, although the level of this metabolite does correlate with cold hardiness in some insects.\textsuperscript{22} However, putrescine and its derivatives, spermine and spermidine are regulatory molecules that play important roles in basic genetic processes, such as DNA synthesis and gene expression.\textsuperscript{46} Putrescine has been shown to modulate the action of juvenile hormone during neural development in crickets.\textsuperscript{47} The potential physiological role of putrescine in \textit{O. nubilalis} larvae remains an open question.

4. Conclusions

Results from the present study strongly suggest that distinct metabolomes function in actively developing versus diapausing larvae: metabolism is suppressed during diapause and, due to a decline in respiration and oxidative metabolism, is diverted to anaerobic processes, especially during exposure to subzero temperatures. The present study provides new insight into mechanisms of cold resistance and diapause in \textit{O. nubilalis} and suggest areas requiring future research, such as the role of polyamines in diapause. Moreover, based on our results, \textsuperscript{1}H-NMR spectroscopy may be a useful and reliable method for studying insect metabolomes.

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