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Metabolic insights into the cold survival strategy and overwintering of the common cutworm, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae)

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ABSTRACT

The common cutworm, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae), is a destructive pest in Asia. Although overwintering in the field has not been reported for this species, their larvae are capable of long-term survival in fluctuating temperatures, i.e., 5 °C (12 h) plus 13 °C (12 h), if food is available. With an increase in climate change due to global warming and the widespread use of greenhouses, further understanding of their cold survival strategy is needed to predict and control their population in the future. In this study, metabolomics was performed to analyze the metabolic features of *S. litura* larvae exposed to two typical low temperatures: 15 °C and 4 °C, at which the development, locomotion and feeding activities are maintained or halted, respectively. The results showed that the strategies that regulate lipid and amino acid metabolism were similar at 15 °C and 4 °C. Cold exposure induced a metabolic shift of energy from carbohydrate to lipid and decreased free amino acids level. Biosynthesis likely contributed to the decrease in amino acids levels even at 4 °C, a non-feeding temperature, suggesting an insufficient suppression of anabolism. This explains why food and high temperature pulses are necessary for their long-term cold survival. Glycometabolism was different between 15 °C and 4 °C. Carbohydrates were used rapidly at 15 °C, while trehalose accumulated at 4 °C. Interestingly, abundant trehalose and serine are prominent features of *Spodoptera exigua* larvae, an overwintering species, when compared to *S. litura* larvae. Exposure to 4 °C also induced up-regulation of carbohydrase and protease in the guts of *S. litura*. Therefore, it is likely that concurrence of food supplement and fluctuating temperatures could facilitate the cold survival of *S. litura* larvae. We also found that exposure to 4 °C could activate the mevalonate pathway in *S. litura* larvae, which might be related to glycometabolism at 4 °C. Overall, our study describes systematically the responses of a cold susceptible insect, *S. litura*, to low temperatures and explains how fluctuating temperatures facilitate their long-term cold survival indicating the possibility for overwintering of *S. litura* larvae with global warming and agricultural reforms.

1. Introduction

The common cutworm, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae), is a destructive insect pest that causes serious damages to economically important crops, especially Leguminosae and Cruciferae, throughout tropical and temperate Asia. In China, the frequency and scale of *S. litura* outbreak has increased with the reform of agricultural industry structure, and the expansion of beans and vegetables planting areas in recent years (Chen et al., 2001; Gao et al., 2013; Zeng et al., 2010). In one year, *S. litura* is capable of producing 4–5 generations in North China, where the average temperature in January (coldest month) is in general between −10 and 0 °C; 5–6 generations across middle and lower Yangtze River areas, where the average temperature in January is generally between 0 and 5.5 °C; 8–9 generations in parts of Fujian, Guangdong, Guangxi and Taiwan provinces (Gao et al., 2004, 2013), where the average temperature in January is higher than 10 °C and *S. litura* can complete generations in all seasons without diapause.

The cold-hardiness and overwintering of *S. litura* are important for predicting their population size in the following year. The optimum temperatures for the development of *S. litura* eggs, larvae, and pupae are around 24–27 °C, and their threshold for lower temperatures is around 10–12 °C (Chen et al., 2001; Fand et al., 2015). No field

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evidence has thus far indicated the overwintering of *S. litura* in areas with average temperatures lower than 10 °C in the month of January in China. This has led to the speculation that migration of adults from the southern provinces each spring is likely the major source of *S. litura* in Yangtze River areas and North China (Chen et al., 2001; Gao et al., 2013; Luo et al., 2012). However, in fluctuating experimental conditions where daily average values are at or even lower than 10 °C, *S. litura* larvae can develop continuously and survive for more than 3 months (typical duration of winters) if food is accessible (Matsuura and Naito, 1991, 1992; Matsuura et al., 1991a,b). These daily fluctuating temperature profiles (for example, 5 °C:13 °C = 12 h:12 h and 0 °C:15 °C = 10 h:14 h) should meet several criteria to guarantee long-term survival, including a daily cumulative temperature (above 10 °C) higher than 1 °C, avoiding exposure to extremely cold temperatures (lower than −5 °C) as well as long-term exposure to temperatures lower than the developmental threshold (Matsuura et al., 1991a). However, how fluctuating temperature profiles and food supply facilitate *S. litura* overwintering is unknown.

Younger *S. litura* larvae (2nd and 3th instars) released artificially could survive the mild winters in the fields at Tateyama and Wada in Japan, and those that survived in the following spring were mostly in their 6th and 5th instars, rather than in the pupal stage or in younger instars (Matsuura et al., 1992). When 4th instar larvae were released into un-heated greenhouses or other artificial facilities, some individuals could even pupate and emerge normally during the winter (Matsuura et al., 1992; Zheng et al., 2005). These phenomena suggested that *S. litura* larvae could grow in mild cold environment, and can likely achieve temperature-dependent overwintering, which could be improved by artificial shelters. In China, it has been reported that the survival strategies and distribution of *S. litura* could have adjusted to global warming and the development of greenhouses, and may cause potential outbreaks of *S. litura* more early and in larger scales (Chen et al., 2001). To devise strategies to better control this pest, it is essential to understand the influences of environmental and agricultural changes on *S. litura* development. Particularly, it is essential to obtain insight from the perspectives of cold adaptive strategies and the underlying molecular mechanisms to understand if *S. litura* may overwinter in the field or in experimental conditions to avoid their unpredicted future outbreaks.

In general, there are two major challenges for insects to live through chilly winter: one is protection from cold injury and the other is long-term metabolic maintenance (Hahn and Denlinger, 2011; Lee, 2010; Storey and Storey, 2004). Two metabolic adjustments, accumulation of cryoprotectants (i.e. inositol, trehalose, glycerol, and sorbitol) and membrane remodeling, are effective cold protective strategies (Storey and Storey, 1983, 1991; Wang et al., 2010; Watanabe, 2002), which are closely associated with the long-term and rapid cold-hardening phenomena in insects (Clark and Worland, 2008; Storey, 1997; Storey and Storey, 1988). For long-term metabolic maintenance in winter, ectotherms may enter into metabolic suppression or sustain activity by thermal compensation strategies (Guderley and St-Pierre, 2002), which aim to slow the consumption of limited resource reserves (Hahn and Denlinger, 2011; Storey and Storey, 2004) and maintain the capacity of resources exploitation from the environment (Bullock, 1955; Horwath and Duman, 1983; Sømme and Block, 1991; Zhu et al., 2016), respectively. Obviously, these physiological strategies should rely on the reorganization of metabolic network. Although the specific metabolic adjustments in response to cold can vary among species based on the accumulation of different cryoprotectants and choice of survival strategy, it is reasonable to postulate that cold protection and metabolic maintenance should be well coordinated by these adjustments in all overwintering insects, especially with respect to resource allocation. For example, glycolysis and Krebs cycle were enhanced and suppressed, respectively, in flesh flies (*Sarcophaga crassipalpis*) during diapause (Michaud and Denlinger, 2007). Such a regulation pattern could promote the synthesis of glycerol, the major cryoprotectant in flesh flies (Yoder et al., 2006), without accelerating the consumption of resource storage. Besides, increasing number of studies also suggest a cold-induced shift in nutrient utilization or rearrangement of nutrient priority as an important mechanism that may likely coordinate cold protection and metabolic maintenance (Hahn and Denlinger, 2011; Kim and Song, 2000; Zhu et al., 2016). In species accumulating trehalose or glucose as major cryoprotectants, it is easy to understand that a shift from carbohydrates to lipids as the metabolic substrate can harmonize cold protection and energy supply. Overall, these advances provide us clear directions for estimating whether *S. litura* larvae have the potential for winter survival and for analyzing how fluctuating temperatures may facilitate their cold tolerance with respect to metabolism.

To deepen our understanding of the cold-hardiness and overwintering in *S. litura*, we investigated and compared the metabolic characteristics in *S. litura* larvae exposed to two typical low temperatures, 15 °C and 4 °C, when their development, locomotion and feeding activities are maintained or halted, respectively. In China, winter temperatures fluctuating between 4 °C and 15 °C is common in wide range of areas in north with 10 °C isotherm in January, as well as in un-heated greenhouses distributed in more northern areas. We also compared the metabolic differences between *S. litura* larvae and *S. exigua* larvae, which has been reported to overwinter in the Yangtze River areas (Jiang and Luo, 2010; Zheng et al., 2015) to analyze the requirements for cold adaption at the metabolic level and thus to predict whether global warming or reform of agricultural industry structure would induce overwintering of *S. litura*.

2. Methods

2.1. Insect rearing and treatments

The laboratory colonies of *S. litura* and *S. exigua* were maintained on artificial diet at 27 °C, 70 ± 10% RH and a photoperiod of 16L:8D. Larvae in the 5th instar were collected one day after molting and treated at the following temperatures: 4 ± 0.5 °C, 15 ± 0.5 °C and 27 ± 0.5 °C (control) for 4 h or 48 h. Larvae were not provided food during the exposures. Whole larvae, hemolymph and guts collected from these larvae were stored at −100 °C in an ultra-cold storage freezer for further tests and analysis.

2.2. Measurement of respiration rate

First, larvae were acclimated at 4 °C (n = 5), 15 °C (n = 5), 27 °C (n = 15) and 37 °C (n = 5) for 4 h. Then, the CO_2 production rate and the O_2 consumption rate were determined by a Sable System (Las Vegas, USA) equipped with an SS-3 subsampler unit, MFC-2 Gas Mixers and Mass Flow Controller, RM8 Gas Flow Multiplexer, CA-10A CO_2 analyzer, FC-10A O_2 analyzer, ExpeData, and UI-2 package as described previously (Zhu et al., 2016). Each larva was measured once, and each measure lasted 5–10 min.

2.3. Metabolomics based on Gas Chromatography-Mass Spectrometer (GC-MS)

First, larvae were acclimated at 4 °C and 15 °C for 4 h (short-term) or 48 h (long-term) (n = 9–11 for each treatment group). Each whole larva was then homogenized in 1200 μl chloroform:methanol:water (2:5:2, v/v) plus 20 μl of 10 mg/ml heptadecanoic acid in methanol (internal standard, TCI) using a glass homogenizer. Then, the homogenate was incubated at 50 °C for 30 min, and centrifuged at 12,000*g* for 15 min (4 °C, Eppendorf 5417R). The resulting supernatant was transferred into a clean 2.0 ml Eppendorf tube, while the residue was resuspended in 400 μl of 70% ethanol for extraction and centrifugation steps again. The supernatants from the above two centrifugation steps were combined, and 400 μl was vacuum-dried at 40 °C to remove water.

Next, 50 µl of methoxyamine hydrochloride (20 mg/ml in pyridine, J & K) was pipetted into each tube and the samples were incubated at 37 °C overnight to oximate the carboxides. Silylation was accomplished by adding 50 µl of N-methyl-N-trifluoroacetamide (MSTFA, Sigma) into each tube and incubating at 37 °C for 30 min. Then, 100 µl of hexane (HPLC grade) was added into the reaction tubes and the samples were centrifuged at 12,000*g* for 15 min at room temperature. The supernatant was used for chromatographic analysis on Agilent 6890N-5973N (Agilent Technologies Inc.), data extraction and normalization according to previously described methods (Zhu et al., 2016). The repeatability of the equipment was verified in pre-experiments, so each sample was measured only once. Quantitative results of detected metabolites (matrixes of samples against metabolites) are presented in Table S1.

2.4. Thin-layer chromatography (TLC)

Larvae were acclimated at 4 °C, 15 °C and 27 °C for 4 h. Then, 15 µl of hemolymph was collected from each larva through an incision on the prolegs. Lipid extraction, TLC, coloration and image capture were performed as described previously (Zhu et al., 2016).

2.5. Enzyme extract preparation

Larvae were acclimated for 4 h at each of the following temperatures: 4 °C, 15 °C and 27 °C and then each larval gut was collected in a 2 ml Eppendorf tube. The guts were then homogenized using a tissue grinder in 1 ml PBS (pH 7.4, 0.02 M) and centrifuged at 13,000*g* for 15 min (4 °C). The resulting supernatants were collected as crude enzyme extracts. The protein concentration of crude enzyme extracts was determined by using the BCA protein assay kit (Beyotime Biotechnology) following the manufacturers' protocol.

2.5.1. Total carbohydrase activity

For each test, 50 µl of the crude enzyme was added to a 0.2 ml Eppendorf tube with 100 µl of 0.5% soluble starch and incubated at 37 °C for 40 min, heated in boiling water for 5 min to deactivate the enzymes, followed by centrifugation at 13,000*g* for 15 min (4 °C). Then, 100 µl of each supernatant was mixed with 100 µl of color developing agent (5*g* 3,5-dinitrosalicylic acid, 5 g NaOH, 1 g phenol and 0.25 g sodium sulfite dissolved in 500 ml H_2O), followed by heating at 95 °C for 5 min in a metal water bath. The optical density (OD) of the mixture was measured at 520 nm. A control test was setup for each crude enzyme sample, in which the sample was heated in boiling water before incubation at 37 °C. The final OD value of each sample was obtained after subtracting the control value. After normalizing for protein concentration the values were compared to obtain the relative activity of total carbohydrase between the different treatment groups.

2.5.2. Total protease activity

For each test, 50 µl of the crude enzyme extract from above was added to a 0.2 ml Eppendorf tube with 50 µl of 1% casein. After incubation at 37 °C for 40 min, the tube was heated in boiling water for 5 min to deactivate the enzymes, followed by centrifugation at 13,000*g* for 15 min (4 °C). Then, 3 µl of each supernatant was mixed with 30 µl of color developing agents (0.4 g ninhydrin and 0.01 g vitamin C dissolved in 5 ml ethanol) followed by heating at 95 °C for 16 min. After cooling, the mixture was added to 30 µl of 0.2% KIO_3, and diluted with PBS to 200 ml. The OD value of the resulting solution was detected at 570 nm. In control tests, enzyme extract was replaced with equal volume of PBS (pH 7.4, 0.02 M). The relative activity of total protease was calculated as described for total carbohydrase.

2.5.3. Total lipase/esterase activity

For each test, 50 µl of the crude enzyme extract from above was added to a 0.5 ml Eppendorf tube with 200 µl of 0.3 mM α-naphthylacetate (α-NA, in 0.02 M pH 7.4 PBS) followed by incubation at 37 °C for 20 min. Then, 120 µl of the reaction mixture was pipetted into 120 µl of color developing agents (0.01 g fast blue B salt dye, 0.5 ml pH 7.4, 0.02 M PBS and 2.5 ml 10% SDS). The OD value at 600 nm was recorded 10 min later, and normalized for protein concentration to compare the relative activity of total lipase/esterase between the different treatment groups.

2.6. Statistical analysis

Basic statistical analyses were done using IBM SPSS v21.0 (SPSS Inc., Chicago, USA). The effect of temperature treatments on the metabolic rate and respiratory quotient (RQ, the ratio between CO_2 production and O_2 consumption), and digestive enzyme activity were analyzed using one-way ANOVA and Student Newman Keuls post hoc tests. The effect of temperatures and treatment durations on metabolite content was analyzed with two-way ANOVA. Variations in metabolites between the groups were evaluated by Student's T-test. Since most analyses revealed whole variation trends in group of metabolites, rather than variations in individual metabolites, no FDR corrections were performed.

Principal component analysis-discriminant analyses (PCA-DA) on whole larvae metabolomes were executed using MarkerView 1.2.1, with weighting and scaling type set as "Sqrt" and "Pareto," respectively. Graphs were drawn using Graphpad prism 5 or ggplot2, an R package (R Core Team, 2014; Wickham, 2009). Metabolic network was created on the Cytoscape_3.2.1 platform.

3. Results and discussion

3.1. Cold-induced metabolic shift in S. litura

At 27 °C, the optimum temperature for *S. litura* larval development, the CO_2 production and O_2 consumption rates were 6.7 ± 1.6 nmol/g/s and 7.8 ± 1.8 (mean ± SD) nmol/g/s, respectively, and RQ was 0.86 ± 0.12. Rising temperature up to 37 °C for 4 h increased the rates of CO_2 production and O_2 consumption that maintained the average RQ around 0.9 (Fig. 1A–C). When larvae were cooled to 4 °C or 15 °C from 27 °C for 4 h, the CO_2 production rate decreased more than the O_2 consumption rate, and this led to a decrease in RQ from the average 0.86 at 27 °C, to 0.67 at 15 °C and to 0.4 at 4 °C. In principle, oxidation of pure carbohydrate, protein and fat results in RQ values of 1.0, 0.8–0.9 and 0.7, respectively. Considering the deviation between measured values and estimated values, the decrease in RQ from 0.86 to 0.67 suggested that lipids are likely being used primarily as metabolic fuel in *S. litura* larvae exposed to 15 °C for 4 h. However, the average RQ of 0.4 at 4 °C could not be explained by shift of metabolic fuels. From the perspective of biochemical reactions, RQ values lower than 0.7 indicate robust partial oxygenation of the carbon, nitrogen and sulfur in metabolites, like gluconeogenesis and other reactions catalyzed by monooxygenases. Results from TLC indicated that cold treatment at 15 °C and 4 °C for 4 h up-regulated diacylglycerol (DAG) in the hemolymph of *S. litura* larvae (Fig. 1D), a typical phenomenon during lipid mobilization. Previously, it was reported that carbohydrates and lipids were preferentially consumed at 25 °C and 5 °C, respectively, in fasted *S. exigua* larvae (Kim and Song, 2000). Our results indicated that exposure to cold induced a metabolic shift from carbohydrate to lipid, a response that exists in both *S. litura* and *S. exigua* larvae.

3.2. Metabolomics in S. litura at 15°C and 4°C

The whole body metabolomes of *S. litura* larvae treated at 15 °C and 4 °C for 4 h and 48 h each were measured. The score plot of PCA-DA (Fig. 2) showed that larvae treated for 48 h at either temperature were separate from those treated only for 4 h along the first principle

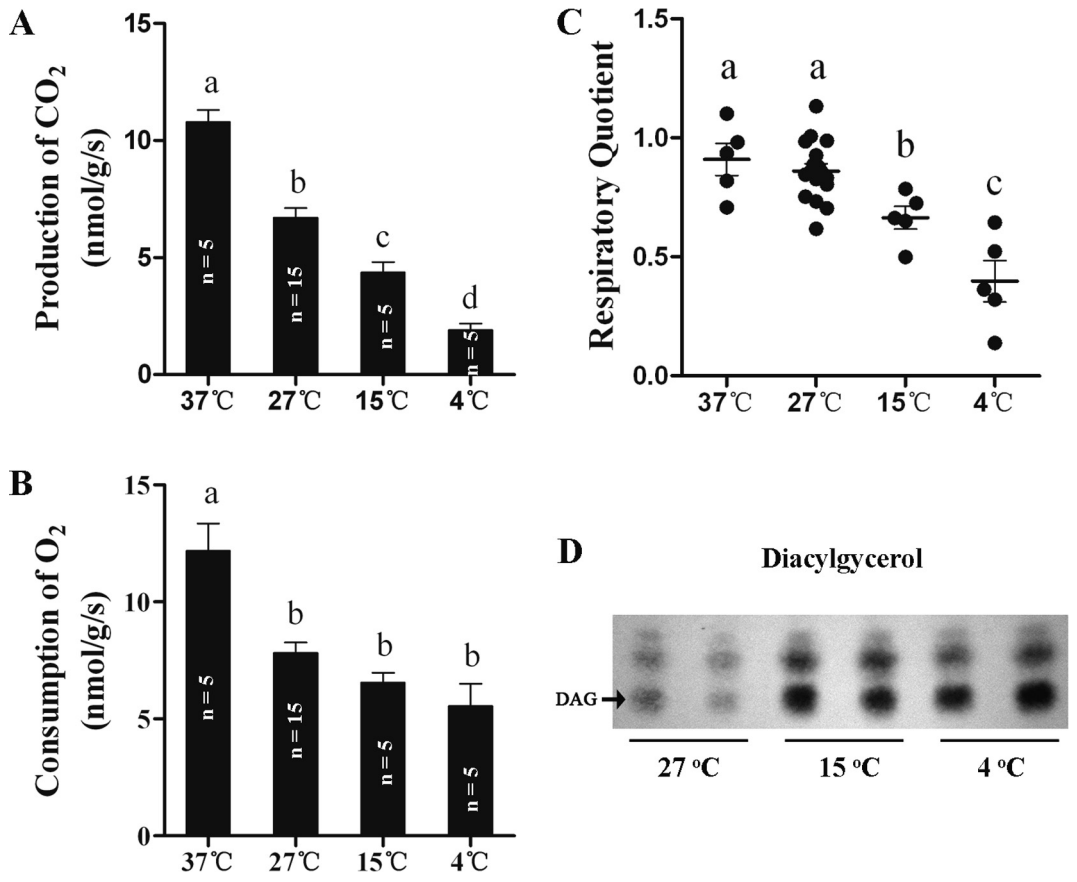

Fig. 1. Metabolic shift in *S. litura* exposed to low temperatures. The effect of temperature on CO_2 production rate (A), O_2 consumption rate (B) and respiration quotient (C) was tested by one-way ANOVA and Student Newman Keuls post hoc test. Different letters indicate significant difference at $p < 0.05$. Each column represents mean ± SE. (D) Effect of temperature on the DAG content in hemolymph measured by TLC. Larvae were acclimated for 4 h at the respective temperatures prior to determining respiration rate and collecting hemolymph for TLC analysis.

component, suggesting that the metabolic profiles drastically varied over time regardless of treatment temperatures. Besides, larvae treated for 48 h at 15 ˚C were separate from those treated at 4 ˚C along the second principle component. However, larvae treated for only 4 h were not segregated, suggesting the differential influence of the two different temperatures on the metabolome of *S. litura* larvae; clearly, effect of the temperatures was not obvious unless a long-term treatment was studied.

3.2.1. *Metabolism of lipids*

A total of 19 metabolites participating in lipid metabolism were identified. Among these, octadecanoic acid, hexadecanoic acid, 9,12-octadecadienoic acid, glycerol 3-monostearoylester, and glycerol 3-monopaimitylester showed the highest peaks in total ion chromatography (data not shown). More than half of these intermediates decreased over time at both 15 ˚C and 4 ˚C, including the five most abundant ones. In contrast, 3-hydroxybutyric acid, a ketone synthesized during robust

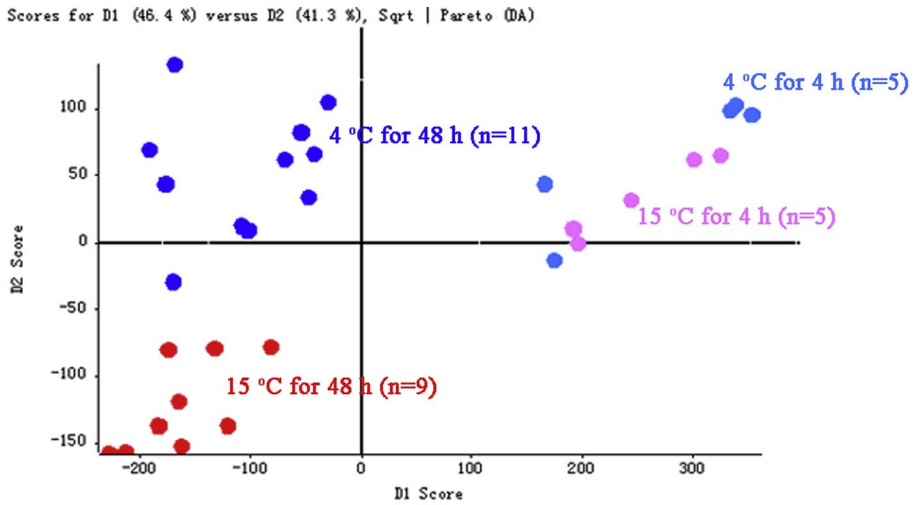

Fig. 2. Multivariate analysis (PCA-DA) of metabolomes from the different treatment groups. Each cycle represents a sample. Treatments: 4 ˚C for 4 h, cyan solid cycles (n = 5); 15 ˚C for 4 h, pink solid cycles (n = 5); 4 ˚C for 48 h, blue solid cycles (n = 11); 15 ˚C for 48 h, red solid cycles (n = 9).

beta-oxidation of fatty acids, was increased during cold treatment. These results suggested a constant consumption of lipids at both 15 °C and 4 °C. There was no major difference in the consumption rate of these lipid intermediates between larvae maintained at 15 °C and 4 °C, suggesting that the 48 h exposure to 4 °C did not change the metabolism pattern of lipids when compared to those exposed to 15 °C. Consistent with the results from respirometry, variation pattern of these metabolites supported a lipid based metabolic maintenance at 15 °C and 4 °C. In many overwintering insects, triacylglycerols are the major fuel for basal metabolism (Adedokun and Denlinger, 1985; Ohtsu et al., 1992, 1993). Metabolism based on lipids, instead of carbohydrates and amino acids, may bring several benefits to insects experiencing cold stress. First, most low molecular cryoprotectants are sugars, amino acids or their derivatives (Storey and Storey, 1991). More sugars and amino acids can be accumulated or used for the synthesis of other cryoprotectants, if lipids are mobilized to sustain the basic metabolism. Second, triacylglycerols with more unsaturated fatty acids are always preferentially mobilized (reviewed by Hahn and Denlinger, 2011). Such a preference likely contributes to the remodeling of membranes by providing more unsaturated fatty acids (Zhu et al., 2016). Interestingly, palmitelaidic acid, a 16-carbon monounsaturated fatty acid, is the only fatty acid that increased over treatment duration in our study (Fig. 3). Increased palmitelaidic acid was widely detected in membrane remodeling induced by diapause (Bashan and Cakmak, 2005) and cold-hardening (Kayukawa et al., 2007; Overgaard et al., 2006) in insects. The sole increment of this fatty acid during the cold treatment indicated that *S. litura* larvae might also implement similar cold-hardening strategies to protect from cold injury.

3.2.2. Metabolism of amino acids

A total of 28 amino acids were identified in *S. litura* larvae. Most of them, including all essential amino acids detected, decreased over treatment duration at both 15 °C and 4 °C (Fig. 4A). Free amino acids may be consumed through three major routes: energy metabolism, gluconeogenesis (Thompson, 1997), and synthesis of cellular components (i.e. protein). As nitrogen detoxication in insects expend ATP (Bursell, 1981), not all amino acids are eligible metabolic fuels. Based on their solubility, convenience in degradation and net ATP yield, proline is the most beneficial amino fuel in insects (Bursell, 1981). However, its' consumption rate was much lower than those with shorter chain, branched chain or benzene ring (Fig. 4A), suggesting that energy metabolism was not a likely factor to cause the overall reduction in amino acids. Gluconeogenesis could explain the decrease in several amino acids, including serine, valine and aspartate. However, this could not have led to the consumption of lysine, leucine and aromatic amino acids, which are not glucogenic amino acids (Thompson, 2000). Besides, gluconeogenesis could not account for the decrease in low abundance-amino acids, such as cysteine, methionine and homoserine, as they were less likely preferred for glucogenesis compared to the abundant amino acids, such as glycine and 5-oxo-proline (pyroglutamic acid) the levels of which did not decrease. Therefore, synthesis of cellular components was likely a major factor that caused the down-regulated trends in amino acids particularly those with low abundance and those that are insulated from glucogenesis. Moreover, why various amino acids decreased to varying degrees could be explained by the differences in their requirement. The increased need for glutamate and alanine could have been provided by the robust transamination between amino acids.

The influence of temperature on the metabolism of amino acids was smaller than treatment duration. Although several amino acids, typically essential ones, kept in significantly higher content at 4 °C than at 15 °C after long-term treatment, most of them were those consumed

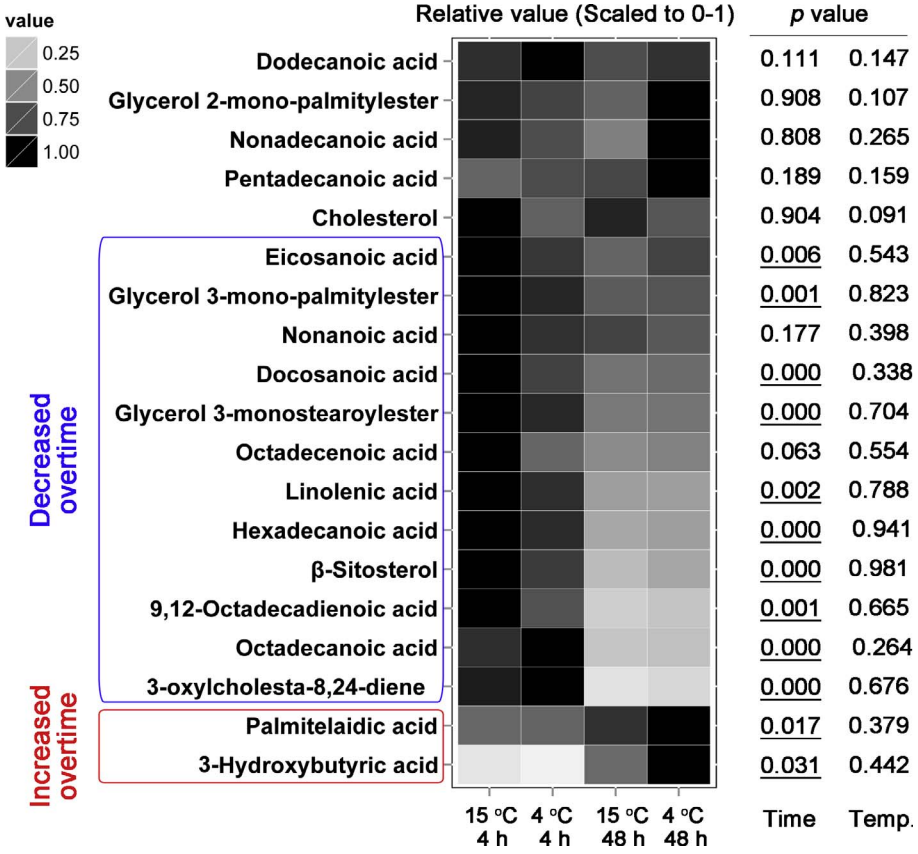

Fig. 3. Relative content of intermediates in lipid metabolism. For each metabolite, the average values of individual groups are represented as shades indicating values on a scale from 0 to 1 by dividing the maximal average value of four groups. Effect of temperature and treatment duration was tested by two-way ANOVA. Statistical significance is indicated by underlined *p* values.

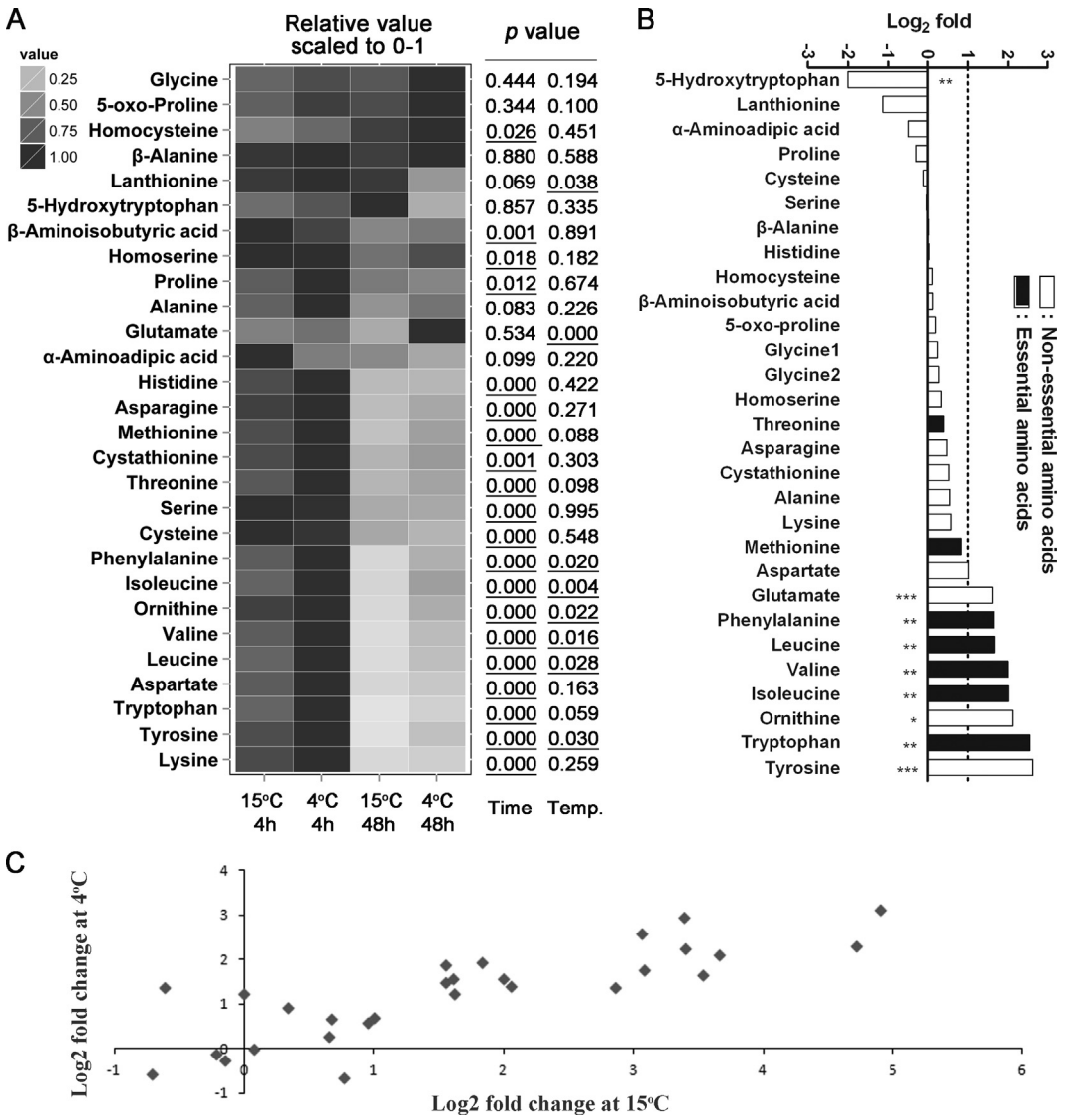

Fig. 4. Amino acids metabolism in *S. litura* at 15 °C and 4 °C. (A) Tile plot represents the average values of each metabolite in each group. Values of each metabolite were ranked based on a scale from 0 to 1 by dividing its maximal average value among treatment groups. Effect of temperature and treatment duration was tested by two-way ANOVA. Statistical significance is indicated by underlined *p* values. (B) Fold difference (log_2 transformed) in the abundance of metabolites between larvae exposed to 15 °C and 4 °C for 48 h. Positive values indicate abundance, and negative values indicate down-regulation of metabolites in larvae at 4 °C. Asterisks indicate significant differences between the two groups (*t*-test). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. (C) Scatter plot showing the relationship between fold changes (log_2 transformed) in metabolites after treatment at 15 °C and 4 °C, and positive values indicate decrease over treatment duration, and negative values represent increase.

drastically at both 15 and 4 °C (Fig. 4A–B). We further calculated the fold changes of amino acids between larvae treated short-term and long-term at 15 °C and 4 °C, and found an obvious correlation (Fig. 4C). These results suggested that the overall metabolic pattern of amino acids was quite similar in larvae treated at 15 °C and 4 °C, and exposure to 4 °C could not effectively suppress anabolism in *S. litura* larvae, even though their feeding activity was lost. This explains why a daily cumulative temperature higher than 1 °C and food supply are necessary for the development of *S. litura* larvae to sustain long-term survival. In some species, amino acids are accumulated as cryoprotectants by the synthesis or breakdown of proteins during cold exposure (Koštál et al., 2011a,b; Lalouette et al., 2007; Michaud et al., 2008; Michaud and Denlinger, 2007; Nieminen et al., 2012). The variation pattern of amino acids in this study ruled out similar cold adaptive mechanisms in *S. litura* larvae. However, it could not rule out the possibility that the synthesis of certain protective proteins, like heat shock proteins (Colinet et al., 2007; Qin et al., 2005; Sinclair et al., 2007), could be induced by cold exposure in these larvae. Notably, synthesis of these proteins are more likely induced by fluctuating temperature profiles, especially the heating-up period (Colinet et al., 2007; Lalouette et al., 2007; Qin et al., 2005; Sinclair et al., 2007; Teets et al., 2012). Therefore, potential cold-adaptive mechanisms at the protein level should be investigated in *S. litura* larvae in future work.

3.2.3. Metabolism of carbohydrates

A total of 26 sugars and their derivatives were identified in *S. litura* larvae. Based on their locations in the metabolic network, they were divided into two categories: metabolic intermediates and sugars/polys, which are carbohydrate reserves and functional products (i.e., trehalose, 3-phosphoglycerol, inositol and glucose), respectively.

After long-term treatment at 15 °C, larvae showed an overall decrease in sugars, polyols and even metabolic intermediates (Fig. 5A–C). This was not likely caused by suppressed utilization and promoted storage of carbohydrates, as the increase in trehalose at 15 °C was not significant (Fig. 5B, C). In fasted larvae, it is also unlikely that trehalose was transformed into glycogen. Therefore, carbohydrates were more likely consumed rapidly at 15 °C within 48 h either through biosynthesis based on carbohydrates, or through metabolic mainte-

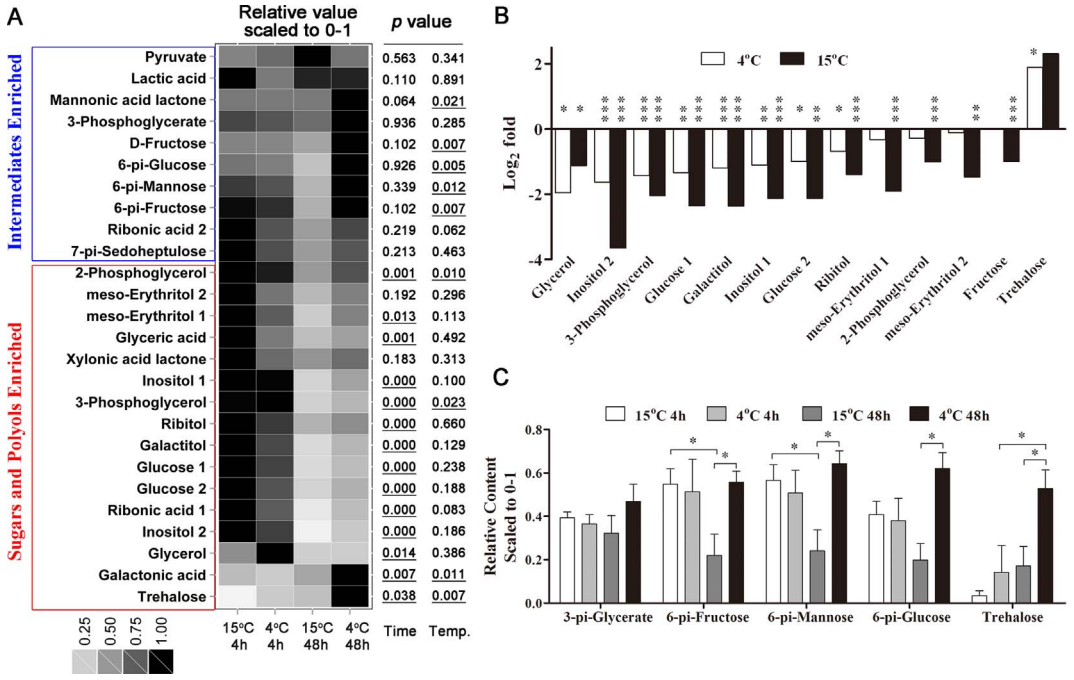

Fig. 5. Carbohydrate metabolism in *S. litura* exposed to 15 °C and 4 °C. (A) Tile plot represents the average values of each metabolite in each group. Values for the same metabolite were scaled from 0 to 1 by dividing its maximal average value among treatment groups. Effect of temperature and treatment duration was tested by two-way ANOVA. Statistical significance is indicated by underlined *p* values. (B) Bar chart shows changes in the abundance of sugars/polyols with treatment duration at 15 °C and 4 °C. Positive values indicate increase and negative values represent decrease over treatment duration. (C) Bar chart shows the relative contents of key intermediates and trehalose among the four treatment groups. For each metabolite, the content in each sample was measured on a scale from 0 to 1 by dividing its maximal value among samples. Each column represents mean ± SE of scaled values. Asterisks indicate significant differences between different treatment durations (*t*-test). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

nance taken over from lipids.

The variation patterns of sugars and their derivatives were quite different between 15 °C and 4 °C (Fig. 5A, C). Although some sugars and polyols showed down-regulated trends after long-term treatment at 4 °C, the degree was much lower (Fig. 5B). Long-term treatment did not lead to a decrease in phosphate hexoses (Fig. 5C), which are obligatory intermediates in almost all glycometabolic pathways, suggesting that flux was maintained throughout certain glycometabolic pathways. More importantly, exposure to 4 °C significantly increased trehalose (Fig. 5C). This might be a cold adaptive strategy of *S. litura* larvae, as high concentrations of trehalose could confer cold tolerance to insects (Jain and Roy, 2009; Storey and Storey, 1991; Thompson, 2003). Previously, it was reported that cold acclimated *S. litura* larvae had higher osmotic pressure in their hemolymph and performed better at lethal temperatures than control larvae (Kim et al., 1997), suggesting that this species could also achieve rapid cold hardening. Because trehalose was the only metabolite with cryoprotective functions to be accumulated in *S. litura* larvae treated at 4 °C (Fig. 4A & Fig. 5A), it is likely that trehalose accumulation plays a key role in cold hardening and regulates the higher osmotic pressure in cold acclimated larvae.

3.3. Metabolic comparison between S. litura and S. exigua larvae

To obtain insight into the mechanisms underlying the different cold tolerance between *S. litura* and *S. exigua* larvae, their metabolomes were analyzed using PCA-DA (Fig. 6A). The first principal component clearly divided *S. litura* (positive axis) and *S. exigua* (negative axis) samples, with trehalose and serine having the largest negative loading scores. Compared with *S. litura* larvae, high abundance of trehalose and serine was the major metabolic feature in *S. exigua* larvae, an overwintering species (Fig. 6B). The contribution of a single or more than one metabolite to the cold tolerance of insects has been well-proven by artificial introduction of these metabolites into organisms. For example, the survival rate of *Plutella xylostella* larvae after cold shock at −10 °C for 1 h was improved by the injection of glycerol (Park and Kim, 2014). Similarly, the performance of *Pyrrhocoris apterus* at −14 °C was improved by injecting a mixture of sorbitol and ribitol (Koštál et al., 2001). Like polyols, trehalose, an efficient cryoprotectant accumulated in insects, has a series of cold protective functions, including anti-freezing, equilibrating osmotic pressure, and stabilizing membrane structures (Jain and Roy, 2009; Overgaard et al., 2007; Storey and Storey, 1991; Thompson, 2003). Although cold treatment at 4 °C for 48 h induced significant accumulation of trehalose in *S. litura* larvae, its abundance was still much lower than in *S. exigua* larvae treated in a similar manner (Fig. 6B). Similar cases have been reported in *Drosophila* species (*D. birchii*, *D. equinoxialis*, *D. melanogaster*, *D. persimilis* and *D. montana*) with different cold tolerance. Higher abundance of trehalose and proline account for a major metabolic characteristic in chill tolerant species compared to cold susceptible ones (Olsson et al., 2016). Serine, an amino acid that is multifunctional in cellular processes, was another metabolite significantly higher in *S. exigua* than in *S. litura* larvae. Serine is one of the most abundant amino acids in both *S. litura* and *S. exigua* and contributes greater to the metabolic pools than most other amino acids, as revealed by the total ion chromatograms (data not shown). Despite its abundance, serine is rarely reported as an effective low molecular cryoprotectant in insects. It is rather well-known as a critical precursor for the synthesis of carbohydrates, nucleotides, phospholipids, vitamins and other amino acids (Nelson and Cox, 2012). In *S. litura* larvae, serine was rapidly consumed at both 15 °C and 4 °C (Fig. 4A), suggesting its essential role in metabolic maintenance. Although it was not clear whether serine could directly contribute to cold protection, high reserves of this multifunctional and dominant amino acid is expected to sustain long-term metabolism.

Interestingly, the enzyme activity in the guts of *S. litura* larvae showed that cold treatment at 4 °C for 4 h up-regulated total carbohydrase and protease, and down-regulated total esterase/lipase (Fig. 6C). This suggested that carbohydrates and amino acids were preferred at 4 °C, an intentional adjustment adopted by *S. litura* larvae in response to cold. Therefore, it can be speculated that daily temperature fluctuations

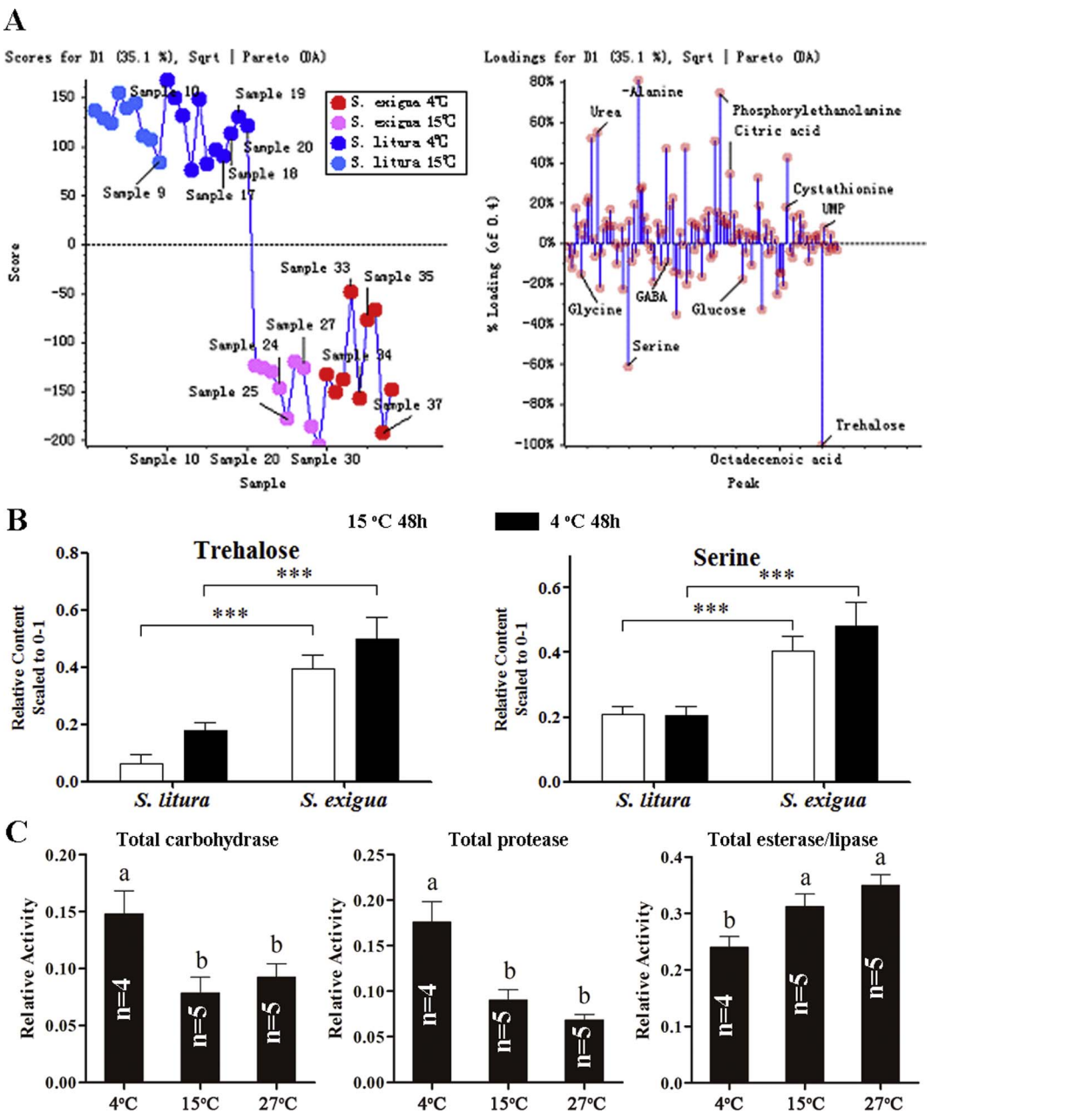

Fig. 6. Analysis of cold hardening ability in *S. litura* larvae. Multivariate analysis of metabolomes in *S. litura* and *S. exigua* larvae. (A) Score plot and loading plot of PCA-DA. Each cycle represents a sample: *S. litura* at 4 °C for 48 h, blue solid cycles (n = 11); *S. litura* at 15 °C for 48 h, cyan solid cycles (n = 9); *S. exigua* at 4 °C for 48 h, red solid cycles (n = 9); *S. exigua* at 15 °C for 48 h, pink solid cycles (n = 9). (B) Bar charts show the relative abundance of trehalose and serine in *S. litura* and *S. exigua* larvae. For each metabolite, the content in each sample was measured on a scale from 0 to 1 by dividing its maximal value among samples. Asterisks indicate significant differences between different treatment durations (*t*-test). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. (C) Relative activity of digestive enzymes in the guts of *S. litura* larvae exposed to the indicated temperatures for 4 h, and different letters indicate significant difference at $p < 0.05$, tested by one-way ANOVA and Student Newman Keuls post hoc test. Each column represents a mean ± SE.

between 4–15 °C likely enhance the capacity of *S. litura* larvae in absorbing carbohydrate and amino acids if food is available and this would likely benefit the accumulation of trehalose and enrich the reserve amino acids, thus improving their cold tolerance in a manner resembling metabolic compensation (Cossins and Bowler, 1987). The benefits of fluctuating temperatures/repeated cold stress, compared to a single sustained bout with the same total cold exposure length, on cold tolerance of insects has long been studied (Chen and Denlinger, 1992; Sinclair and Marshall, 2010; Teets et al., 2011). Besides inducing the synthesis of heat shock proteins, the high temperature pulses of fluctuating temperature profiles could also repair the oxidative damage (Lalouette et al., 2011), reestablish the disrupted ion gradients across cell membranes (Koštál et al., 2007), regenerate exhausted energy reserves (Dollo et al., 2010) and rebuild the disrupted energy homeostasis (Colinet et al., 2007; Colinet, 2011). Our study on *S. litura* larvae implies that fluctuating temperatures may also facilitate overwintering of insects by accelerating the accumulation of required resources. It should be noted that the rates of most enzymatic processes tend to increase exponentially as functions of temperature within certain temperature ranges. As a result, the accumulated efficiency of these processes is higher under fluctuating temperature regimes than under fixed average temperatures, as indicated by Jensen's inequality (Ruel and Ayres, 1999). Accordingly, in natural conditions where daily temperature fluctuations are common, cold protective processes, i.e., damage repair, cryoprotectant accumulation and resource exploitation, may function more efficiently in insects than anticipated at average

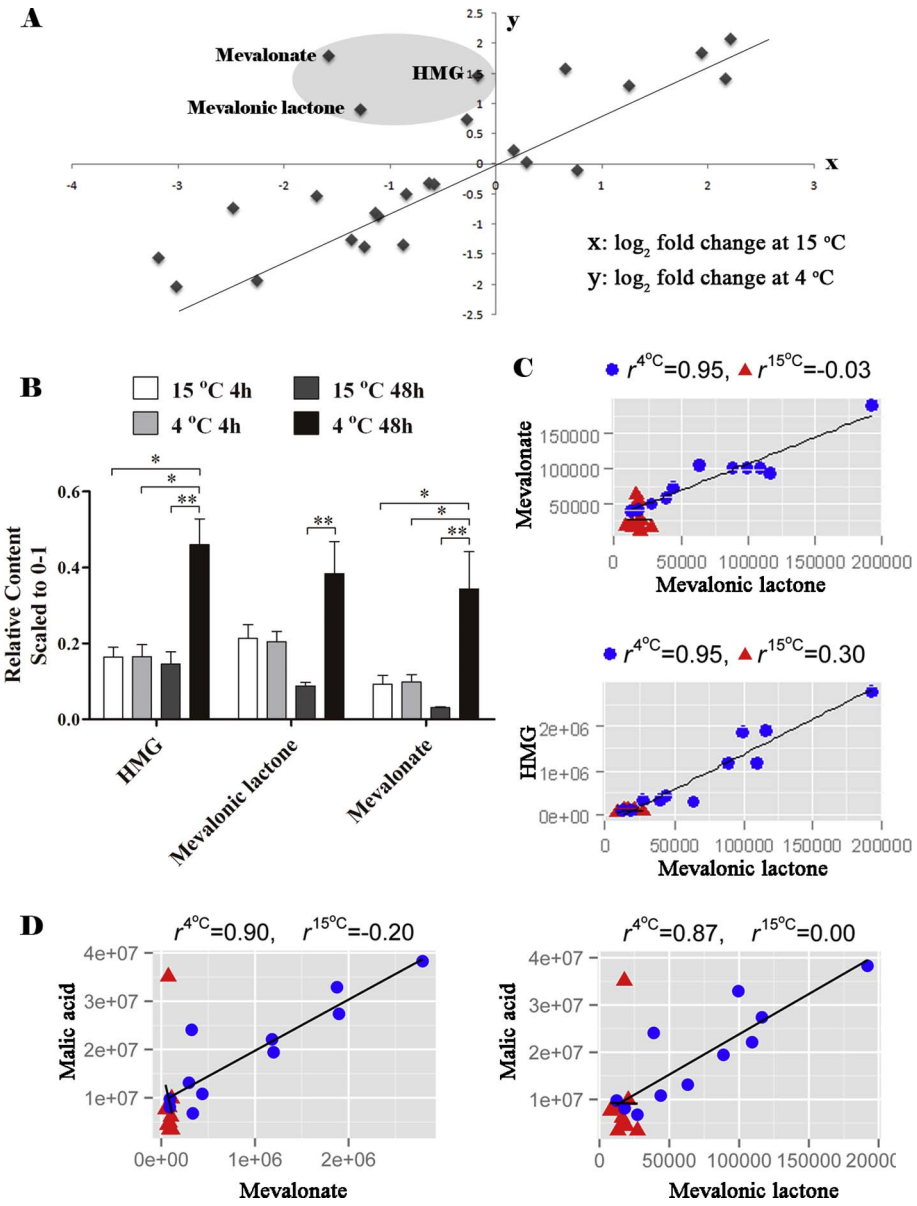

Fig. 7. Influence of cold exposure on organic acids in *S. litura* larvae. (A) Scatter plot shows the relationship between fold changes (log_2 transformed) in metabolites at 15 °C and 4 °C. Positive values indicate increase over treatment duration, and negative values indicate decrease. (B) Bar chart shows relative content of HMG, mevalonic acid and mevalonate among the four treatment groups. For each metabolite, its content in each sample was quantified on a scale from 0 to 1 by dividing its maximal value among samples. Each column represents mean ± SE of scaled values. Asterisks indicate significant differences between different treatment durations (*t*-test). $^{*}p < 0.05$; $^{**}p < 0.01$. Correlations between metabolites are also shown (C, D).

temperatures.

Our study has thus revealed evidence at the molecular level for the possibility of overwintering by *S. litura* larvae in the field and experimental conditions, and suggested that feeding activity was not only necessary for metabolic maintenance, but also played an important role in adopting cold hardening strategies by *S. litura* larvae. More importantly, these results implied that autumn and winter vegetables rich in sugars and serine more likely facilitate the survival of *S. litura* larvae in winter. This speculation was, to some extent, supported by interesting research on *D. melanogaster* (Koštál et al., 2012), in which proline-augmented diet improved the proline abundance in *D. melanogaster* larvae and thus helped convert this chill susceptible species to a freeze tolerant organism. Therefore, our results suggest that more attention should be given to the interactive effects of agriculture facilities, temperature and vegetables when considering the future of pest monitoring and control.

3.4. Potential metabolic regulation in *S. litura* larvae at 4°C

Trends in the variation of organic acids were similar at 15 °C and 4 °C, except for 3-hydroxy-3-methylglutarate (HMG), mevalonic lactone and mevalonate (Fig. 7A), all of which are involved in the mevalonate pathway. Mevalonate and its lactone form, mevalonic lactone, are produced from HMG-CoA by HMG-CoA reductase. In our study, these three metabolites showed significant increase and correlation with each other after long-term exposure to 4 °C (Fig. 7B, C), suggesting that mevalonate pathway and particularly the activity of HMG-CoA reductase, was robust. The products of mevalonate pathway have been widely reported to facilitate cold tolerance in organisms, for example, cholesterol in fish (Irvine et al., 1957) and insects (Shreve et al., 2007), abscisic acid and isoprenoids in plants (Ayub et al., 2010; Swamy and Smith, 1999), and isoprenoids in yeast (Rodriguez-Vargas et al., 2002) and archaea (Nichols et al., 2004). As insect do not synthesize cholesterol *de novo*, increase in cholesterol synthesis and the subsequent

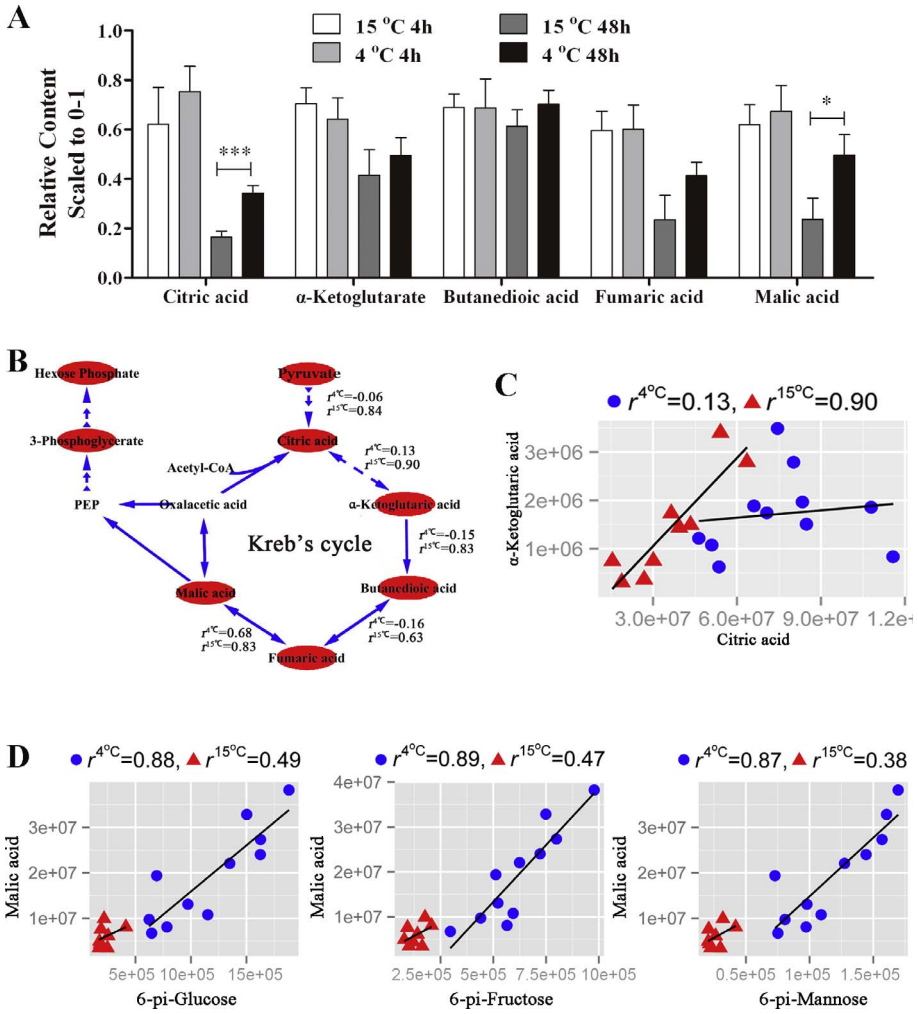

Fig. 8. Potential gluconeogenesis in *S. litura* larvae. (A) Bar chart shows the relative content of intermediates in the Kreb's cycle among the four treatment groups. For each metabolite, its content in each sample was measured based on a scale from 0 to 1 by dividing its maximal value among samples. Each column represents mean ± SE of scaled values. Asterisks indicate significant differences between groups (*t*-test). $^{*}p < 0.05$; $^{***}p < 0.001$. (B) Diagram indicating the metabolic flux throughout Kreb's cycle and gluconeogenesis. Circled metabolites were identified by metabolic profiling, and vice versa. Solid arrows between metabolites represent one-step reactions, while dashed arrows represent multiple-step reactions. Correlations between metabolites are also shown (C, D).

benefits to cold tolerance could not be expected from the robust mevalonate pathway in *S. litura* larvae. However, in insects the unique sesquiterpenoid hormones (juvenile hormones, JHs) are synthesized from mevalonate, and they have been widely reported to participate in cold tolerance by regulating the synthesis of anti-freeze proteins and polyols (Duman, 2001; Hamilton et al., 1984, 1986; Rojas et al., 1987; Xu and Duman, 1991). Moreover, parallelisms exist between the profiles of HMG-CoA reductase activity and the JH production during insect development. Addition of precursors such as mevalonate, farnesol or farnesoic acid increased JH production in the corpus allatum while the use of HMG-CoA reductase inhibitors such as compactin or mevilonin decreased it (Noriega, 2014). Therefore, increased synthesis of JH could be expected in *S. litura* larvae treated at 4 °C for 48 h, and might play a role in cold adaptation.

In *S. litura* larvae treated long-term at 4 °C, we found that close correlations existed between mevalonate/mevalonic lactone and malic acid (Fig. 7D), an intermediate in Kreb's cycle, and phosphate hexoses (data not shown). A total of 5 Kreb's cycle intermediates were identified in *S. litura* larvae. After long-term treatment, larvae at 4 °C had higher concentrations of citric acid and malic acid than larvae treated at 15 °C, while the other three metabolites showed no significant difference (Fig. 8A). This variation pattern might be better explained by the reorganization of metabolic fluxes. In larvae treated at 15 °C, citric acid was highly correlative to pyruvate, and intermediates of the Kreb's cycle also maintained strong correlation with each other (Fig. 8B, C), suggesting robust and dominant metabolic flux throughout the Kreb's cycle. However, corresponding correlations did not exist at 4 °C, suggesting the lack of robust Kreb's cycle at the lower temperature. Instead, these larvae maintained a strong correlation between malic acid and phosphate hexoses (Fig. 8D) that may not likely resulted from metabolic flux throughout glycolysis, pyruvate dehydrogenase and Kreb's cycle according to the correlation analysis. Given that malic acid is also involved in gluconeogenesis, these metabolic correlations might be resulted from gluconeogenesis in larvae at 4 °C (Fig. 8B), which was consistent with the accumulation of trehalose and could explain the low RQ value < 0.7 and the persistently high levels of phosphate hexoses in these larvae. Gluconeogenesis, in response to low temperature, has been observed in plants (Sasaki et al., 1998), frogs (Costanzo et al., 1993) and also in other insects (Teets et al., 2012), and it could be a likely cold adaptive adjustment in species accumulating carbohydrates as cryoprotectants. Thus, it appears that gluconeogenesis may have likely contributed to the increased trehalose level in *S. litura* larvae at 4 °C and therefore this could be a cold adaptive strategy in this species. However, its direct contribution to trehalose accumulation is unknown. Amino acids are common substrates for gluconeogenesis in most animals. As discussed above, anabolism based on amino acids was maintained in *S. litura* larvae at 4 °C, which would likely restrict available substrates for gluconeogenesis. This further emphasized the

necessity of food supply for overwintering *S. litura*.

The metabolic and/or regulatory mechanisms that account for the correlations between the intermediates in mevalonate pathway and gluconeogenesis are unknown. It is likely that the relationship between mevalonate pathway and JH synthesis could regulate this mechanism. However, whether JH synthesis was promoted at 4 °C was inconclusive in our study and quantitative data of JH is essential for further insight. Regulation of the mevalonate pathway can be considered as a cold adaptive mechanism in *S. litura* larvae only when JH is proven to benefit trehalose accumulation and cold tolerance. Finally, the cold-induced synthesis of mevalonate may promote future research on cold adaptive metabolic regulation in *S. litura* larvae.

4. Conclusions

The regulation strategies of lipids and amino acids metabolism were similar in *S. litura* larvae exposed to 15 °C and 4 °C. Cold exposure induced an energy metabolic shift from carbohydrate to lipid and decreased free amino acids levels. Free amino acids were most likely consumed as substrate for biosynthesis, suggesting no sufficient suppression of anabolism induced at 4 °C, a temperature at which larvae lost their feeding activity. This could explain why a daily cumulative temperature higher than 1 °C and food supply are necessary for the development of *S. litura* larvae to sustain long-term survival. Glycometabolism was quite different between 15 °C and 4 °C. Carbohydrates were consumed rapidly at 15 °C, with a general decrease in monose, polyols and intermediates after treatment for 48 h. In contrast, exposure to 4 °C induced a significant accumulation of trehalose after 48 h, and the content of key intermediates in glycometabolism did not decrease over treatment. A comparative analysis found that differences in the abundance of trehalose and serine might underpin the difference in cold tolerance between *S. litura* and *S. exigua*. Cold exposure at 4 °C induced up-regulation of total carbohydrase and protease in the guts of *S. litura* larvae. This is expected to facilitate the accumulation of carbohydrates and amino acids. Besides, correlative analysis implied that gluconeogenesis could be induced in larvae at 4 °C. Taking these into consideration, it is likely that temperature fluctuations between 4 °C to 15 °C could enhance the cold hardening of *S. litura* and improve their possibility to overwinter in the fields and/or experimental conditions if food was available. Interestingly, we found that *S. litura* larvae exposed to 4 °C showed increased mevalonate pathway activity that was likely related to gluconeogenesis, and thus implying potential metabolic regulation might be involved during cold exposure. Overall, our study revealed the conditions necessary for overwintering of *S. litura* larvae at the metabolic level, which could be used to predict the influence of global warming and agricultural reforms on the breakouts of *S. litura*. Also, our study systematically described the metabolic variations in the cold response of an insect species with limited cold tolerance, which could be a meaningful contrast to investigating the mechanisms underlying cold adaptive strategies in insects.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2017.05.008.

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