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# Proteomic research on diapause-related proteins in the female ladybird, *Coccinella septempunctata* L.

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## Abstract

In the experiments reported here, we used the female ladybird *Coccinella septempunctata* L. as a model to identify diapause-associated proteins using proteomics technology. Our results indicated that protein expression patterns of diapausing and nondiapausing individuals were highly differentiated. A total of 58 spots showed significant differences in abundance (Ratio > 2 and $P < 0.05$) according to two-dimensional electrophoresis and GE Image Scanner III analysis. Sixteen protein spots were further investigated using mass spectrometry. Eight proteins were characterized, including chaperones and proteins involved in glucose metabolism, lipid metabolism, and the tricarboxylic acid cycle. Among these proteins, five proteins were upregulated in diapausing female adults, including a chaperone (Symbionin symL), malate dehydrogenase (putative), two proteins linked to lipid metabolism (unknown and conserved hypothetical protein) and phosphoglyceromutase (partial). By contrast, isocitrate dehydrogenase (RH49423p), fumarylacetoacetate hydrolase (AGAP001942-PA), and a putative medium chain acyl-CoA dehydrogenase were downregulated. These results contribute to the understanding of diapause mechanisms of the ladybird *C. septempunctata* and may suggest methods for improving the application of this natural enemy insect.

**Keywords:** diapause associated proteins, *Coccinella septempunctata*, female adults, two-dimensional electrophoresis

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## Introduction

Aphids are economical pests that, via asexual reproduction, can reach large numbers in a short time and can transmit viruses, causing significant crop loss (Jaouannet *et al.*, 2014; Schwessinger *et al.*, 2015). With insecticide use, aphids have developed multiple resistance mechanisms to insecticides, making these pests difficult to control (Han *et al.*, 1998; Puinean *et al.*, 2010; Jaouannet *et al.*, 2014). Therefore, biological control of aphids has become urgent.

The widely distributed seven-spotted ladybird, *Coccinella septempunctata* L., is one of the important natural enemies in farmland ecosystem, preying on several species of aphids, including *Aphis glycines, Aphis gossypii*, and *Lipaphis erysimi*. *C. septempunctata* has colonized most states (Sakurai *et al.*, 1983; Hodek & Michaud, 2008; Simelane *et al.*, 2008). Since 1980, it has been used as a commodity and has been mass-cultured in Europe (van Lenteren, 2012). As a special commodity, storage method (van Lanteren, 1988) is one of the key factors for augmentative rearing, and diapause may offer an effective method to overcome this problem. Photoperiod and temperature are the main factors in regulating diapause (Tauber & Tauber, 1973, 1976; Cho *et al.*, 2008). With the combination of

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short day-length and low temperature, *C. septempunctata* can enter winter diapause (Wang *et al*., 2013; Zhang, 2014), substantially extending its longevity. The most significant features of diapausing individuals are halted ovarian development, accompanied by transparent ovarian tubules and the accumulations of hypertrophic fat bodies in the abdomen. The content of total lipid in diapausing adults is more than twice that in nondiapausing individuals, and levels of both glycogen and total sugars are much higher in the diapausing group. In addition, the supercooling point in the diapause group is lower than in nondiapausing adults. The changes in these biochemical substances could ensure an energy supply for diapause development and thus guarantee *C. septempunctata* survival in a hostile environment. This ladybeetle has two important advantages for successful regulation: long-distance shipping and long-term storage, providing benefits for manufacturing.

Diapause is a dynamic developmental stage (Tauber & Tauber, 1976; Denlinger, 2002; Koštál, 2006). In accordance with the special needs of diapause, intracorporal indicators will change markedly, including in physiology, biochemistry, gene expression, and protein expression (Hahn & Denlinger, 2007; Rinehart *et al*., 2007). Adult Colorado potato beetles, *Leptinotarsa decemlineata*, will store lipids in fat bodies for energy needs during diapause and accumulate proteins known as 'storage proteins' (De Kort, 1990), which can preserve amino acids (Hahn & Denlinger, 2007). In diapausing flesh flies, *Sarcophaga crassipalpis*, the expression patterns of heat shock proteins (HSPs) show changes: Hsp70, Hsp23, and SmHsp are upregulated, and Hsp90 is downregulated (Li *et al*., 2007; Rinehart *et al*., 2007) Further research using RNA interference (RNAi) demonstrated that Hsp70 and Hsp23 can improve the ability of organisms to survive in low temperatures (Rinehart *et al*., 2007).

In this paper, we used proteomic technologies, including two-dimensional gel electrophoresis (2DE) and electrospray ionization (ESI), quadrupole (QUAD), and time-of-flight (TOF) mass spectrometry, to search for diapause-associated proteins, with the aim of providing background information regarding *C. septempunctata* diapause mechanisms.

## Materials and methods

### *Insect rearing*

The *C. septempunctata* were captured in wheat fields at the Chinese Academy of Agricultural Sciences, and reared on fresh *Aphis glycines* Matsumura every day (Wang, 2012).

For culturing nondiapause adults, eggs, larvae, nondiapause pupae, and nondiapause adults were cultured under normal developmental conditions, with a photoperiod of 16L:8D and normal temperature (24 ± 1)°C and humidity (70 ± 10)%.

For culturing diapause adults, when the fourth instar larvae pupated, the pupae were moved into diapause conditions with a short day-length (14L:10D), low temperature (18 ± 1)°C and normal humidity (70 ± 10)%.

For nondiapause groups, we chose 4-day-old female adults. For diapause groups, after adults had been under diapause conditions for 40 days, we dissected females and observed their ovaries and fat bodies. If the ovarioles were empty and fat bodies developed well, we considered that they were in diapause. Developmental conditions of nondiapause adults are equivalent to diapause individuals. We selected samples of nondiapause and diapause females, and those samples were then washed with phosphate buffer (pH 7.4), dried with filter paper and frozen with liquid nitrogen.

### *Protein extraction*

The method of total protein extraction was based on Wang (Wang *et al*., 2006) with some improvements. Fifteen nondiapause/diapause female adults were weighed and put into a glass homogenizer filled with phosphate buffer (pH 7.4). Adults were fully homogenized for 10 min on ice and sonicated 5 times for 3 s at 10-second intervals. The glass homogenizers were kept on the ice for 10 min and vibrated every 5 min. The supernatant was transferred to a 2.0 ml centrifugal tube and centrifuged at 4°C and 15,000 rpm for 20 min. The supernatant was carefully transferred to another 2.0 ml tube, avoiding the fat layer, and was centrifuged at 4°C and 15,000 rpm for 30 min. The supernatant was again transferred to new 2.0 ml tubes, and 100% tricarboxylic acid (TCA) was added to the supernatant, slowly adjusting the final TCA content to 10%. The tubes were kept on −20°C for 5 min and then moved to ice for 15 min. Samples were centrifuged at 4°C and 15,000 rpm for 10 min, and the supernatant was discarded. The precipitation was washed twice with −20°C pre-cooled acetone and then centrifuged at 15,000 rpm for 10 min (4°C). The precipitation was air-dried at room temperature. A 1:1 (v:v) mixture of Phenol: Tris was added to suspend the precipitation (add 0.4–0.8 ml mixture per 0.1 g starting sample), was mixed well and incubated. The sample was then centrifuged for 10 min at 15,000 rpm (4°C). The phenol layer was moved to new 2.0 ml tubes and centrifuged once more. Ammonium acetate methanol (prepared immediately before use) was added to the phenol layer in a 5:1 ratio, mixed thoroughly and stored at −20°C overnight, and a white precipitate appeared the next day. Samples were centrifuged 15,000 rpm for 20 min (4°C). The supernatant was discarded, and the precipitate was washed with −20°C pre-cooled methanol and then washed twice with 80% acetone (pre-cooled in −20°C). The pellet was air-dried at room temperature and then resuspended in lysis buffer (7 M urea, 2 M thiourea, 4% 3-(3-(cholamidopropyl) dimethylammonio) propanesulfonate (CHAPS), 65 mM dithiothreitol (DTT), 2% Pharmalyte 3–10) and stored at −80°C.

Protein quantitation was achieved using a 2D-Quant Kit made by GE Healthcare production.

### *Two-dimensional gel electrophoresis*

The samples were passively hydrated for 14 h. For the rehydration of samples, 1000 μg of protein was mixed with rehydration buffer [7 M urea, 2 M thiourea, 2% CHAPS (w/v), 15 mM DTT, 0.5% immobilized pH gradient (IPG) Buffer pH 3-10 L (v/v)] to a final volume of 420 μl and was applied to 24 cm IPG strip (GE), pH3-10. Focusing protocols were as follows: 100 V for 1 h, 500 V for 4–6 h, 1000 V for 1–2 h, 1000 V for 3–4 h, 10,000 V for 95,000 V·hr. The applied current was no more than 50 μA per strip.

After the first dimensional electrophoresis finished, the strips were equilibrated for 15 min in equilibration buffer I [6 M urea, 75 mM Tris-HCl, 29.3% glycerol (v/v), 2% SDS (w/v), 1% bromophenol blue (v/v), 1% DTT (w/v)]. Then, the strips were moved to equilibration buffer II [6 M urea, 75 mM Tris-HCl, 29.3% glycerol (v/v), 2% SDS (w/v), 1% bromophenol blue (v/v), 2.5% iodoethanamide (IAA) (w/v)] for 15 min. The second dimensional electrophoresis was

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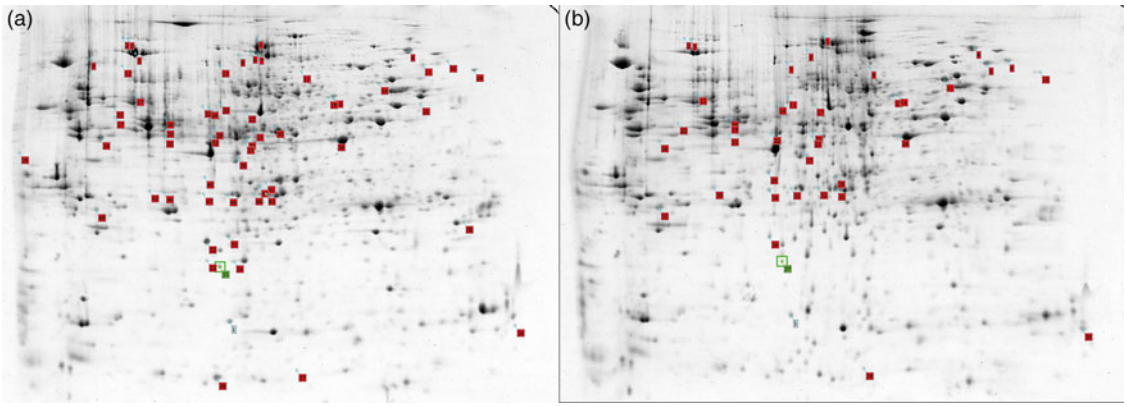


Fig. 1. Two-dimensional gel electrophoresis map of total proteins from adult female *C. septempunctata* L. Proteins were separated by first dimension isoelectric focusing (IEF) (pH3–10) and second dimension SDS-PAGE. Then, 58 individual spots were marked on the map based on Coomassie Blue staining. (A) Nondiapause; (B) diapause.

implemented on an Ettan DALTsix system with 12% sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE) gels. The initial running condition of the electrophoresis was 1 W per gel for 30 min, after which 5 W per gel was applied until the bromophenol blue reached the bottom of the gel.

Gels were fixed in fixation fluid (40% ethanol, 10% acetic acid, and 50% Mill-Q water) overnight and then washed with deionized water and stained for 24 h with Coomassie G-250 solution (10% ammonium sulfate, 10% phosphoric acid, 20% methanol, 0.12% G-250). The gels were destained in deionized water.

Both nondiapause and diapause treatments included three biological repetitions and three technical repetitions.

### *Image analysis*

The gels were scanned with a GE ImageScanner III imaging system, and the differential proteins spots (nondiapause and diapause) were analyzed with ImageMaster 2D Platinum 7.0, including protein spot detection, background subtraction, normalization, and spot matching. Three nondiapause gel images and three diapause gel images were analyzed. In the expression abundance analysis, differentially expressed protein spots appeared repeatedly on nine nondiapause and nine diapause gels, and intensities that changed by more than threefold (analysis of variance (ANOVA), $P < 0.05$) were selected. We used the nondiapause gel images as a baseline to determine the up- or down-regulation of protein spots on diapause images.

### *Mass spectrometry analysis and protein identification*

Sixteen differentially expressed spots with twofold changes (ANOVA, $P < 0.05$) were excised. The protein spots were washed with deionized water three times, and the water was changed every 2–3 h and then sent to the Beijing Protein Innovation Company, Ltd. ESI-QUAD-TOF was used to obtain peptide mass fingerprints (PMF). The PMF data were analyzed using Mascot software and searched against the NCBI nr database using the following parameters: enzyme by trypsin, fixed modifications by carbamidomethyl (C), variable modification by methionine oxidation, mass values = monoisotopic; peptide tolerance by ± 0.1 Da. The significance threshold was $P < 0.05$.

## Results

### *Protein images of nondiapause and diapause female adults*

Total proteins were extracted from nondiapause/diapause *C. septempunctata* using phenol extractions, separated with 24 cm IPG liner strips (pH 3–10) and stained with Coomassie blue. Through image analysis, 1531 individual spots from diapause female adults' gels were matched with spots from nondiapause female adults' gels, among which 58 protein spots were marked for significant differential expression (Ratio > 2, ANOVA, $P < 0.05$) (fig. 1). Sixteen abundant protein spots were selected for excision for mass spectrometry analysis.

### *Differential protein expression between diapausing and nondiapausing adult females*

Sixteen individual spots were cut from gels and sent for identification by mass spectrometry. Eight proteins were annotated in the National Center for Biotechnology Information (NCBI) nr database as searching using Mascot software (table 1), and corresponding 3D profiles were analyzed in detail using a GE ImageScanner III imaging system (fig. 2). The eight proteins can be divided into four categories: chaperone (1-D11, 4-D35), glucose metabolism (1-D26), tricarboxylic acid cycle (8-D21, 20-N31, 18-N34), and lipid metabolism (10-D8, 11-D6, 17-N35). The 5 diapause-upregulated proteins were symbionin symL (1-D11, 4-D35), malate dehydrogenase (putative) (8-D21), unknown (10-D8), phosphoglyceromutase partial (1-D26), and conserved hypothetical protein (11-D6); the three diapause-downregulated proteins were putative medium chain acyl-CoA dehydrogenase (MCAD) (17-N35), AGAP001942-PA (18-N34), and RH49423p (20-N31).

## Discussion

Diapause is a stage of arrested development that entails a series of changes, including morphology, behavior, physiology, and molecular biology (Lees, 1956; Harvey, 1962;

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Table 1. List of differentially expressed proteins identified by mass spectrometry.[1]

| Spot no.[2] | Protein name | Mascot score | Species | Protein MW(kDa) \| PI | Accession no. | D/ND[3] |
|---|---|---|---|---|---|---|
| 1-D11 | Symbionin symL | 3094 | *Acyrthosiphon pisum* | 58.0 \| 5.1 | gi \| 285430 | 6.0 |
| 4-D35 | Symbionin symL | 451 | *Acyrthosiphon pisum* | 58.0 \| 5.1 | gi \| 285430 | 3.1 |
| 8-D21 | Malate dehydrogenase, putative | 279 | *Pediculus humanus corporis* | 37.5 \| 8.5 | gi \| 212508346 | 3.1 |
| 10-D8 | Unknown | 147 | *Dendroctonus ponderosae* | 62.2 \| 5.0 | gi \| 332374222 | 3.0 |
| 1-D26 | Phosphoglycero-mutase, partial | 325 | *Curculio pyrrhoceras* | 15.2 \| 8.1 | gi \| 33088811 | 2.9 |
| 11-D6 | Conserved hypothetical protein | 97 | *Culex quinquefasciatus* | 57.0 \| 4.9 | gi \| 167867241 | 2.3 |
| 17-N35 | Putative medium chain acyl-CoA dehydrogenase, MCAD | 3519 | *Rhodnius prolixus* | 46.1 \| 8.6 | gi \| 485218590 | −2.4 |
| 18-N34 | AGAP001942-PA | 111 | *Anopheles gambiae str. PEST* | 24.7 \| 5.3 | gi \| 157012464 | −3.4 |
| 20-N31 | RH49423p isocitrate dehydrogenase | 288 | *Drosophila melanogaster* | 44.6 \| 6.3 | gi \| 21392222 | −2.5 |

[1]Spots not identified are not shown.
[2]Spot numbers are marked in fig. 1 as D, representing the diapause map, or as N, representing the nondiapause map.
[3]The diapause (D)/nondiapause (ND) expression ratios of the indicated spots.

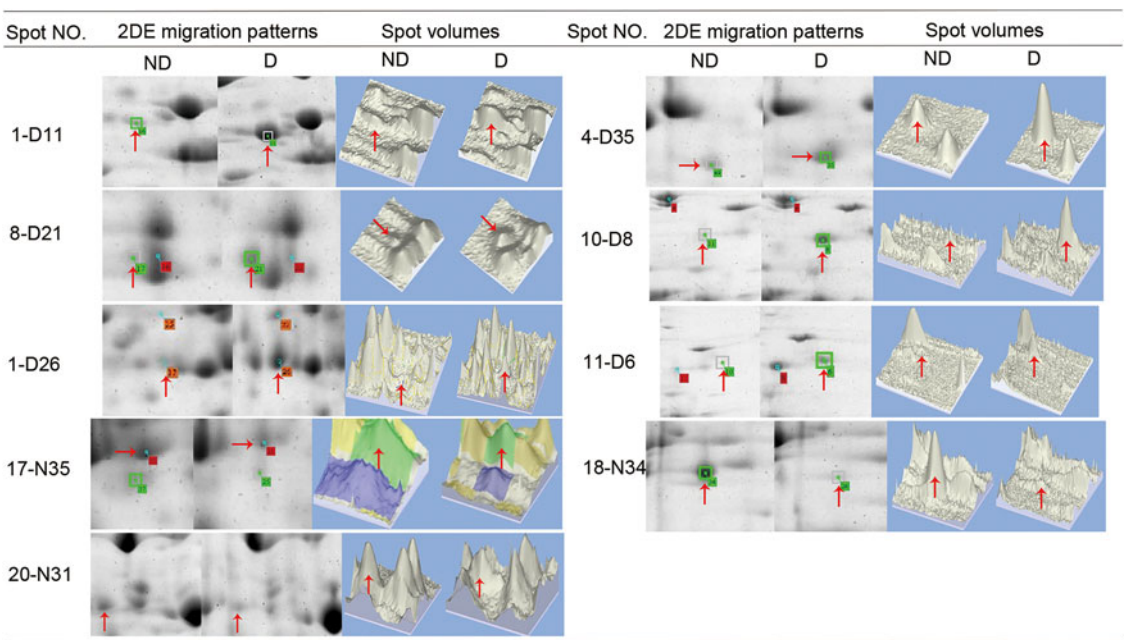


Fig. 2. Selected areas of 2D gels and 3D views of selected differential expression proteins identified in diapause (D) and nondiapause (ND) adult females of *C. septempunctata*.

Tauber & Tauber, 1976; Renfree & Shaw, 2000; Zhang, 2009; Hahn & Denlinger, 2011; Denlinger & Armbruster, 2014), and all of these changes ensure that insects can survive afterward. For example, diapausing *Culex pipiens* has thicker cuticular hydrocarbons than nondiapause individuals, which contributes to a reduction in water loss. In addition, the metabolic rate during diapause is lower than during nondiapause, which increases the desiccation tolerance of *C. pipiens* (Benoit & Denlinger, 2007; Denlinger & Armbruster, 2014). In diapausing *Sarcophaga crassipalpis*, most Hsps are upregulated, which increases their cold tolerance during winter (Li *et al.*, 2007; Rinehart *et al.*, 2007). In this study, we detected four categories of proteins—chaperone (1-D11, 4-D35), glucose metabolism (1-D26), tricarboxylic acid cycle (8-D21, 20-N31, 18-N34), and lipid metabolism (10-D8, 11-D6, 17-N35)—and this will contribute to elucidating the mechanism of *C. septempunctata* diapause.

Molecular chaperones are essential cellular machinery for correct protein folding, transmembrane transport, and preventing aggregation of polypeptides (Bukau & Horwich, 1998; Wandinger *et al.*, 2008). In the present study, 1-D11 and 4-D35 were upregulated in diapause adults and matched with symbionin symL coming from *Acyrthosiphon pisum*. Symbionin symL is a member of the Hsp60 family. In numerous diapause studies, the Hsps are important protective agents in environmental stress tolerance. During diapause in

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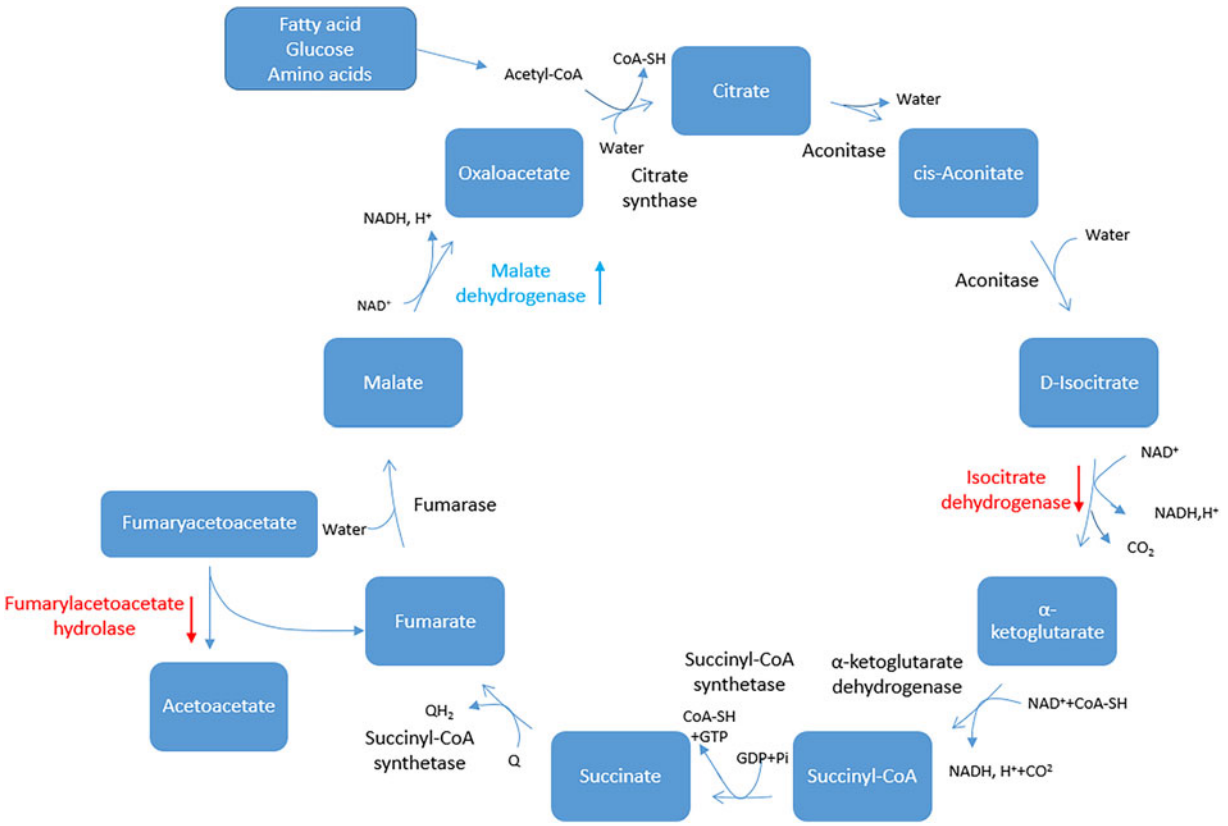


Fig. 3. TCA cycle. IDH and fumarylacetoacetate hydrolase were downregulated in the diapause female ladybeetles, and malate dehydrogenase was upregulated.

the flesh fly *S. crassipalpis*, upregulated *Hsp23* and *Hsp70* profoundly improved the pupa's ability to survive in low temperatures (Rinehart *et al.*, 2007). In this study, where a diapausing *C. septempunctata* was placed under a low temperature (18°C), chaperones may keep proteins in native states, ensuring that the protein activity is preserved during diapause (Mayer, 2010; King & MacRae, 2015).

Diapause exhibits low or halted development, altering the normal developmental pathway into an alternative one, and lipids, glycogen, and storage proteins will accumulate (Lees, 1956; Hahn & Denlinger, 2007; Li *et al.*, 2007; King & MacRae, 2015). In accordance with the metabolic depression, we observed the downregulation of isocitrate dehydrogenase (IDH, 20-N31), which plays an important role in the TCA cycle. IDH is a key enzyme regulating the reaction between isocitric acid and NAD, which is a rate-limiting step of the TCA cycle. Glucose, lipids, and proteins are degraded completely in the TCA cycle (Balazs *et al.*, 1970; Jafri *et al.*, 2001). In this study, IDH was less abundant in diapause, indicating that metabolism in diapausing *C. septempunctata* is suppressed (fig. 3). Another protein spot that showed less abundance in diapause adults was 18-N34, which showed high identity to AGAP001942-PA, a fumarylacetoacetate hydrolase (FAH) that hydrolyzes fumarylacetoacetate into fumarate and acetoacetate in tyrosine catabolism (Lindblad *et al.*, 1977; St-Louis & Tanguay, 1997). Both fumarate and acetoacetates are members of the TCA cycle, so we categorized FAH as a TCA cycle enzyme. Moreover, pigment precursors of melanin come from tyrosine (True, 2003; Wittkopp *et al.*, 2003). In general, the elytron of aestivation adults is paler, and in our research, the elytron color of diapausing adults is also lighter than that of nondiapausing adults. Another TCA protein, malate dehydrogenase (8-D21, MDH), which catalyzes the conversion of oxaloacetate and malate following oxidation/reduction of dinucleotide coenzymes (NAD), was abundant in diapausing adults (Goward & Nicholls, 1994; Ying, 2008). The downregulation of IDH indicated that the TCA cycle is arrested, and the conversion of malate to oxaloacetate is constrained with higher NAD levels. Because NAD plays an important role in biological processes such as cellular respiration and immunological functions (Berger, 2004; Peter *et al.*, 2007), as well as mediating protection against neurodegeneration (Peter *et al.*, 2007), we predicted that the upregulated MDH may lead to NAD accumulation and improve stress tolerance of diapausing adults.

The putative MCAD is a member of the acyl coenzyme A dehydrogenase family plays an important role in fatty acid oxidation. This protein was downregulated in diapause, indicating that in diapause female adults, lipid metabolism was depressed. Both the upregulated unknown and the conserved hypothetical protein have the same structural domains, CBM_14 and LDLa domains, respectively affecting the peritrophic membrane (PM) and the low-density lipoprotein receptor in hemolymph. The function of the PM varies based on the components of the PM and pressure, but they include protecting the midgut epithelium from mechanical damage

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and microorganism invasion, enzyme immobilization and toxin binding (Lehane, 1997; Terra, 2001). LDLa is a domain of the low-density lipoprotein receptor (LDLp). *In vitro*, the high-density lipoprotein receptor (HDLp) can be transformed into low-density lipophorin (LDLp) in the presence of diacylglycerol (Lum & Chino, 1990; Surholt *et al*., 1992), providing energy for the needs of physiological activities and maintaining the homeostasis of the hemolymph. Moreover, another upregulated protein in diapausing adult females is phosphoglyceromutase (partial), with a histidine phosphatase domain and showing phosphoglycerate mutase activity, which regulates the reversible conversion reactions between 3-phosphoglycerate and 2-phosphoglycerate in glycometabolism. Because the TCA cycle is inhibited, the speed of gluconeogenesis may be higher than that of glycolysis.

Diapausing *C. septempunctata* has obvious changes in protein expressions. Both upregulated proteins and downregulated proteins were important for diapause. In conclusion, diapausing seven-spotted ladybirds may change their metabolic patterns to survive the rigors of the environment and to subserve the particular requirements of diapause. This research will help us understand the mechanism underlying diapause and assist us to utilize this natural enemy for further research.

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## Disclosure statement

The authors X. Y. Ren and L. S. Zhang contributed equally to this paper. And the authors declare no competing financial interest.

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