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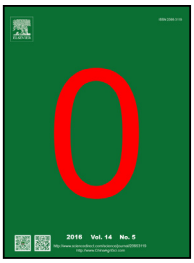

RESEARCH ARTICLE

Identification of diapause-associated proteins in migratory locust, *Locusta migratoria* L. (Orthoptera: Acridoidea) by label-free quantification analysis

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Abstract

Maternal photoperiodic response is a key factor that affects offspring diapause in migratory locust, *Locusta migratoria* L. (Orthoptera: Acridoidea). Although many aspects of insect diapause have been studied, little is known about the molecular mechanisms of maternal photoperiodic response that influence diapause regulation. To gain insight into the possible mechanisms of maternal photoperiod influence on diapause regulation, proteomics data by label-free quantification analysis were generated from non-diapause and diapause eggs. A total of 175 proteins were differentially expressed between diapause and non-diapause eggs. Among them, 24 proteins were upregulated, and 151 proteins were downregulated. Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichments were performed on all differentially expressed proteins (DEPs) and showed that peroxisome, insect hormone biosynthesis, and longevity regulating pathway may be related to diapause of migratory locust. Furthermore, we used qRT-PCR to verify some results of the proteomic analysis. Proteins such as hexamerin-like protein 4, juvenile hormone epoxide hydrolase 1 (JHEH1), cytochrome P450 and heat shock protein (Hsp) 20.7 were predicted to be involved in diapause. This study provides an important reference for future research that will explore the mechanisms of diapause induced by maternal effects in migratory locust.

Keywords: *Locusta migratoria*, diapause, proteome, maternal effect, molecular mechanism

1. Introduction

Diapause is a specific strategy of insect survival under unfavorable conditions including short photoperiods, cool temperature and drought (Denlinger and Armbruster 2014). There are two forms of insect diapause: facultative diapause and obligatory diapause. Facultative diapause is programmed by environmental tokens such as daylength, while obligatory diapause, a mandatory, fixed component of ontogeny, is not relevant to the environmental condition

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(Denlinger 2002). Depending on the species, insect diapause can occur in embryos, larvae, pupae or adults (Tatar and Yin 2001). The migratory locust, *Locusta migratoria* L., a facultative diapause insect, enters diapause in the egg stage (Uvarov 1966, 1977).

The factors that influence diapause in the migratory locust are temperature, photoperiod and maternal (Tanaka 1992; Tanaka 1994; Tu *et al*. 2014). The effects of temperature and photoperiod on diapause have been widely reported (Sawchyn and Church 1973; Kobayashi and Numata 1975; Yi *et al.* 2007; Saunders 2010) including studies on the regulatory mechanisms of temperature and photoperiod (Williams and Adkisson 1964; Saunders 2014; Meuti *et al.* 2015). Maternal control of diapause in a few insects and mites has been reported (Mousseau and Fox 1998). Generally speaking, females experiencing deteriorating environmental conditions are more likely to produce diapausing offspring (Mousseau and Fox 1998). In particular, maternal photoperiodic response on offspring diapause has been reported. In *Trichogramma*, the percentage of diapausing prepupae was significantly dependent on the photoperiodic conditions of the preimaginal development of the maternal generation (Vaghina *et al.* 2013). Maternal photoperiodic response on diapause incidence of the parasitoid has also been reported in *Cotesia plutellae* (Guo *et al.* 2007).

The migratory locust is an economically important species of insect because it threatens agricultural production. Maternal photoperiodic response affects embryo diapause in *L. migratoria* L. For example, in the laboratory, female migratory locusts produced non-diapause eggs when exposed to long photoperiods (16 h L:8 h D) and diapause eggs when exposed to short photoperiods (10 h L:14 h D or 12 h L:12 h D) (Tanaka 1994). In order to explain the regulatory mechanism of diapause in migratory locust, the previous studies analyzed the transcriptome and proteomics of the locust eggs at different embryonic states. The results showed that “juvenile hormone biosynthesis, insulin and PPAR signaling pathways” may play a critical role on diapause regulation in migratory locust (Tu *et al.* 2015; Hao 2017). How the female adults of migratory locust transfer the environmental information to offspring and induce egg diapause is still unclear. To reveal the molecular regulatory mechanisms influenced by maternal photoperiod on locust diapause, we performed a label-free protocol for a comparative analysis of proteomes between diapause and non-diapause eggs isolated from the ovaries of females exposed to long or short photoperiods. The present study can improve our understanding of the genetic and molecular mechanisms underlying diapause in an agriculturally important insect pest.

2. Materials and methods

2.1. Insect materials

Eggs of migratory locust were collected from the field at Tianjin, China (38°49′N, 117°18′E). Eggs were dug out from the soil, gently sieved and placed into a re-sealable bag before placing into an insulated container to keep cool. Eggs were then transferred to the Langfang Field Station of the Institute of Plant Protection, Chinese Academy of Agricultural Sciences.

2.2. Insect growth conditions

The eggs were placed in vermiculite with 20–30% water content and then hatched at 30°C and 60% relative humidity in an artificial climate box (PRX-250B-30, Haishu Saifu Experimental Instrument Factory, Ningbo, China). The emerged nymphs were transferred to wooden rearing chambers (60 cm×50 cm×60 cm), and fed with wheat seedlings. Second instars were exposed to either long-day or short-day conditions. We used two artificial climate boxes to mimic the natural daily photoperiod cycles for the long and short days. The photoperiodic regime used for non-diapause locusts in the experiment was 16 h L:8 h D. The temperature was 27°C and relative humidity was 60%. To induce diapause, locusts were reared at 10 h L:14 h D, 27°C and 60% relative humidity (Tanaka 1994; Wang *et al.* 2014). We recorded temperature data per hour for each 24-h day for each artificial climate box using a HOBO U23 Pro v2 Temperature/Relative Humidity Data Logger (Onset, USA).

2.3. Production of non-diapause and diapause eggs

We reared adults under long and short photoperiods to obtain non-diapause and diapause egg-producing females, respectively. Females were dissected two weeks after adults and eggs from the ovaries were collected. The ovaries of 15-days-old adult females were typically at the 4th developmental stage with an approximate size of about 15 mm (L) and 10 mm (W) (Guo *et al.* 1991). Dissection was performed on ice, and the extracted eggs were immediately put into liquid nitrogen and stored at –80°C for further analysis. Three independent biological replicates were prepared for each photoperiod treatment. Every replicate involved 60 eggs dissected from three female locusts.

2.4. Diapause rate

The remnant locusts were reared at 27°C in both long or short photoperiods until they laid eggs. After oviposition, we

monitored daily for neonate emergence in both photoperiod treatment groups. We counted the total number of hatchlings (D1) and unhatched eggs (D2) to calculate the diapause rate of locust eggs by the following formula:

Diapause rate (%)=D2/(D1+D2)×100

2.5. Label-free quantification

Three independent biological replicates were prepared in each treatment with 60 eggs per replicate. The egg samples were homogenized by lysis buffer that was composed of lysogeny broth, 8 mol L^{-1} urea, 2 mol L^{-1} thiourea, 4% CHAPS, 20 mmol L^{-1} Tris-base, 30 mmol L^{-1} dithiothreitol (DTT), and 2% Biolyte pH 3−10. The sample was sonicated for 20 s every 5 min for a total of 30 min while on ice. Then it was centrifuged at 1 200×g for 10 min at 4°C and further centrifuged at 15 000×g for 10 min at 4°C. We added three volumes of acetone to the supernatant in order to precipitate proteins and desalt the sample. Next, the mixture was centrifuged two times at 15 000×g for 10 min. The supernatant was removed and the pellet was dissolved using lysogeny broth. After 5 min of incubation at 4°C, the sample was sonicated for 2 min. Protein concentrations were quantified by the Bradford method (Bradford 1976). LC−MS/MS analysis, identification and label-free quantitation of proteins were carried out as described previously (Han 2017). The fold change of proteins between the two photoperiod treatments was calculated by the ratio of diapause to non-diapause group means.

2.6. Bioinformatics analyses

Gene-set enrichment was completed by KOBAS 3.0 (Xie *et al.* 2011). To enrich the identified proteins involved in the canonical pathway, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used. The GO database was used to facilitate the biological interpretation of the identified proteins in this study. GO enrichment analysis was carried out as described previously (Tu *et al.* 2015).

2.7. Quantitative real-time PCR (qRT-PCR)

To further investigate the diapause mechanism at the gene level, total RNA was extracted from non-diapause and diapause eggs using a Quick-RNA™ MicroPrep Kit (Zymo, USA) according to the manufacturer's instructions. Total RNA quantification was performed by a Nano Photometer (Implen, BRD) and the quality of RNA was evaluated by 1.0% denaturing agarose gel electrophoresis and compared to the bands of the 28S and 18S rRNA. Reverse transcription was performed using a 5X All-In-One RT MasterMix Kit (ABM, Canada) according to the manufacturer's instructions. The mRNA levels of four important proteins, hexamerin-like protein 4, JHEH1, cytochrome P450, and HSP20.7 were checked. qRT-PCR was performed with the SYBR Premix *Ex Taq* (TaKaRa, Japan) on the ABI 7500 Real-Time PCR System (ABI, USA). The actin gene was used as a reference control. Each plate was repeated four times in independent runs for all reference and selected genes. Gene expression was evaluated by the $2^{-\Delta\Delta C_T}$ method. The primers used in this paper are shown in Table 1.

2.8. Statistical analysis

The differences in diapause rates and mRNA levels of four proteins were tested for statistical significances by independent sample *t*-test where $P<0.05$ was the threshold of significance. Results were expressed as mean±SE. Statistical analyses were performed using SPSS16.0 software.

3. Results

3.1. Diapause rate

The data showed that the long photoperiod regime produced virtually 100% non-diapause eggs, whereas the short photoperiod regime produced approximately 85% diapause and 15% non-diapause eggs (Fig. 1). Although samples from the diapause group contained a moderate number of non-diapause eggs, the high percentage of diapause eggs in these samples (~85%) still allowed us to identify DEPs between the two groups during molecular analyses.

3.2. Identification of DEPs between non-diapause eggs and diapause eggs

The total number of sequences identified by mass spectrometry of locust egg proteomes was 1 090. Among them, 175 proteins (representing 1 385 peptides) were differentially expressed between diapause and non-

Table 1 Primers used in this study

Genes	Primers (5′→3′)
Hexamerin-like protein 4-F	CATCACAGCCAACTACACGG
Hexamerin-like protein 4-R	AACATCTGCCTGAGGGAGTG
JHEH1-F	CTCGGGCACAGCAAGTTCTA
JHEH1-R	CAGCAGTATGAGATTGAGCCAA
Cytochrome P450-F	CATTGCTCCTGAACTCTCTG
Cytochrome P450-R	GACTTGCTGGTTGTTTCTGAG
HSP20.7-F	CAGCCACAGTTCCTTTACTTTC
HSP20.7-R	TGCTTCCCTTCAATAACGAC
Actin-F	GTTACAAACTGGGACGACAT
Actin-R	AGAAAGCACAGCCTGAATAG

diapause eggs at $P<0.05$ (≥1.5-fold change, FDR≤0.01) including 84 proteins at $P<0.01$ (Fig. 2). Among the 175 DEPs, 24 proteins were upregulated, and 151 were downregulated (Fig. 3). The 24 significantly upregulated and the top 20 significantly downregulated proteins are shown in Tables 2 and 3, respectively.

3.3. GO classification of DEPs

Proteins were annotated by the GO database based on their molecular function, cellular component and biological process to further analyze the biological variability of the DEPs (Tu *et al.* 2015). The most enriched GO terms are shown in Fig. 4. Under the category of biological processes, small molecule metabolic process (19, 10.9%), organic acid metabolic process (12, 6.9%) and carboxylic acid metabolic process (12, 6.9%) were the most represented. Within cellular components, proton-transporting two-sector ATPase complex (4, 2.3%), ribonucleoprotein complex (12, 6.9%) and macromolecular complex (18, 10.3%) were highly represented. For molecular functions, oxidoreductase activity (20, 11.4%), oxidoreductase activity acting on the CH-CH group of donors (4, 2.3%) and acyl-CoA dehydrogenase activity (3, 1.7%) were the most represented.

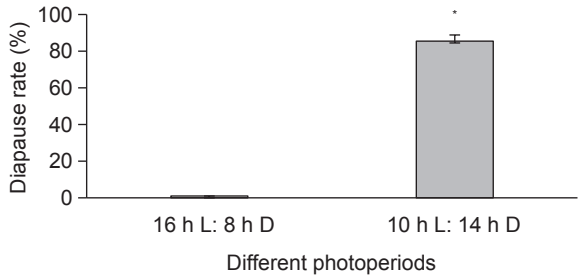

Fig. 1 Diapause rates of egg produced from females exposed to the long 16 h L: 8 h D and short 10 h L: 14 h D photoperiods.

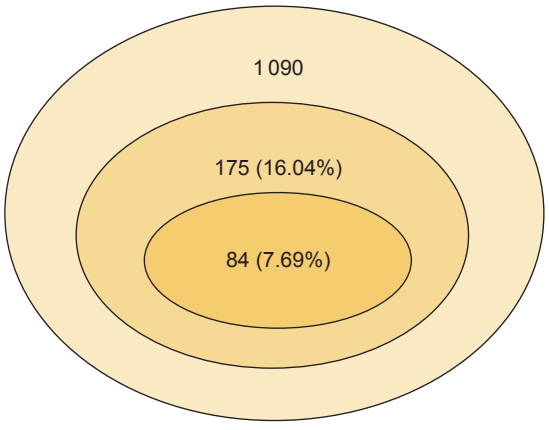

Fig. 2 Statistics of differentially expressed proteins. Out of the 1 090 identified proteins, 175 (16.05%) revealed significant differences ($P<0.05$) including 84 (7.69%) proteins that were significant at $P<0.01$.

3.4. KEGG pathway analysis of DEPs

In order to determine the biological processes participating in diapause, we used KOBAS 3.0 Software to test the statistical enrichment of DEPs in KEGG pathways (Xie *et al.* 2011). The 175 DEPs between non-diapause eggs and diapause eggs were mapped to 69 pathways in the KEGG database. But only 40 pathways were substantially enriched ($P<0.05$) (Table 4). Metabolic pathways (KEGG: dme01100), carbon metabolism (KEGG: dme01200) and protein processing in endoplasmic reticulum (KEGG: dme04141) were among the top three pathways identified by the pathway analysis.

3.5. Validation by qRT-PCR

To detect changes in mRNA levels, four important proteins, hexamerin-like protein 4, JHEH1, cytochrome P450, and HSP20.7, were used for relative quantitative analysis. Results showed the relative expressions of hexamerin-like protein 4, JHEH1, cytochrome P450, and HSP20.7 were higher in non-diapausing eggs compared to diapausing eggs ($P<0.05$; Fig. 5). The trends in relative quantitative expression of JHEH1, cytochrome P450, and HSP20.7 were similar to the proteomics results. However, the relative quantitative expression of hexamerin-like protein 4 was contrary to the proteomics.

4. Discussion

Photoperiod and incubation temperature are critical induction factors in facultative diapause of insects, somehow associated with maternal line (Delinger 2002; Timer *et al.* 2010). For *L. migratoria* L., the maternal photoperiodic response induces embryonic diapause allowing the eggs

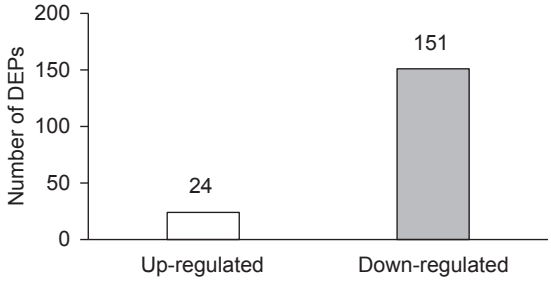

Fig. 3 Number of differentially expressed proteins (DEPs) in diapause eggs treated at short photoperiod (10 h L:14 h D) as compared to non-diapause eggs (control) treated at long photoperiod (16 h L:8 h D).

Table 2 Significantly up-regulated proteins in diapause eggs

No.	Protein	Significance	Fold change
1	Protein arginine N-methyltransferase 3	60.69	27.53
2	Eukaryotic translation initiation factor 4E transporter partial	13.16	4.83
3	Hexamerin-like protein 4	26.03	3.29
4	Xanthine dehydrogenase partial	28.56	3.11
5	ABC transporter G family member 23	20.77	2.87
6	Probable small nuclear ribonucleoprotein E	25.86	2.53
7	60S ribosomal protein L10	13.21	2.41
8	Transcription factor HES-4	21.23	2.38
9	Multidrug resistance-associated protein 4-like	14.83	2.11
10	60S ribosomal protein L23	21.49	1.87
11	Protein MEMO1	20.74	1.79
12	Adenosylhomocysteinase putative	20.05	1.79
13	Small nuclear ribonucleoprotein Sm D3	22.52	1.75
14	60S ribosomal protein L6	16.40	1.68
15	IQ domain-containing protein E	22.39	1.65
16	Histone H1-III	51.26	1.64
17	Tropomyosin-2 isoform X7	13.41	1.61
18	40S ribosomal protein S25	14.40	1.60
19	QN1-like protein	20.40	1.60
20	Eukaryotic translation initiation factor 3 subunit J	20.64	1.57
21	Lysosome-associated membrane glycoprotein 1	18.31	1.54
22	Hypothetical protein TcasGA2	18.11	1.53
23	Hypothetical protein L798	22.21	1.53
24	ABCC4-like protein	13.07	1.51

Table 3 Significantly down-regulated proteins in diapause eggs

No.	Protein	Significance	Fold change
1	Very long-chain specific acyl-CoA dehydrogenase mitochondrial	38.62	0.03
2	Transforming acidic coiled-coil-containing protein 3-like	18.80	0.06
3	Greglin	26.54	0.08
4	Transformer-2 protein-like protein beta	28.43	0.09
5	Serine/threonine-protein kinase OSR1	19.5?	0.10
6	Actin-interacting protein 1	22.94	0.12
7	Asparagine synthetase	17.00	0.13
8	Synaptic vesicle membrane protein VAT-1 homolog isoform X2	17.87	0.14
9	Hrp65 protein	15.57	0.14
10	Carboxylesterase	23.43	0.15
11	Aromatic-L-amino-acid decarboxylase	14.26	0.15
12	Protein TFG	24.69	0.17
13	Nicotinate phosphoribosyltransferase isoform X3	46.74	0.17
14	Cytochrome P450 307A1	13.88	0.17
15	Heterogeneous nuclear ribonucleoprotein A1 putative	17.38	0.18
16	Mannose-1-phosphate guanyltransferase beta	18.44	0.19
17	Sorting nexin-2	20.33	0.20
18	Variable lymphocyte receptor partial	19.95	0.20
19	ATP-binding cassette sub-family E member 1	13.78	0.21
20	WD and tetratricopeptide repeats protein 1 partial	13.98	0.23

to survive under unfavourable environmental conditions (Tanaka 1994). How female adults transfer the environmental information to their offspring and induce egg diapause is still unclear. Tu *et al.* (2015) and Hao *et al.* (2017) have studied the effect of temperature on diapause in migratory locusts and they determined the optimum temperature of 27°C for locusts to enter diapause. We primarily examined the maternal photoperiodic response on oocytes while Tu *et al.* (2015) and Hao *et al.* (2017) focused on the embryonic stage of locust. As part of developing a better understanding of the underlying biochemical processes to diapause induction, we performed a comparative analysis of proteomes of both non-diapausing and diapausing eggs *via* a label-free approach and qRT-PCR was used for verification.

Most heat shock proteins (HSPs) are synthesized in response to various stresses, and some of them are essential to insects' development (Chapuis *et al.* 2011) and have been associated with insect diapause (Denlinger 2002; Rinehart *et al.* 2007). Unlike upregulated HSPs in response to stress, HSPs vary considerably during diapause among different species and different physiological stages of the insect. At the transcriptional level, Hsp20.7 and Hsp90 in diapausing embryos of the cricket, *Allonemobius socius*, were significantly decreased as compared to the non-diapausing cohort, while Hsp70 transcripts were unchanged (Reynolds and Hand 2009). Using qRT-PCR to verify the transcriptional level of HSP20.7, we observed a significantly lower level in diapause eggs than that in non-diapause eggs, which was consistent with the change in protein levels. Additionally, HSP70 was upregulated in diapause eggs but the difference was insignificant. In diapause and non-diapause eggs, HSP90, HSP20.5, HSP23.8, HSP19.8, and HSP21.1 were expressed but without any significant differences between the two groups.

Insects are able to survive through a long diapause period by increasing their energy reserves. However, previous studies on insect storage proteins show that not all proteins exhibit the same trends in expression during diapause (Miura *et al.* 1998; Godlewski *et al.* 2001, Lewis *et al.* 2002, Sonoda *et al.* 2006, Spyliotopoulos *et al.* 2007, Tungjitwitayakul *et al.* 2008). For example, in diapausing

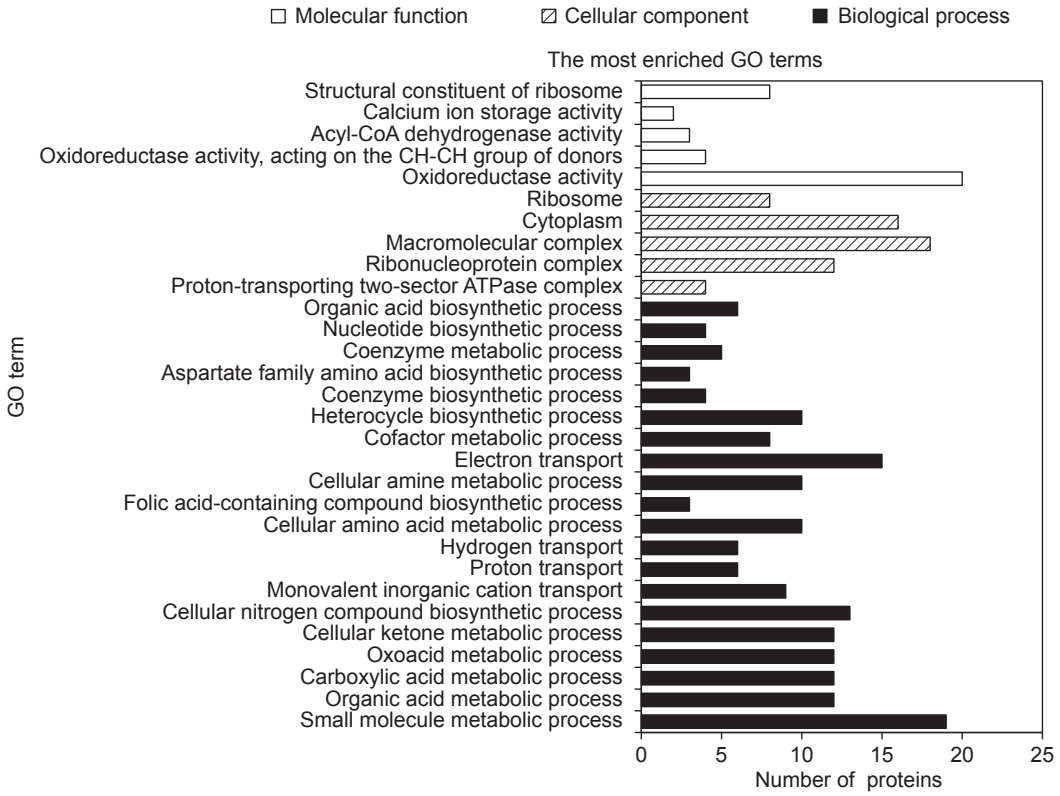

Fig. 4 Gene Ontology (GO) classification of differentially expressed proteins (DEPs) between non-diapause and diapause locust eggs. The DEPs are grouped into three hierarchically structured GO terms, biological process, cellular component, and molecular function.

larvae of rice stem borer, *Chilo suppressalis*, Sonoda *et al.* (2006) found the up-regulation of storage protein 1 when the temperature of cold acclimation was shifted to 5 from 10°C. In *Sesamia nonagrioides*, mRNA levels of a storage protein were extremely low in larvae at the pre-diapause stage, but levels dramatically increased at the maintenance and termination stages of diapause (Spyliotopoulos *et al.* 2007). In our study, hexamerin-like protein 4 was significantly upregulated in diapause eggs while hexamerin-like protein 1 was significantly downregulated in diapause eggs in comparison to non-diapause eggs. Hexamerin-like protein 2 showed no significant difference between diapause and non-diapause eggs. Furthermore, verification of transcriptional levels by qRT-PCR showed that the level of hexamerin-like protein 4 in diapause eggs was significantly lower than that in non-diapause eggs, which was contrary to the change trend observed of protein levels. We speculate that the female locusts of the diapause treatment group synthesized hexamerin-like protein 4 and passed it onto their offspring, in order to help their offsprings survive the long diapause period.

Studies revealed that juvenile hormone (JH) and its analogue are involved in diapause regulation (Yin and Chippendale 1976; Singtripop *et al.* 2002). Tawfik *et al.* (2002) found that the content of JH in diapause eggs was significantly lower than that in non-diapause eggs of migratory locust, with at least three types of enzymes, JH esterase, JH epoxide hydrolase and JH diol kinase, involved in JH degradation (Morisseau and Hammock 2005; Kamita and Hammock 2010; Saito *et al.* 2015). Wolschin and Gadau (2009) found the expression level of a JH epoxide hydroxylase increases with time in *Nasonia* diapause larvae. In our research, we found that the expression of JHEH1 was downregulated in diapause eggs, but the difference from non-diapause eggs was not significant. Moreover, the qRT-PCR results showed that transcript expression of JHEH1 in diapause eggs was significantly lower than that of the non-diapause eggs, which was consistent with the difference observed in the protein result.

Ecdysone also plays an important role in diapause regulation (Sonobe *et al.* 1999) and the cytochrome P450 gene is critical in the synthesis of ecdysone. Cytochrome P450 enzymes catalyze biosynthesis of ecdysone in *Drosophila melanogaster* (Scott *et al.* 1998; Gilbert and Warren 2005) and in the fall webworm, *Hyphantria cunea* Drury, cytochrome P450 is involved in detoxification and

Table 4 Substantially enriched pathways identified by KEGG pathway analysis

No.	Pathway	ID	DEPs number	Percentage (%)[1)]	*P*-value
1	Metabolic pathways	dme01100	44	25.14	3.31E-18
2	Carbon metabolism	dme01200	11	6.29	2.84E-08
3	Protein processing in endoplasmic reticulum	dme04141	11	6.29	7.60E-08
4	Beta-Alanine metabolism	dme00410	5	2.86	2.46E-06
5	Glutathione metabolism	dme00480	7	4.00	4.89E-06
6	Fatty acid degradation	dme00071	5	2.86	2.16E-05
7	Valine, leucine and isoleucine degradation	dme00280	5	2.86	2.85E-05
8	Drug metabolism-cytochrome P450	dme00982	6	3.43	5.30E-05
9	Metabolism of xenobiotics by cytochrome P450	dme00980	6	3.43	5.30E-05
10	Biosynthesis of amino acids	dme01230	6	3.43	6.25E-05
11	Fatty acid metabolism	dme01212	5	2.86	9.27E-05
12	Histidine metabolism	dme00340	3	1.71	1.43E-04
13	Phagosome	dme04145	6	3.43	1.62E-04
14	Cysteine and methionine metabolism	dme00270	4	2.29	4.07E-04
15	Arachidonic acid metabolism	dme00590	3	1.71	5.65E-04
16	Oxidative phosphorylation	dme00190	7	4.00	5.86E-04
17	Proteasome	dme03050	4	2.29	1.91E-03
18	Glycolysis/Gluconeogenesis	dme00010	4	2.29	2.33E-03
19	RNA transport	dme03013	6	3.43	3.04E-03
20	Fructose and mannose metabolism	dme00051	3	1.71	3.07E-03
21	Phenylalanine metabolism	dme00360	2	1.14	3.27E-03
22	mRNA surveillance pathway	dme03015	4	2.29	5.97E-03
23	Peroxisome	dme04146	4	2.29	5.97E-03
24	Ribosome	dme03010	7	4.00	8.64E-03
25	Spliceosome	dme03040	5	2.86	9.05E-03
26	Fatty acid elongation	dme00062	2	1.14	9.18E-03
27	Citrate cycle (TCA cycle)	dme00020	3	1.71	1.02E-02
28	Pyruvate metabolism	dme00620	3	1.71	1.15E-02
29	Insect hormone biosynthesis	dme00981	2	1.14	1.17E-02
30	Pentose and glucuronate interconversions	dme00040	3	1.71	1.22E-02
31	Glycerolipid metabolism	dme00561	3	1.71	1.29E-02
32	Butanoate metabolism	dme00650	2	1.14	1.31E-02
33	Tyrosine metabolism	dme00350	2	1.14	1.31E-02
34	Lysosome	dme04142	4	2.29	1.51E-02
35	2-Oxocarboxylic acid metabolism	dme01210	2	1.14	1.61E-02
36	Tryptophan metabolism	dme00380	2	1.14	1.77E-02
37	Propanoate metabolism	dme00640	2	1.14	2.10E-02
38	Pentose phosphate pathway	dme00030	2	1.14	2.28E-02
39	Glycine, serine and threonine metabolism	dme00260	2	1.14	3.46E-02
40	Alanine, aspartate and glutamate metabolism	dme00250	2	1.14	3.67E-02

[1)] Percentage (%)=The number of differentially enriched proteins in the pathway/The total number of differentially expressed proteins

metabolism as indicated by downregulated mRNA levels of cytochrome P450 in diapausing pupae (Tijet *et al.* 2001; Deng *et al.* 2018). In this research, cytochrome P450 was found in both non-diapausing and diapausing eggs, with expression in diapausing eggs significantly downregulated. Moreover, at the transcriptional level, cytochrome P450 in diapausing oocytes was significantly lower than the non-diapausing cohort, which was consistent with the data at the protein level.

KEGG pathway analysis showed that peroxisome was a highly and significantly enriched pathway. Peroxisome, a subcellular organelle, can catalyze various metabolic reactions, such as fatty-acid β-oxidation, glyoxylate detoxification and plasmalogen biosynthesis (Wanders 2014). Our studies showed that the expression of proteins related to peroxisome differed between the diapausing and non-diapausing eggs in migratory locust. It is evident that genes involved in β-oxidation were strongly suppressed in early diapause (Poelchau *et al.* 2013; Hao *et al.* 2016). Multifunctional enzymes are critical in the second and third reactions of the peroxisomal β-oxidation cycle. In *D. melanogaster*, multifunctional enzyme type 2, a peroxisomal protein, is active in the β-oxidation of fatty acyl-CoAs. In our study, we observed the significant down-regulation of peroxisomal multifunctional enzyme type 2 isoform X1 in diapause eggs. Fatty acyl-CoA reductase 1,

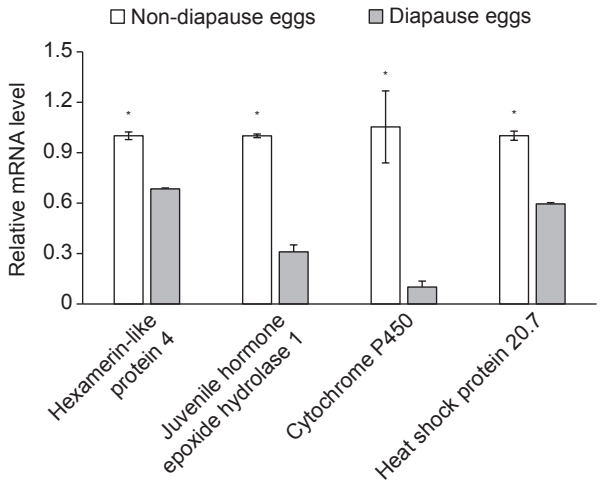

Fig. 5 The relative quantitative expression of four specific proteins using $2^{-\Delta\Delta C_T}$ methods. * indicate a significant difference of mRNA expression between non-diapause eggs and diapause eggs (independent sample *t*-test, $P<0.05$).

a critical enzyme in plasmalogen biosynthesis, can reduce fatty acids into their respective fatty alcohols (Cheng and Russell 2004; Honsho *et al.* 2013). The significant increased expression of five genes encoding the fatty acyl-CoA reductase 1 has been observed in oocytes of *Bombyx mori* producing diapausing eggs (Chen *et al.* 2017). The putative fatty acyl-CoA reductase was upregulated in diapausing eggs in this study. However, the difference was insignificant. Additionally, superoxide dismutase (Cu-Zn)-like precursor and superoxide dismutase (Mn), two typical antioxidant enzymes were less abundant in diapausing eggs than that in non-diapause eggs but with no significant difference. Similar results were also observed in diapausing flesh fly, *Sarcophaga crassipalpis*, and bivoltine silkworm, *B. mori* (Ragland *et al.* 2010; Chen *et al.* 2017).

5. Conclusion

Through proteome analysis of both diapausing and non-diapausing eggs, we found that maternal effects may regulate diapause through hormone biosynthesis, the longevity regulating pathway and peroxisome pathway. Our study could be informative for further future research on the mechanisms of diapause induced by maternal effects in migratory locust.

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