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Comparative RNA-Seq analysis of *Ephestia elutella* (Lepidoptera: Pyralidae) reveals genes in response to low temperature

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Abstract
Ephestia elutella, the tobacco moth, is regarded as a serious pest in the storage. *E. elutella* at low temperatures is conducive to parasitoid reproduction. Identifying the genes that respond to low temperature has important guiding significance for the breeding of parasitoid wasps. Transcriptome sequencing analysis of last instar *E. elutella* at normal and low temperature using RNA-seq. This study found that the expression of several genes in *E. elutella* changed significantly under low temperature. Among them, *larvae cuticle protein*, *alkaline phosphatase* and *apyrase* were significantly up-regulated, while *Hsp70* and *Hsp90* were significantly down-regulated. GO functional analysis showed that the genes may be involved in structural constituent of cuticle, catalytic activity, and metabolic process. KEGG functional analysis showed that the genes may be involved in glycolysis/gluconeogenesis and pentose phosphate pathway. These genes and pathways form the basis for subsequent breeding of parasitoid wasps and provide a reference for studying the mechanism of insect response to low temperature.

Keywords: *Ephestia elutella*, transcriptome, low temperature, biological control

Introduction
Ephestia elutella (Lepidoptera: Pyralidae), a storage pest distributed worldwide, primarily feeds on tobacco leaves but also infests stored products such as coffee, cereal, and dried fruits [1]. The prevention and control of *E. elutella* is an important issue that needs to be addressed in warehouse management. Traditionally, fumigants have been used to control *E. elutella*. Fumigation with a certain concentration of phosphine can effectively kill the diapausing larvae of *E. elutella* [2]. Methyl bromide is an effective fumigant and can provide ideal control at certain concentrations and fumigation exposure times [3]. In addition, sublethal concentrations of deltamethrin inhibit the growth and reproduction of *E. elutella* [4]. Due to the long-term use of fumigants as the main method of killing insects, most insects have also developed some degree of resistance [5]. Some pesticides can also cause harm to humans and natural enemies, which is not conducive to maintaining ecological stability [6]. Unfortunately, low lethal concentrations of deltamethrin significantly reduced the pre-adult survival, life table parameters and paralysis rate parameters of *H. hebetor* [7]. To solve the problem of drug resistance caused by traditional prevention and control, various technical means such as joint prevention and control are adopted [8].
Biological control, as a key part of IPM, has played an important role in pest control [9]. For example, *H. hebetor* is commonly used to control *E. elutella* and *Plodia interpunctella* [10, 11]. *Plodia interpunctella* produces more parasitoid wasps under diapause conditions [12]. This is most likely because the insects develop more slowly and accumulate more lipids at low temperatures [12]. Studies have shown that *E. elutella* that has been refrigerated at 4 °C for 30 days can better reproduce *H. hebetor* [14]. In addition, the diapausing larvae of *E. elutella* can survive for 22 days at temperatures as low as -10 °C, showing strong cold tolerance [15]. How *E. elutella* responds to environmental changes at the level of gene expression in low temperature conditions deserves further study. It may provide guidance for better control of *E. elutella* under extreme climatic conditions, while also providing more reference information for expanding parasitoid wasp resources.

In this study, transcriptomics technology was used for the first time to sequence *E. elutella* at low and normal developmental temperatures. Gene sequences were assembled using a de novo assembly strategy, followed by functional annotation and expression analysis. Gene expression quantification screened for DEGs in response to low temperature. Functional enrichment analysis was performed on the DEGs to find pathways and DEGs that respond to changes in low temperature conditions.

Materials and Methods

Insect Rearing, Exposure Temperatures and Sample Preparation

The insect source was obtained from the Natural Enemy Insect Biology Laboratory of Qingjiang Park, Enshi, Hubei, China. A stable laboratory population was selected as the parent species of *E. elutella*. Approximately 50 adults were placed in the egg collection device and fed with 10% honey water. *E. elutella* eggs were collected after 24 hours. Feed ratios for *E. elutella* were based on Bell's method [16]. A total of two treatments were set up, the normal temperature group and the low temperature group, with three replicates for each treatment (Table 1). A 10 cm diameter petri dish was used as the rearing container for each replicate. Place 10 eggs in the petri dish and finally cover the eggs with an appropriate amount of food. After covering the filter paper and the petri dish lid, place the petri dish in an artificial climate box. The temperature of the artificial climate box is 28 ℃±0.5 ℃, the relative humidity is 70%±5% and the photoperiod is L:D=16:8. After 24 hours of culture, three of the culture dishes were transferred to the low temperature group for culture.

Collect last instar larvae from both treatments, at least five from each replicate. The larvae were first cleaned of surface contaminants with PBS, then placed in a threaded tube and frozen in liquid nitrogen for 10 minutes. They were then stored in an ultra-low temperature air conditioner at -80°C for the following experiments.

Table 1: *E. elutella* feeding and handling conditions.

Number	Category	Feeding conditions	Processing conditions
1	N	28°C±0.5°C, RH=70%±5%, L:D=16:8	28°C±0.5°C, RH=70%±5%, L:D=16:8
2	L		20°C±0.5°C, RH=70%±5%, L:D=16:8

RNA Extraction, Illumina Sequencing and De Novo Assembly

Agilent 4200 TapeStation (Agilent Technologies) was used to electrophorese the total RNA extracted from the sample using the MJzol animal RNA Extraction Kit (Majorbio). Total RNA was purified using RNAClean XP Kit (Beckman Coulter) and RNase-Free DNase Set (QIAGEN) after passing the quality inspection. After passing RNA quality control, library construction is performed. The Qubit® 2.0 Fluorometer was used to determine concentration and the Agilent4200 to determine library size. Libraries that passed quality control were sequenced on the Illumina NovaSeq6000 using the PE150 sequencing mode. Use seqtk [17] to filter raw reads to obtain clean Reads. Apply the scaffolding contig algorithm of CLC Genomics Workbench (version: 6.0.4) to perform de novo splicing (word size=45, minimum contig length>=200) to obtain primary UniGene. Use CAP3 [18] splicing software to splice the primary UniGene for the second time to obtain the final UniGene.

Gene Functional Annotation

Blastx [19] compared the final UniGene sequence with the UniProt and NR databases. UniGene was then compared to the CDD database [20] (E-value≤1E-5) and the top five results were selected for KOG functional classification prediction. Next, the UniGene sequence was compared with the Gene Ontology (GO) database using Blastx (E-value≤1E-5) and classified according to molecular function, cellular component, and biological process. Finally, the KEGG KAAS online pathway comparison analysis tool was used to perform KEGG mapping analysis on UniGene.

Gene Expression Quantification

The reads of each sample were aligned with the UniGene reference. The read coverage of each sample on each UniGene was determined. Gene expression quantification was performed using eXpress [21] to obtain the read counts matrix.

Differential Expression Gene Analysis

Differential gene analysis between samples is performed using edge R [22]. The initial p-values undergo correction for multiple hypothesis testing to manage the false discovery rate and generate the q-values. Q-value≤0.05 and |log2Fold-Change|≥1 are the criteria for differential gene screening.

Gene Functional Enrichment Analysis

Analysis of gene function enrichment and visualization of DEGs using clusterProfiler [23].

Results

Sequencing and Assembly Results

A total of 339,827,272 raw reads were obtained by sequencing the six samples, and 314,313,125 clean reads were obtained after filtering and trimming. The first splice yielded 131,506 unigenes, and the second splice yielded 98,465 unigenes. The average length is 786 bp, and the GC content is 40.56%.

Table 2: Transcriptome sequencing and assembly.

Transcriptome sequencing and assembly	Counts
Raw reads	339,827,272
Clean reads	314,313,125
Contigs	151,495
Primary UniGene	131,506
Final UniGene	98,465
Average length	786

Gene Functional Annotation and Classification

The final spliced unigenes were compared and annotated using NR and UniProt. In NR, a total of 41,191 unigenes were annotated, accounting for 41.83%. In UniProt, a total of 38,701 unigenes were annotated, accounting for 39.30%. Among the species distribution annotated by UniProt, *Danaus plexippus*, *Bombyx mori* and *Pararge aegeria* account for more (Fig1A). The E-value distribution is shown in Fig1B. UniProt is a detailed resource for protein sequence and

functional information, and subsequent analyses are based on this database.

Table 3: Gene functional annotation.

aaDatabase type	Unigenes	Annotation rate
NR	41,191	41.83%
UniProt	38,701	39.30%
KOG	45,399	46.11%
GO	25,228	25.62%
KEGG	17,058	17.32%

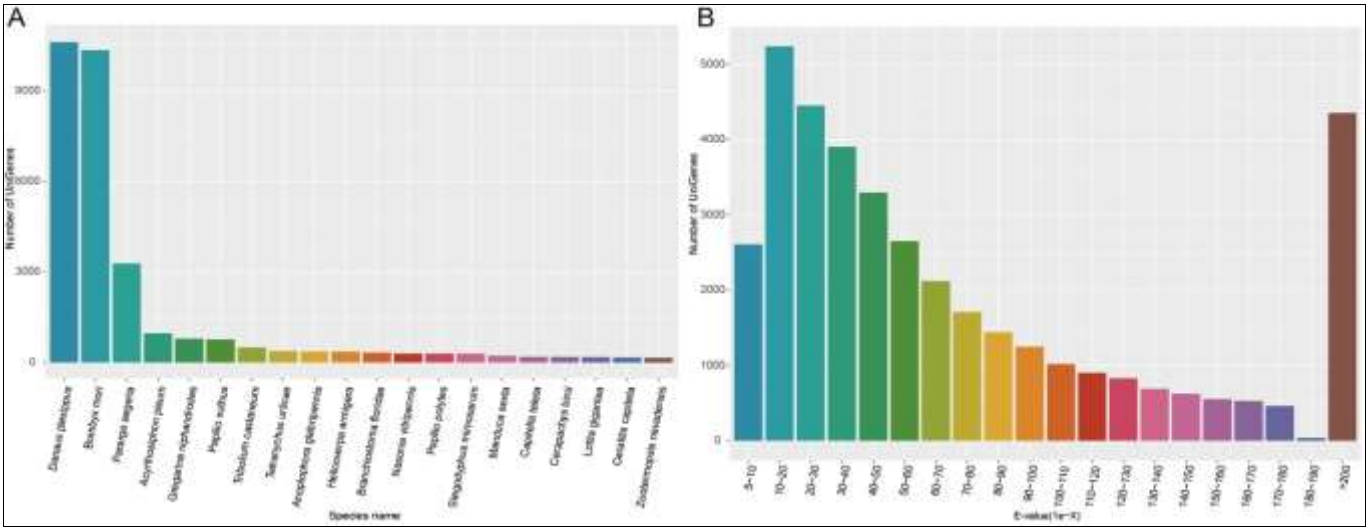

Fig 1: UniProt annotated species distribution map (A) and UniProt E-value distribution map (B)

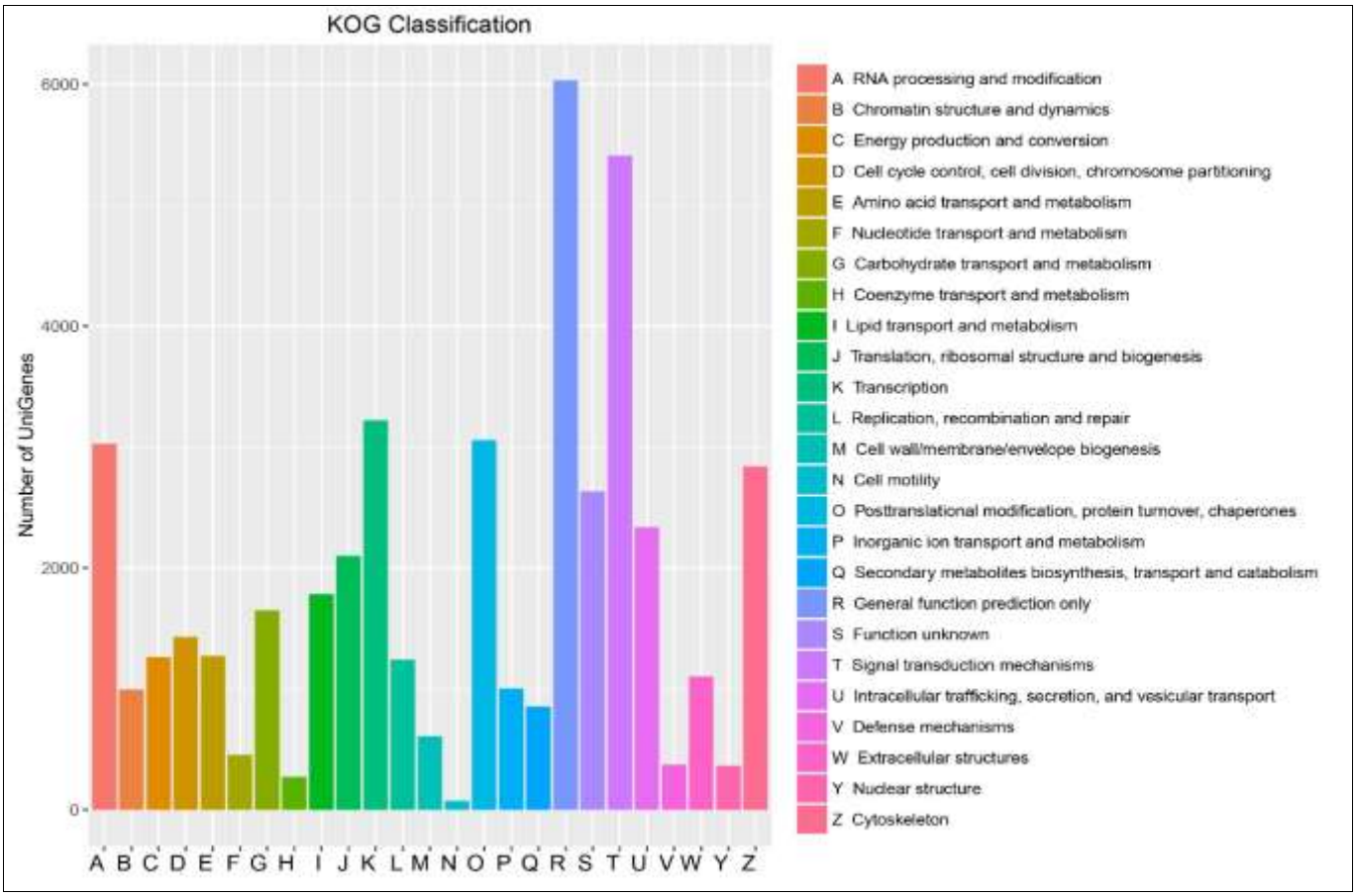

Fig 2: KOG classification of *E. elutella*

In KOG, 45,399 unigenes were assigned to KOG clusters by function (Fig 2). Among them, the three clusters with the most unigenes are General function prediction only, Signal transduction mechanisms, and Transcription, which contain 6,031, 5,410, and 3,222 unigenes respectively. The smallest one is Cell motility, with only 74 unigenes.

In the GO functional classification, the unigenes were grouped into three main GO categories (Fig 3). The three GO categories are biological process, cellular component, and molecular function. Among them, more than 10,000 unigenes were classified into four GO terms (metabolic process, binding, cellular process, catalytic activity). In addition, presynaptic process involved in synaptic transmission, nutrient reservoir activity, metallochaperone activity and protein tag, there is only one unigene among these four GO terms.

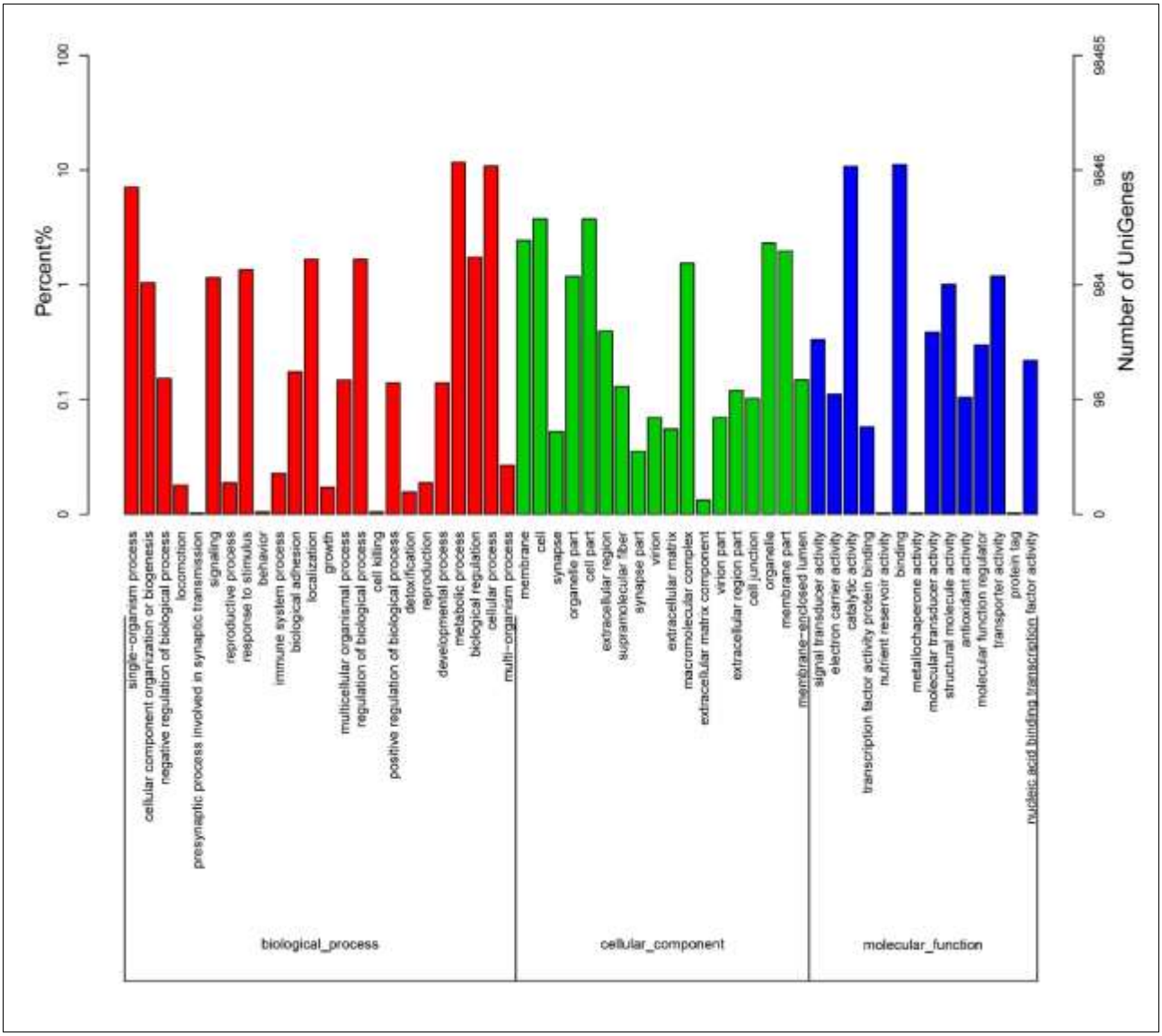

Fig 3: GO classification of *E. elutella*

In the KEGG pathway classification, 17,058 unigenes were classified into 233 pathways, and 32 pathways belonged to 31 secondary pathways (Fig 4). Carbohydrate metabolism accounts for the largest proportion, with a total of 11,259 unigenes. Signaling molecules and interaction account for the smallest proportion, with only 44 unigenes.

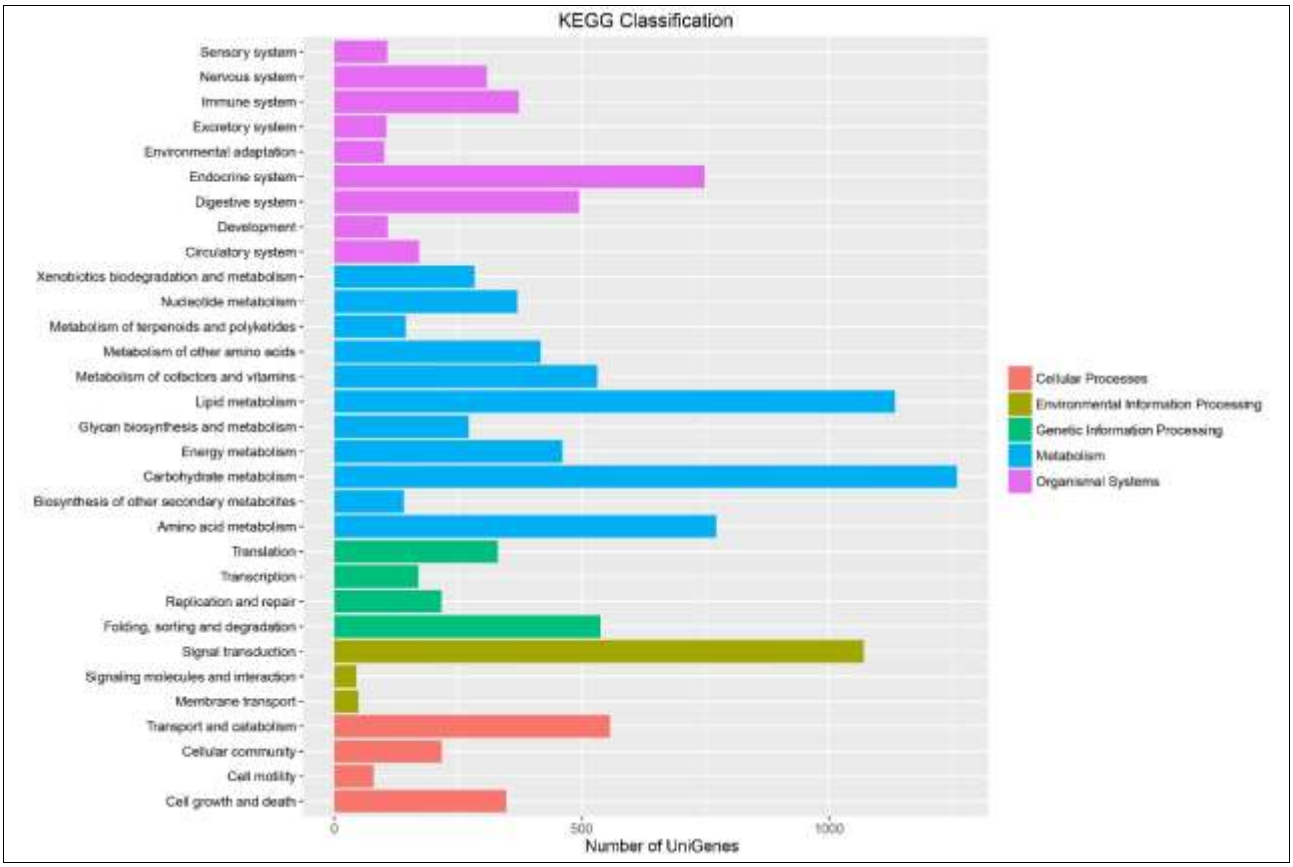

Fig 4: KEGG pathway classification of *E. elutella*

Differentially Expression Gene at Low Temperature

Correlation analysis between samples showed that the difference between groups under the two temperature treatments was greater than the difference within the group (Fig 5A). Comparing the transcriptome data at different temperatures, a total of 3,700 DEGs were obtained, including 536 up-regulated genes and 3,164 down-regulated genes (Fig 5B). Among the up-regulated genes, the most significantly differentially expressed gene was *Contig_2323*, which was annotated as larvae cuticle protein (log_2FC=16.23, Q-value=2.08E-17). The most significantly down-regulated gene was *Contig_58444*, annotated as inner membrane complex protein (log_2FC=-15.81, Q-value=2.41E-17). There are two important down-regulated genes: *Hsp90-like protein* (*First_Contig11053*, log_2FC=-19.13, Q-value=2.92E-17) and *Heat shock protein 70* (*First_Contig7185*, log_2FC=-19.06, Q-value=2.92E-17). In addition to the most significantly expressed genes, we also found some other genes in the top 10 significant DEGs. For example, *alkaline phosphatase*, chymotrypsin-like serine protease, apyrase, NAD (P) transhydrogenase, etc. The top 10 up- and down-regulated DEGs are shown in Table 4.

Table 4: The top 10 DEGs with between L and N of *E. elutella*.

Unigene id	Annotation	Q-value	updown
Contig_2323	Larvae cuticle protein	2.08E-17	UP
Contig_763	Putative cuticle protein	1.20E-15	UP
Second_Contig1240	Alkaline phosphatase	2.03E-13	UP
Second_Contig823	Putative cuticle protein	2.87E-12	UP
Second_Contig762	Cuticular protein CPFL4A	5.10E-10	UP
Contig_3500	Apyrase	6.20E-10	UP
Contig_13048	Cuticular protein PpolCPH6	7.06E-10	UP
Contig_28154	Putative cyclopropane fatty acid synthase	1.47E-09	UP
First_Contig754	Chymotrypsin-like protease C2	2.87E-09	UP
Contig_5260	Putative cuticle protein	3.70E-09	UP
Contig_58444	Inner membrane complex protein	2.41E-17	DOWN
Contig_112099	Alpha-1,4 glucan phosphorylase	2.92E-17	DOWN
Contig_112114	Putative thrombospondin type 1 domain protein	2.92E-17	DOWN
First_Contig11053	Hsp90-like protein	2.92E-17	DOWN
First_Contig7185	Heat shock protein 70	2.95E-17	DOWN
First_Contig11025	NAD(P) transhydrogenase	2.95E-17	DOWN
Contig_112250	Fructose-bisphosphate aldolase	3.02E-17	DOWN
Contig_75103	Sugar transporter	3.02E-17	DOWN
Contig_112299	AAA domain protein	3.02E-17	DOWN
First_Contig11231	Adaptor-related complex 1 mu 1 subunit	3.02E-17	DOWN

Note: The rankings are sorted by Q-value, excluding unannotated unigenes

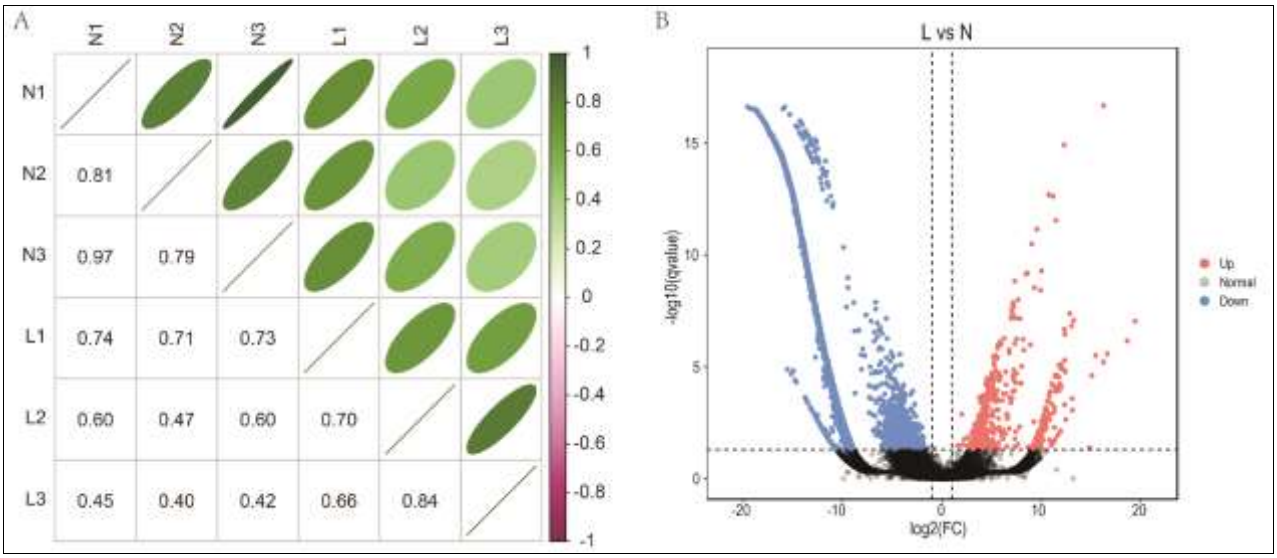

Fig 5: Gene expression correlation plot (A) and volcano plot of DEGs (B)

GO and KEGG Enrichment Analysis

To further understand the biological functions and pathways associated with DEGs, we performed GO and KEGG enrichment analyses. DEGs were enriched in 1962 GO terms, of which 89 GO terms were significantly enriched pathways (Q-value≤0.01). There were 31, 43, and 15 significantly enriched pathways in biological process (BP), molecular function (MF) and cellular component (CC), respectively. In BP, metabolic process (1144 unigenes), protein metabolic process (373 unigenes) and organonitrogen compound metabolic process (197 unigenes) enriched more DEGs. In CC, 330 unigenes were enriched in intracellular part. In MF, more unigenes are enriched in catalytic activity (995 unigenes), hydrolase activity (484 unigenes), small molecule binding (385 unigenes) and nucleotide binding (375 unigenes). The top 10 significantly enriched GO are shown in the Table 5.

Table 5: The top 10 significantly enriched GO terms.

GO ID	GO term	Type	DEGs	Q-value
GO:0003824	catalytic activity	molecular function	995	7.12E-19
GO:0016787	hydrolase activity	molecular function	484	4.46E-14
GO:0036094	small molecule binding	biological process	385	3.40E-09
GO:0000166	nucleotide binding	biological process	375	5.45E-09
GO:1901265	nucleoside phosphate binding	biological process	375	5.45E-09
GO:0005840	ribosome	biological process	78	1.29E-08
GO:0030529	intracellular ribonucleoprotein complex	biological process	94	3.69E-08
GO:1990904	ribonucleoprotein complex	molecular function	94	3.69E-08
GO:0008152	metabolic process	molecular function	1144	7.06E-08
GO:0043168	anion binding	molecular function	346	2.45E-07

In the KEGG enrichment analysis results, 3337 unigenes were enriched in 325 KEGG pathways, of which 10 were significantly enriched (Q-value≤0.05). Galactose metabolism was the most significantly enriched pathway (ko00052, Q-value=1.59E-02). 31 DEGs were enriched in this pathway, including 22 up-regulated genes and 9 down-regulated genes. The significantly enriched Top10 pathways are shown in Table 6.

Table 6: Top10 KEGG enrichment analysis.

ko ID	Pathway	DEGs	Q-value
ko00052	Galactose metabolism	31	1.59E-02
ko00730	Thiamine metabolism	11	1.59E-02
ko00500	Starch and sucrose metabolism	27	1.59E-02
ko04974	Protein digestion and absorption	22	1.59E-02
ko00030	Pentose phosphate pathway	27	2.09E-02
ko00051	Fructose and mannose metabolism	28	2.17E-02
ko00010	Glycolysis / Gluconeogenesis	42	3.87E-02
ko00480	Glutathione metabolism	34	3.87E-02
ko04910	Insulin signaling pathway	31	3.87E-02
ko04341	Hedgehog signaling pathway - fly	10	3.87E-02

Discussion

This study used RNA-seq to analyze *E. elutella* at normal and low temperature and found some significant DEGs. Up-regulated genes include encoding *larvae cuticle protein*, *alkaline phosphatase*, and *apyrase*. Down-regulated genes include *Hsp70* and *Hsp90*.

Insects, as cold-blooded animals, have body temperatures and metabolic activities that are affected by changes in environmental temperature [24]. Insect exoskeletons are composed of chitin and cuticle proteins, and genes encoding cuticle proteins have been implicated in the molecular mechanisms of ecdysteroids and juvenile hormone signaling [25]. In addition, the larval cuticle can protect the larvae from surviving in harsh environments, especially by preventing the loss of body water [26, 27]. In this study, upregulation of many *larvae cuticle protein* genes (*LCP*) was detected in *E. elutella* under low temperature. We believe that this helps *E. elutella* to better protect itself from the external environment at low temperatures, ensuring that individuals can survive the cold winter. Similarly, Carrasco *et al*. also found that the expression of larval cuticle protein A2B was up-regulated in winter samples of *Cucujus clavipes puniceus* and speculated that the up-regulation of this protein can strengthen the cuticle to prevent cross-cuticle ice nucleation [28]. In GO enrichment analysis, 24 DEGs were significantly enriched in structural constituents of cuticle (GO:0042302, Q-value=5.10E-03). Overall, *LCP* plays an important role in cold hardiness in the larval stage of *E. elutella*.

Another key up-regulated gene is *Alkaline phosphatase* (*ALP*). ALP exists in some glands [29], Malpighian ducts [30], alimentary tract [31] and storage tissue of insects. ALP plays a key role in the toxic effects of Cry1Ac δ-endotoxin on *Helicoverpa armigera* larvae [32]. Moreover, ALP also holds significance in resistance of insect. Under heat stress, ALP activity is reduced in *Drosophila* of line 101 [33]. Low temperature significantly increased the activity of phosphatase (acidic and alkaline) in fifth instar larvae of *Philosamia ricini* [34]. In summary, the *ALP* gene is significant in the heat stress and cold resistance of insects. Apyrases are enzymes that hydrolyze nucleotide di- and triphosphates to orthophosphate and mononucleotides [35]. Gilmour and Calaby discovered a higher concentration of apyrase in the muscles of insects, and that its form is more active [36]. Insects in lower chill-coma temperatures have higher apyrase activity and lower temperature coefficient, which is beneficial for insects to maintain their physiological functions (muscle contraction) by regulating ATP hydrolysis under low temperature conditions [37, 38]. In *E. elutella* larvae under low temperature, the *apyrase* gene also showed a significant down-regulation trend. It is worth mentioning that in this experiment, *E. elutella* did not enter the diapause state at 20 °C, and still maintained basic life activities in a relatively slow developmental state. This is consistent with Bell's findings, which showed that laboratory populations require short days of light at 20 °C to enter diapause [39]. We speculate that the up-regulated genes contribute to the adaptation of the laboratory population to low temperatures of 20 °C.

In addition to the up-regulated genes, we also noticed two genes that were down-regulated (*Hsp70*, *Hsp90*). *Hsp* is related to stress in insects, especially in response to low temperature and diapause [40]. In diapausing pupae of *Rhagoletis pomonella*, *Hsp70* is highly up-regulated but *Hsp90* is down-regulated [41]. During the diapause of *Sesamia nonagrioides*, *Hsp70* gene is down-regulated while *Hsp90* is up-regulated [42, 43]. In this study, it was found that both *Hsp70* and *Hsp90* were down-regulated in *E. elutella* larvae. The results suggest that *Hsp70* and *Hsp90* may have different functions in different species.

Insects utilize several strategies to tolerate cold, including ice nucleating agents, polyhydroxy alcohols (polyols), and sugars [44]. The synthesis of polyol metabolism is jointly regulated by multiple pathways including glycolysis, gluconeogenesis, and pentose phosphate [45]. KEGG enrichment analysis revealed that DEGs were significantly enriched in processes such as glycolysis/gluconeogenesis and pentose phosphate pathway. This suggests that *E. elutella* produces polyol substances in low temperatures to protect itself from freezing damage. Further research is needed.

Conclusion

This study found that under a low temperature of 20°C, some genes (such as *larvae cuticle protein*, *alkaline phosphatase*, and *apyrase*) are up-regulated in *E. elutella*. Two heat shock protein genes (*Hsp70* and *Hsp90*) showed a down-regulation trend. These results show how *E. elutella* responds to low temperature conditions at the genetic level. In addition, through GO and KEGG enrichment analysis, it was found that DEGs at low temperature are involved in molecular functions and biological processes such as structural constituent of cuticle, glycolysis/gluconeogenesis, and pentose phosphate pathway. Although this study preliminarily explored the differences in transcriptomics of *E. elutella* at low temperature, there are still certain limitations. In particular, the natural population of *E. elutella* can still survive normally at extremely low temperatures [15]. Lower temperatures should be set in the future to study its adaptation mechanism to low temperatures.

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Availability of data

The raw datasets generated during the sequencing of the current study are available in SRA under the BioProject PRJNA1093250 (https://www.ncbi.nlm.nih.gov/sra/PRJNA1093250).

Conflict of interest declaration

The author declare that they have no competing interests.

References

1. Berry BJL, Freese F, Shepherd H, Baker JA, Dowden PB, Botts RR, *et al*. Stored Tobacco Insects: Biology and Control. Agricultural Research Service, US Department of Agriculture, Washington, D.C.; c1962.
2. Bell CH. The efficiency of phosphine against diapausing larvae of *Ephestia elutella* (Lepidoptera) over a wide range of concentrations and exposure times. Journal of Stored Products Research. 1979;15(2):53-58.
3. Bell CH, Glanville V. The effect of concentration and exposure in tests with methyl bromide and with phosphine on diapausing larvae of *Ephestia elutella* (Hübner) (Lepidoptera, Pyralidae). Journal of Stored Products Research. 1973;9(3):165-170.
4. Yuan M, Wei ZY, Yang H, Ou HD, Yang MF, Jin X, *et al*. Effects of sublethal concentrations of deltamethyrin on the biological characteristics of *Ephestia elutella* Hiibner. Journal of Environmental Entomology. 2020;42(2):459-470.
5. Bell CH. A review of insect responses to variations encountered in the managed storage environment. Journal of Stored Products Research. 2014;59:260-274.
6. Pathak VM, Verma VK, Rawat BS, Kaur B, Babu N,

Sharma A, *et al*. Current status of pesticide effects on environment, human health and it's eco-friendly management as bioremediation: A comprehensive review. Frontiers in Microbiology; c2022. p. 13.
7. Wang XQ, Ou HD, Li HX, Wei L, Huang Y, Yang MF, *et al*. Adverse impacts of development on the deltamethrin-exposed host *Ephestia elutella* (Lepidoptera: Pyralidae) on population parameters of *Habrobracon hebetor* (Hymenoptera: Braconidae). Crop Protection. 2023;167:106211.
8. Trematerra P. Combined control of *Lasioderma serricorne* (F.) and *Ephestia elutella* (Hbn.) in a tobacco processing facility by attracticide method. Journal of Applied Entomology. 2020;144(7):598-604.
9. Scholler M. Forager in the rye: biological control of *Ephestia elutella* in bulk grain. IOBC wprs Bulletin. 2000;23(10):149-160.
10. Pezzini C, Zilch KCF, Jahnke SM, Köhler A. Effect of *Habrobracon hebetor* (Hymenoptera: Braconidae) Release on Moth Infestation in Stored Tobacco. Brazilian Archives of Biology and Technology. 2023;66.
11. Adarkwah C, Schöller M. Biological control of *Plodia interpunctella* (Lepidoptera: Pyralidae) by single and double releases of two larval parasitoids in bulk stored wheat. Journal of Stored Products Research. 2012;51:1-5.
12. Sanower W, Mbata GN, Payton ME. Improvement of reproductive performance of *Habrobracon hebetor*: Consideration of diapausing and non-diapausing larvae of *Plodia interpunctella*. Biological Control. 2018;118:32-36.
13. Ferguson LV, Kortet R, Sinclair BJ. Eco-immunology in the cold: the role of immunity in shaping the overwintering survival of ectotherms. Journal of Experimental Biology. 2018;221(13):jeb163873.
14. Wei L, Ou HD, Wang XQ, Wang B, Yu XF, Yang MF, *et al*. Effects of cold storage of the tobacco moth *Ephestia elutella* on the fitness of ectoparasitoid wasp *Habrobracon hebetor*. Journal of Plant Protection. 2023;50(1):119-128.
15. Bell CH. Diapause and cold tolerance of larvae of *Ephestia elutella*. Postharvest Biology and Technology. 1991;1(1):81-93.
16. Bell CH, Walker DJ. Diapause induction in *Ephestia elutella* (Hübner) and *Plodia interpunctella* (Hübner) (Lepidoptera, Pyralidae) with a dawn-dusk lighting system. Journal of Stored Products Research. 1973;9(3):149-158.
17. Li H. seqtk Toolkit for processing sequences in FASTA/Q formats. GitHub. 2012;767:69.
18. Huang X, Madan A. CAP3: A DNA sequence assembly program. Genome research. 1999;9(9):868-877.
19. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. Journal of Molecular Biology. 1990;215(3):403-410.
20. Marchler-Bauer A, Panchenko AR, Shoemaker BA, Thiessen PA, Geer LY, Bryant SH, *et al*. CDD: A database of conserved domain alignments with links to domain three-dimensional structure. Nucleic Acids Research. 2002;30(1):281-283.
21. Roberts A, Pachter L. Streaming fragment assignment for real-time analysis of sequencing experiments. Nature Methods. 2013;10(1):71-73.
22. Robinson MD, Mccarthy DJ, Smyth GK. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2010;26(1):139-140.
23. Wu TZ, Hu EQ, Xu SB, Chen MJ, Guo PF, Dai ZH, *et al*. clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. The Innovation, 2021, 2(3).
24. Mellanby K, Gardiner JS. Low temperature and insect activity. Proceedings of the Royal Society of London Series B - Biological Sciences. 1939;127(849):473-487.
25. Charles JP. The regulation of expression of insect cuticle protein genes. Insect Biochemistry and Molecular Biology. 2010;40(3):205-213.
26. Moussian B. Recent advances in understanding mechanisms of insect cuticle differentiation. Insect Biochemistry and Molecular Biology. 2010;40(5):363-375.
27. Chapman RF. The insects: structure and function, Cambridge university press, Cambridge; c1998.
28. Carrasco MA, Buechler SA, Arnold RJ, Sformo T, Barnes BM, Duman JG, *et al*. Elucidating the biochemical overwintering adaptations of larval *Cucujus clavipes puniceus*, a nonmodel organism, via high throughput proteomics. Journal of proteome research. 2011;10(10):4634-4646.
29. Funk CJ. Alkaline phosphatase activity in whitefly salivary glands and saliva. Archives of Insect Biochemistry and Physiology. 2001;46(4):165-174.
30. Cabrero P, Pollock VP, Davies SA, Dow JaT. A conserved domain of alkaline phosphatase expression in the Malpighian tubules of dipteran insects. Journal of Experimental Biology. 2004;207(19):3299-3305.
31. Day M. The distribution of alkaline phosphatase in insects. Australian Journal of Biological Sciences. 1949;2(1):31-41.
32. Upadhyay SK, Singh PK. Role of alkaline phosphatase in insecticidal action of Cry1Ac against *Helicoverpa armigera* larvae. Biotechnology Letters. 2011;33(10):2027-2036.
33. Sukhanova MZ, Grenback LG, Gruntenko NE, Khlebodarova TM, Rauschenbach IY. Alkaline phosphatase in Drosophila under heat stress. Journal of Insect Physiology. 1996;42(2):161-165.
34. Singh A, Sharma RK, Sharma B. Low temperature induced alterations in certain biochemical constituents of 5th instar larvae of *Philosamia ricini* (Lepidoptera: Satunidae). Open Access Insect Physiology. 2010;2:11-16.
35. Valenzuela JG, Belkaid Y, Rowton E, Ribeiro JM. The salivary apyrase of the blood-sucking sand fly *Phlebotomus papatasi* belongs to the novel Cimex family of apyrases. Journal of Experimental Biology. 2001;204(2):229-237.
36. Gilmour D, Calaby JH. The magnesium-activated apyrase of insect muscle. Archives of Biochemistry and Biophysics. 1952;41(1):83-103.
37. Mutchmor JA, Richards AG. Low temperature tolerance of insects in relation to the influence of temperature on muscle apyrase activity. Journal of Insect Physiology. 1961;7(2):141-158.
38. Thiessen CI, Mutchmor JA. Some effects of thermal acclimation on muscle apyrase activity and mitochondrial number in *Periplaneta americana* and *Musca domestica*. Journal of Insect Physiology. 1967;13(12):1837-1842.
39. Bell CH. Factors governing the induction of diapause in *Ephestia elutella* and *Plodia interpunctella*

(Lepidoptera). Physiological Entomology. 1976;1(2):83-91.
40. King AM, Macrae TH. Insect heat shock proteins during stress and diapause. Annu Rev Entomol. 2015;60:59-75.
41. LOPEZ-MARTINEZ G, Denlinger DL. Regulation of heat shock proteins in the apple maggot *Rhagoletis pomonella* during hot summer days and overwintering diapause. Physiological Entomology. 2008;33(4):346-352.
42. Gkouvitsas T, Kontogiannatos D, Kourti A. Cognate *Hsp70* gene is induced during deep larval diapause in the moth *Sesamia nonagrioides*. Insect Molecular Biology. 2009;18(2):253-264.
43. Gkouvitsas T, Kontogiannatos D, Kourti A. Expression of the *Hsp83* gene in response to diapause and thermal stress in the moth *Sesamia nonagrioides*. Insect Molecular Biology. 2009;18(6):759-768.
44. Bale JS. Insects in the cold. Endeavour. 1993;17(3):132-137.
45. Storey KB, Storey JM. Insect cold hardiness: metabolic, gene, and protein adaptation. Canadian Journal of Zoology. 2012;90(4):456-475.