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Characterization and Developmental Expression Patterns of Four Hexamerin Genes in the Bumble Bee, *Bombus terrestris* (Hymenoptera: Apidae)

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Abstract

Hexamerins are members of the hemocyanin superfamily and play essential roles in providing amino acids and energy for the nonfeeding stages of insects. In this study, we cloned and analyzed the expression patterns of four hexamerin genes (*hex 70a*, *hex 70b*, *hex 70c*, and *hex 110*) at different worker development stages and queen diapause statuses in the bumble bee, *Bombus terrestris*. The results of this study showed that *hex 110* has the longest open reading frame (ORF; 3,297 bp) compared to the ORFs of *hex 70a* (2,034 bp), *hex 70b* (2,067 bp), and *hex 70c* (2,055 bp). The putative translation product of Hex 70a, Hex 70b, Hex70c, and Hex 110 has 677, 688, 684, and 1,098aa with predicted molecular mass of 81.13, 79.69, 81.58, and 119 kDa. In the development stages of workers, the expression levels of *hex 70a*, *hex 70b*, and *hex 70c* increased gradually from the larval stage and exhibited high expression levels at the pink eyed and brown eyed pupae stage, whereas *hex 110* exhibited the highest expression level at the larval period. Four hexamerin genes were highly expressed at the prediapause status of queen ($P < 0.05$), and compared to the eclosion queen, the lowest upregulation was 3.7-fold, and the highest upregulation was 1,742-fold. The expression levels of *hex 70b*, *hex 70c*, and *hex 110* at diapause were significantly higher than those at postdiapause ($P < 0.05$). In conclusion, hexamerins may play important roles in queen diapause and metamorphosis of larval and pupal stages.

Key words: *Bombus terrestris*, hexamerin gene, diapause, expression profile, development

Bumble bees are important pollinators for maintaining plant biodiversity in the wild and providing pollination services to crops (Garratt et al. 2014, Carvell et al. 2017). As annual insects, the colony of bumble bees was established by one queen in the early spring and collapsed during the autumn (Alford 1969). Storage proteins, as an amino acid reserve, are essential for the queen founded colony of social insects (Wheeler et al. 1996). In bumble bees, young fertilized queens need to accumulate enough storage protein and energy to ensure their survival during winter (Woodard et al. 2019). Moreover, the queen also needs to acquire sufficient protein for laying eggs and feeding offspring during the solitary stage in the early spring (Votavová et al. 2015, Bogo et al. 2017). Storage proteins were also proven to provide amino acids for the worker development of other social insects (Sorensen et al. 1981). Thus, analyzing the expression patterns of hexamerins during the nonfeeding period is very important for colony founding and success diapause of bumble bees.

Hexamerins, as an important storage protein, are widely present in insects (Telfer et al. 1991, Tang et al. 2010), and the native molecular mass of those proteins is approximately 500 kDa, which consists of six subunits each of approximately 80 kDa (Burmester, 2002). Hexamerins belong to the Arthropod hemocyanin superfamily, which includes hemocyanins, prophenoloxidases, and arylphorin-receptor proteins (Decker et al. 2000). During evolution, hexamerins lost their ability to bind oxygen molecules and gradually became nutrient storage proteins (Beintema et al. 1994). Most hexamerins are predominantly synthesized by fat body cells and are secreted into the hemolymph during nonfeeding (Tang et al. 2010). Hence, the well-known function of hexamerins is to provide amino acids and energy during nonfeeding periods, which play vital roles in the metamorphic development of insects. Moreover, hexamerins affect the reproduction of insects. Hexamerins are related to the egg production in *Plutella xylostella* (Wheeler et al. 2000). In termite species, hexamerins have an impact on reproductive plasticity (Zhou et al. 2006) and are closely related to colony foundation (Martinez et al. 1994, Johnston et al. 2007).

Four hexamerins were identified in honeybees in 1998, namely, hexamerin 70a (*hex 70a*), hexamerin 70b (*hex 70b*), hexamerin 70c

(*hex 70c*), and hexamerin 110 (*hex 110*; Danty et al. 1998). The *hex 70b* was observed to be closely related to honey bee larval and prepupal development. Moreover, *hex 70b* can be utilized to compensate for the lack of dietary protein, which responds to the regulation of juvenile hormones (Ryan et al. 1984, Danty et al. 1998). The *hex 110* was found to have similar expression characteristics to *hex 70b* during the development stage (Bitondi et al. 2006). The cellular localization showed that *hex 110* was in the cytoplasm and nucleoli of ovaries in adult females (Martins et al. 2016). The structure, molecular evolution, and functional prediction of four hexamerin genes were found to be regulated by JH in honeybees. The *hex 70a* and *hex 110* genes were determined to be expressed in adults, and the *hex 110* gene was observed to be highly expressed in the ovaries of queen (Martins et al. 2010). In other hymenopteran insects, such as *Solenopsis invicta*, four hexamerin genes were identified, and their expression levels were associated with reproductive division of labor and JH titer (Hawkings et al. 2019). In bumble bee, four hexamerin genes were found in the *B. terrestris* genome and three of these genes were found to be conserved by genomic clustering (Sadd et al. 2015). The *hex 70b* was found to have a relatively higher expression in *B. terrestris* larva using transcriptomic analyses (Colgan et al. 2011). Moreover, the expression of hexamerin genes would be upregulation in worker-destined larvae of *B. terrestris* (Pereboom et al. 2005).

In this study, four hexamerin genes from bumble bees, *B. terrestris*, were cloned, and their cDNA/gene structures were compared. The conserved domain of putative proteins were analyzed. Homologs of hymenopteran insect hexamerins were employed to evaluate the phylogenetic relationships. In addition, we analyzed the expression profiles of four hexamerin genes at different worker development stages and queen status. Our results expand the existing knowledge regarding the hexamerin gene family in social insects and may ultimately provide insights for the factory rearing of bumble bee, *B. terrestris*.

Materials and Methods

Samples

Bumble bee, *Bombus terrestris*, colonies were obtained from the Institute of Apicultural Research, Chinese Academy of Agricultural Sciences, Beijing, China. The bumble bee colony before the competition stage is used to collect samples which contains one queen and about 50 workers. The bees were maintained in nest-boxes under a constant darkness room with a temperature of 29 ± 0.5°C and relative humidity 55–65%. Bumble bees were supplied ad libitum with 50% sugar solution of Korean refined sugar (TS corporation, Korean) and fresh frozen pollen collected from honey bee colonies.

To understand the expression patterns of four hexamerin genes at various developmental stages of workers, nine ontogenetic stages of workers were collected, including eggs within 24 h of laying (egg), larvae (eggs hatched approximately 96 h, L), white eyed pupae with an unpigmented cuticle (Pw), pink eyed pupae with an unpigmented cuticle (Pp), brown eyed pupae with an unpigmented cuticle (Pb), brown eyed pupae with thoracic pigmentation (Pb1), brown eyed pupae with a dark pigmented cuticle (Pbd), dark eyed pupae with a dark pigmented cuticle (Pdd), and dark eyed pupae with a dark pigmented and hair (Pdh; Tian et al. 2018, Guan et al. 2019, Dong et al. 2020). Samples were collected from the initial stage of the colony to ensure that bees were of the worker caste. Six eggs were pooled as a biological replicate. Three biological replicates of each stage were sampled.

To research the expression feature of four hexamerin genes at the queen life cycle, eclosion (newly emerged within 24 h), prediapause (six days after mating), diapause (diapause 15 d), and postdiapause (15 d after diapause) queens were collected. The fat body of per status queen were dissected under a stereomicroscope (Olympus, Japan), and three individuals per status were mixed as separate biological replicates. Then, the fat body was frozen in liquid nitrogen immediately after the dissection. All the tissues were stored at −80°C until use. Three biological replicates of each status were sampled.

Molecular Cloning Full Length of Four Hexamerin Gene

Total RNA was isolated from the larvae of *B. terrestris* using TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. The concentration and quality of RNA were quantified using Nanodrops (ND-2000, USA), and the integrity of RNA was assessed by agarose gel electrophoresis. Primers were designed from the predicted hexamerin gene sequence of *B. terrestris* with GenBank accession numbers: XM_003401730, XM_012314274, XM_003401733, and XM_003401734 (Supp Table 1 [online only]). The 5′ UTRs of hexamerin genes were cloned using a Smart RACE cDNA amplification kit (Clontech, USA) according to the manufacturer's instructions. Amplification was performed for 5′ UTR as follows: 35 cycles of 30 s at 94°C, 30 s at 68°C and 3 min at 72°C and a final extension step at 72°C for 5 min. The 3′ UTR of hexamerin genes was cloned using Takara RNA PCR Kit (AMV) Ver.3.0 (Takara, China) according to the manufacturer's instructions. Thermal cycling conditions were as follows: 35 cycles of 30 s at 94°C, 30 s at 55°C and 2 min at 72°C and a final extension step at 72°C for 5 min. To obtain the four hexamerin gene fragments between the 5′ UTR and 3' UTR, La-Taq DNA polymerase (Takara, China) was used in PCRs. PCR parameters were as follows: 94°C for 5 min, 35 cycles of 94°C for 30 s, 55–62°C for 30 s, and 72°C for 3 min followed by extension at 72°C for 10 min. The PCR products were analyzed by agarose gel electrophoresis and purified using an Agarose Gel DNA recovery kit (Real-Times, China) according to the manufacturer's protocol. Then, gel-purified PCR fragments were cloned into pMD19-T vectors (Takara, China) and transformed into Trans-T1 competent cells (Transgen, China). Positive colonies were sequenced using universal M13 primers from both ends by commercial sequencing company.

Real-Time Quantitative PCR Analysis

Total RNA of all samples was extracted using TRIzol reagent (Invitrogen, USA). One microgram of total RNA was used as a template for cDNA synthesis using the PrimeScript RT reagent kit with gDNA Eraser (Takara, China) according to the manufacturer's protocol. Amplification was performed on an Mx3000p system (Agilent, USA) and carried out in 20-µl reaction volumes containing 10 µl SYBR Premix Ex Taq II kit (Takara, China), 1 µl first-stranded cDNA, 1 µl upstream primer, 1 µl downstream primer, and 7 µl ddH_2O (Supp Table 1 [online only]). The expression levels of four hexamerin genes were normalized to the geometric mean of reference genes, β-*actin* and *rp49* (Lourenço et al. 2008). PCR conditions were 94°C for 5 min followed by 40 cycles of 94°C for 10 s and 60°C for 34 s. Three technical replicates were performed. The relative expression levels of the four hexamerin genes were estimated using the $2^{-\Delta\Delta Ct}$ method (Livak et al. 2001). Three biological replicates and three technical replicates per sample were employed.

Sequence and Molecular Phylogenetic Analysis

The nucleotide sequences were verified, merged, and aligned using BioEdit (version 7.2.1; Hall 1999). Open reading frame (ORF) detection was performed using the NCBI online tools (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The protein isoelectric point values (pI),

amino acid composition, and molecular weight (Mw) were calculated using the sequence manipulation suite (Version 2; http://www.detaibio.com/sms2; Stothard 2000). Conserved domains of *hex 70a*, *hex 70a*, *hex 70c*, and *hex 110* were predicted by the NCBI conserved domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi/). The site of a signal peptide was predicted by the web program SignalP 5.0 (http://www.cbs.dtu.dk/services/SignalP/; Almagro Armenteros et al. 2019). Putative protein sequences of the four hexamerin genes were used as queries to search for other homologs using the BLASTP programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The protein sequences were aligned using MUSCLE. The JTT+G model, which had the lowest values of Akaike information criteria and Bayesian information criterion, was chosen for subsequent phylogenetic analyses. Neighbor-joining trees were built using MEGA X (Kumar et al. 2018) using the bootstrap (1,000 replications) method. The sequence alignment and identity of the exon was performed by DNAMAN software version 9 (https://www.lynnon.com/dnaman.html).

Statistical Analysis

All data were statistically analyzed using IBM SPSS Statistics 20. One-way ANOVAs were employed for the analyses followed by Tukey's test. The data were determined to have homogeneity of variances with the Levene test, and data were log-transformed where necessary (Huang et al. 2009). The data are presented as the means ± SEM of at least three independent experiments, and P-values < 0.05 were considered to indicate significant differences.

Results

Structural and Sequential Characteristics of Four *B. terrestris* Hexamerin Genes

Four full-length cDNAs of hexamerin genes were obtained from bumble bee *B. terrestris* by RACE PCR. Results demonstrated that the full-length cDNA of *hex 70a* (GenBank accession number: MW067149) was 2,155 bp and contained a 35-bp 5′ UTR and 86 bp 3′ UTR with polyadenylation signal (AATAAA), the full-length cDNA of *hex 70b* (GenBank accession number: MW067150) was 2,193 bp and contained a 15-bp 5′ UTR and 111-bp 3′ UTR with polyadenylation signal (AATAAA), the full-length cDNA of *hex 70c* (GenBank accession number: MW067151) was 2,201 bp and contained a 52-bp 5′ UTR and 94-bp 3′ UTR with polyadenylation signal (AATAAA), and the full-length cDNA of *hex 110* (GenBank accession number: MW067152) was 3,445 bp and contained a 26-bp 5′ UTR and 122-bp 3′ UTR with nonclassical polyadenylation signal (ATTAAA; Fig. 1A). The coding sequences of four hexamerin genes aligned to the reference genomes (GenBank accession number: GCA_000214255.1) and showed that *hex 70a* and *hex 70c* contained six exons and five introns, and *hex 70b* and *hex 110* contained seven exons and six introns (Fig. 1B). In addition, the similarities of hexamerins at the level of nucleotide and amino acids between the obtained sequences and predicted sequences was a little difference (Supp Fig. 1 [online only]). Four hexamerins have conserved domains according to the alignment of homologous regions of four gene exons (Supp Fig. 2 [online only]).

Amino Acid Composition of Four Hexamerins in *B. terrestris*

The number of amino acids of Hex 70a, Hex 70b, Hex 70c, and Hex 110 was 677, 688, 684, and 1,098 amino acids, and their predicted molecular weights were 81.13, 79.69, 81.58, and 119 kDa with theoretical isoelectric point values of 6.34, 6.74, 6.64, and 6.70, respectively

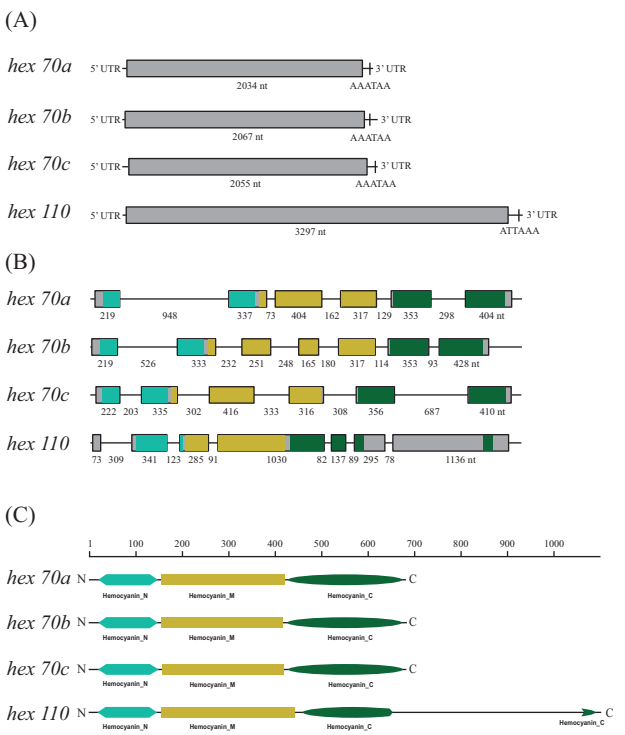

Fig. 1. Structure of cDNA, gene, and protein of four hexamerins in bumble bee, *B. terrestris*. (A) cDNA structure of four hexamerin genes. The 5′ UTR and 3′ UTR are represented by horizontal lines. The number of nucleotides (nt) indicates the length of the CDS. The position of the polyadenylation sequence is represented by a vertical line. (B) Gene structure of four hexamerin genes. The number of nucleotides (nt) indicates the length of exons and introns. Exons and introns are represented by gray boxes and black lines. (C) The conserved protein domains of the deduced hexamerin protein. The length of the amino acid is represented by the number on the axis. The hemocyanin N domain is represented by blue–green boxes; the hemocyanin M domain is represented by yellow boxes; and the hemocyanin C domain is represented by green boxes.

(Table 1). Compared with Hex 70a, Hex 70b, and Hex 70c proteins, Hex 110 protein has lower aromatic amino acid content and high content of Glx (glutamic acid and glutamine). The amino acid composition of four hexamerins was list in Supp Table 2 (online only). All hexamerin genes were composed of conserved hemocyanin N, M, and C domains (Fig. 1C). The cleavage site of the signal peptide from Hex 70a and Hex 70c was between positions 18 and 19 amino acids. The cleavage site of the signal peptide from Hex 70b was between positions 17 and 18 amino acids, and the cleavage site of the signal peptide from Hex 110 was between positions 16 and 17 amino acids.

Phylogenetic Analysis of Four Hexamerin Genes

The evolutionary relationships of hexamerin amino acid sequences from hymenopteran insects were investigated. Bootstrap support values for nodes on the tree range from 75 to 100%. The results showed that the hexamerin gene family was clustered into two clades in Hymenoptera. One of the clades is Hex 110, and the other clades are divided into Hex 70a, Hex 70b, and Hex 70c branches. Moreover, Hex 70a was clustered more closely with Hex 70c (Fig. 2).

Expression Profile of Four Hexamerin Genes at Different Developmental Stages of *B. terrestris* Worker

The expression-level analysis showed that the four hexamerin genes have different expression levels at different developmental

Table 1. Characteristics of four hexamerin proteins of *Bombus terrestris*

Gene name	Amino acids	Molecular weight	Theoretical pI value	Aromatic amino acids (%)	Leucine (%)	Methionine (%)	Glutamic acid and glutamine (%)
hex 70a	677aa	81.13 kDa	6.34	19.65	10.34	2.07	10.04
hex 70b	688aa	79.69 kDa	6.74	13.21	9.29	3.63	9.43
hex 70c	684aa	81.58 kDa	6.64	19.27	6.86	6.57	8.32
hex 110	1098aa	119 kDa	6.70	7.29	8.83	1.00	21.40

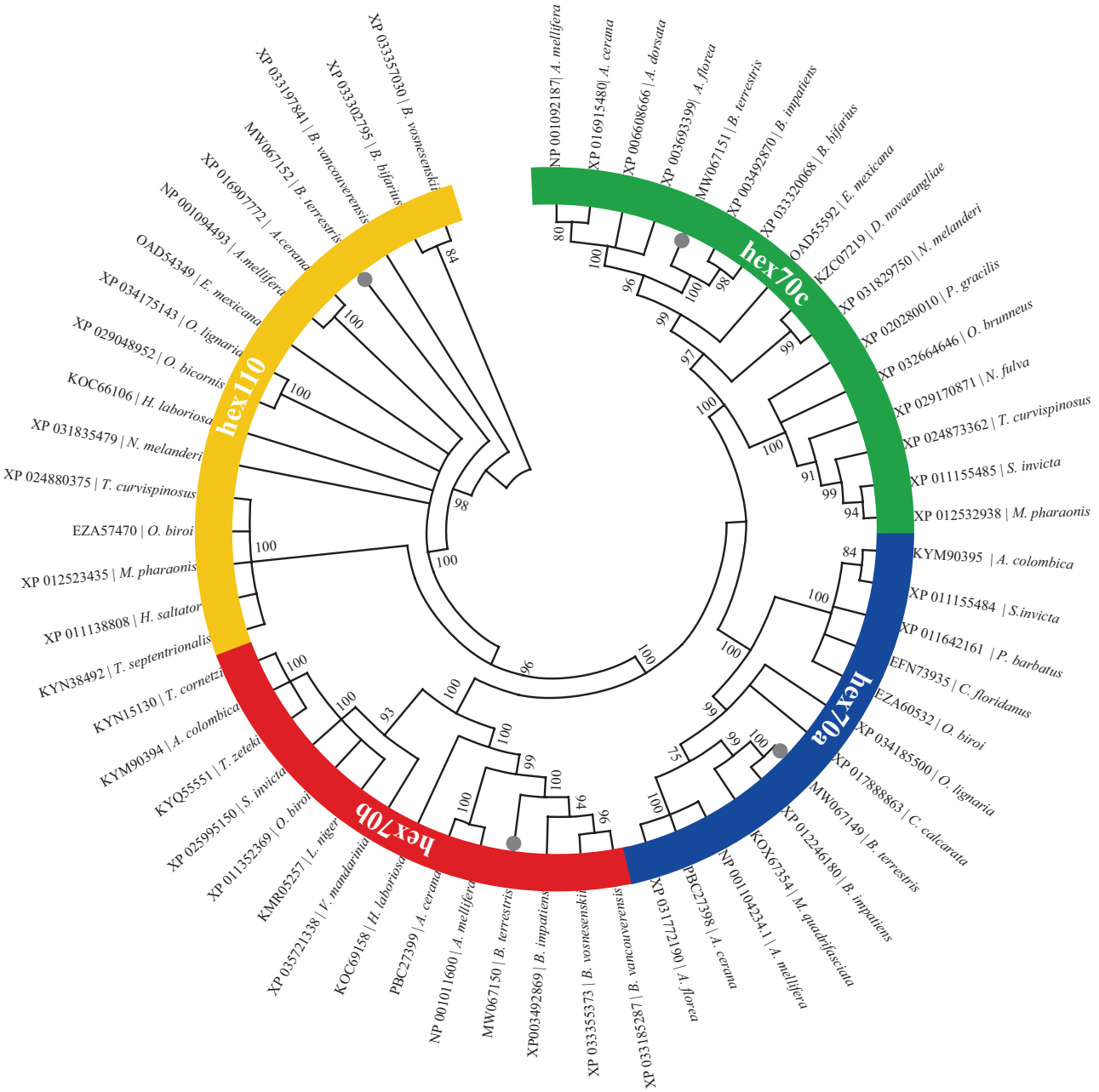

Fig. 2. Neighbor-joining phylogenetic tree of hexamerins using MEGA X. Hexamerins of *B. terrestris* labeled with gray dots. Each branch has bootstrap values based on 1,000 replicates. The hexameric sequences used in this study are listed in (SuppTable 3 [online online]).

stages. The expression of the *hex 70a* gene was significantly increased in the larva stage and reached a high level in the Pp, Pb, and Pb1 stages; next, the expression level significantly decreased at the Pbd and Pdd stages and reduced again in the Pdh stages ($F_{8, 18}$ = 398.38, P < 0.001; Fig. 3A). The expression of the *hex 70b* gene gradually increased from the L stage and reached the highest

level in the Pb stage, later significantly decreased at the Pb1 stage and decreased significantly to very low levels at the Pbd, Pdd, and Pdh stages ($F_{8, 18}$ = 304.95, P < 0.001; Fig. 3B). The expression of the *hex 70c* gene was significantly increased in the L stage and reached a peak in the Pp stage and gradually significantly decreased thereafter until the Pdh stage ($F_{8, 18}$ = 364.35, P < 0.001; Fig. 3C). The *hex 110* gene achieved the highest expression level at the L stage, significantly decreased at the Pw, Pp, Pb, and Pb1 stages, and later decreased significantly at the Pbd, Pdd, and Pdh stages ($F_{8, 18}$ = 415.22, P < 0.001; Fig. 3D).

Expression Profile of Four Hexamerin Genes in Eclosion, Prediapause, Diapause, and Postdiapause *B. terrestris* Queens

The expression-level analysis showed that the expression of the four hexmerin genes varied at different queen statuses. The *hex 70a* gene was significantly increased at prediapause compared to the eclosion queens (*hex 70a*: $F_{3, 8}$ = 191.25, P < 0.001), but there were no significant differences compared to the diapause and postdiapause queens (Fig. 4A). The expression of *hex 70b* gene reached a peak at prediapause and later significantly decreased in the diapause and postdiapause queens, but the postdiapause queens exhibited significantly higher levels than the eclosion queens (*hex 70b*: $F_{3, 8}$ = 1,009.15, P < 0.001; Fig. 4B). The expression of the *hex 70c* gene was significantly increased at the prediapause stage, significantly decreased at the postdiapause stage and again exhibited a significant reduction at postdiapause (*hex 70c*: $F_{3, 8}$ = 54.25, P < 0.001; Fig. 4C). The expression of the *hex 110* gene was significantly increased in the prediapause and diapause queens compared to the eclosion and postdiapause queens (*hex 110*: $F_{3, 8}$ = 587.49, P < 0.001; Fig. 4D).

Discussion

Hexamerins, as storage proteins, are essential for maintaining energy metabolism during the nonfeeding period (Cunha et al. 2005). In this study, we obtained four hexamerin genes from *B. terrestris* and mastered the expression characterization of bumble bee workers at the developmental stage and four stages of the life cycle of bumble bee queens. Four hexamerin genes were demonstrated to have high expression levels in the larvae and prepupae of workers and prediapause queens. These genes are involved in sustaining pupal development and the diapause status of bumble bees. To the best of our knowledge, this study is the first to identify and characterize four hexamerin genes in bumble bees.

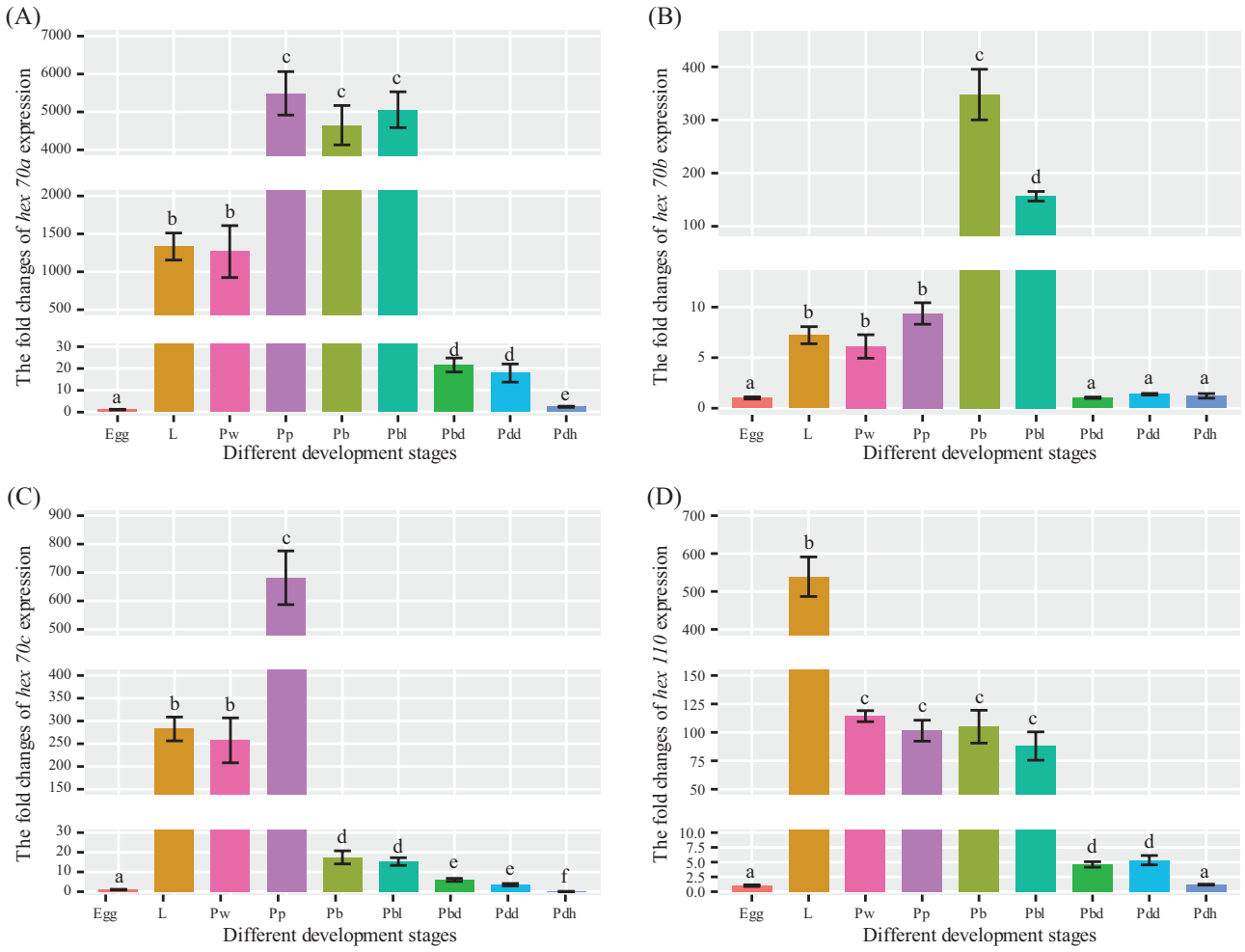

Fig. 3. Relative expression of the four hexamerin genes at different developmental stages of *B. terrestris* workers. (A) Relative expression of *hex 70a*. (B) Relative expression of *hex 70b*. (C) Relative expression of *hex 70c*. (D) Relative expression of *hex 110*. Data represent the mean ± SE for three replications (n = 3). Different lowercase letters indicate significant differences (P < 0.05).

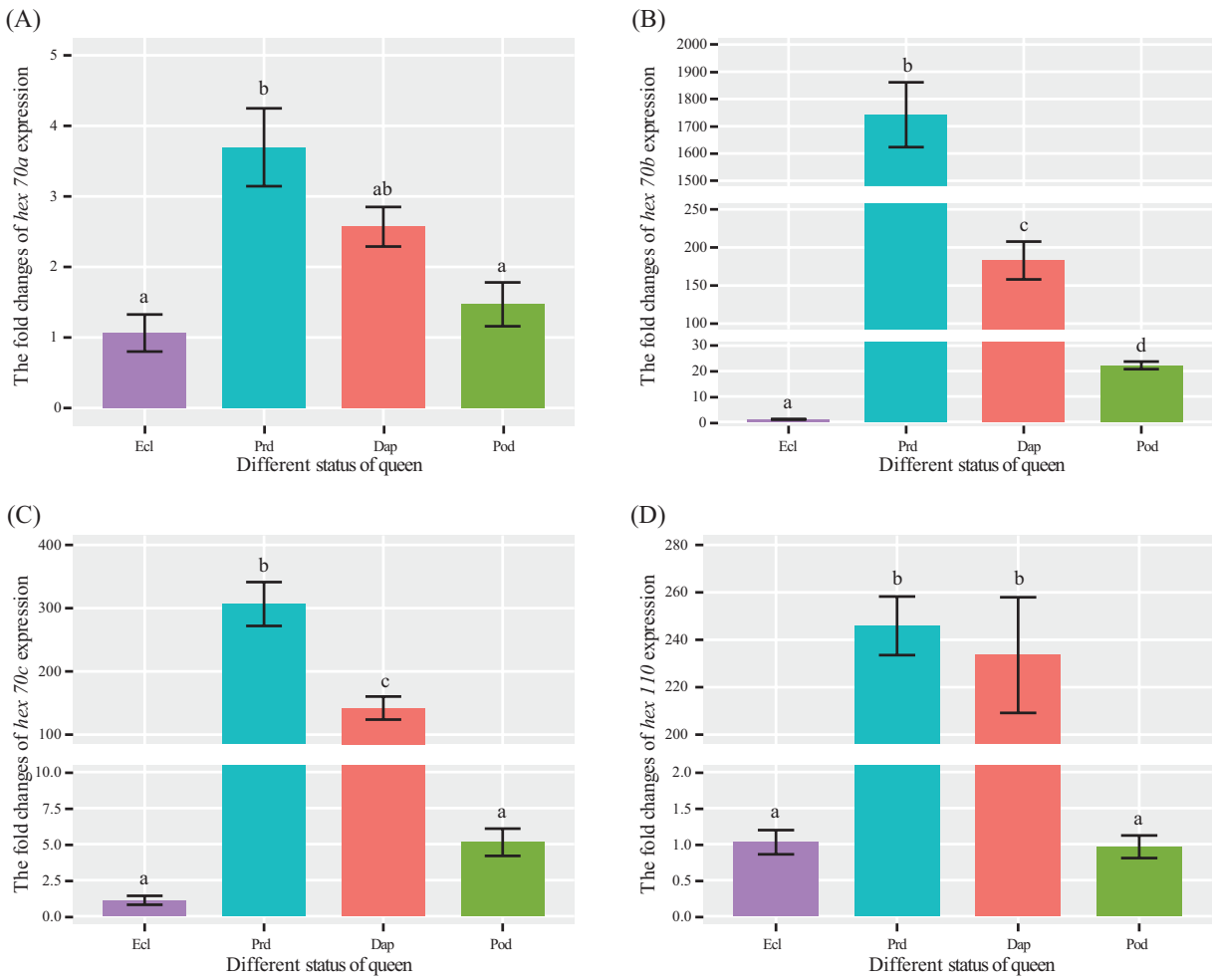

Fig. 4. Expression profile of four hexamerin genes at four statuses of bumble bee queens. Relative expression levels of *hex 70a* (A), *hex 70b* (B), *hex 70c* (C), and *hex 110* (D) at eclosion (Ecl), prediapause (Prd), diapause (Dap), and postdiapause (Pod) status. Data represent the mean ± SE for three replications ($n = 3$). Different lowercase letters indicate significant differences ($P < 0.05$).

Four hexamerin genes have transcript stop signals that are slightly different. The 3′ UTRs of *hex 70a*, *hex 70b*, and *hex 70c* contain a polyadenylation signal (AATAAA), whereas a nonclassical polyadenylation signal (ATTAAA) was observed in *hex 110*. This nonclassical polyadenylation signal was also detected in Liriomyza sativa (Huang et al. 2009, Chang et al. 2019). Four putative hexamerin proteins contained the hexamerin family conserved domains hemocyanin N, hemocyanin M, and hemocyanin C, as in other insects (Martins et al. 2010, Liu et al. 2019). Hex 70a and Hex 70c belong to the class of aromatic amino acid-rich proteins that contain a relatively high quantity of aromatic amino acids (phenylalanine, tryptophan, and tyrosine > 15%). The percentage of methionine in Hex 70c is 6.57%, which can be recognized as methionine-rich protein (>4% methionine; Martins et al. 2010, Hawkings et al. 2019). Hex 110 contains very rich glutamine and glutamic acid (Glx = 21.4%), which is similar to homologous protein of honey bee (Glx = 20.9%; Martins et al. 2010) and lower than ants (Glx = 25.4%; Hawkings et al. 2019). The phylogenetic analysis suggested that Hex 70a, Hex 70b, and Hex 70c clustered into one branch, which separated from the Hex 110 branch. This clustering pattern was also recorded in ants and honeybees, suggesting that the hexamerin family of social insects was conserved (Martins et al. 2010, Hawkings et al. 2019).

During worker development, the expression of four hexamerin genes was increased at the larval stages. High expression of hexamerin genes during the larval stage will ensure sufficient amino acids and energy for the metamorphosis of workers during the nonfeeding period (Moreira et al. 2004). The *hex 70a*, *hex 70b*, and *hex 70c* has a highly expression from larval to the midpupal stage (Pw, Pp, and Pb) and was notably depleted during adult ecdysis (Pdh). This expression profile of accumulation and depletion is common to most holometabolous insects (Korochkina et al. 1997, Hunt et al. 2007, Martins et al. 2012, Liang et al. 2019). *Hex 110* exhibited a different expression profile. It reached a peak in the larval stage and decreased at the pupal stage in bumble bees, which was in keeping with the findings obtained in honeybees (Martins et al. 2012). The highest expression of *hex 70a* and *hex 70b* during cuticle pigmentation stages (Pb and Pb1) indicated that they may be related to the formation of insect epidermis.

Hexamerins have been proven to play vital roles in insect diapause (Salama et al. 1992, Lewis et al. 2002, Wolschin and Gadau 2009, Mishra et al. 2011). It was demonstrated that four hexamerin genes of *B. terrestris* express clear increases in prediapause status. This will promote the accumulation of hexamerin proteins for diapause (Vaudo et al. 2017, Woodard et al. 2019, Costa et al. 2020). Therefore, this process could help queens resist cold stress and

nutrient sequestration. The increased expression levels of the four hexamerin genes in prediapause were significantly different (*hex 70a* upregulated 3.7-fold, *hex 70b* upregulated 1,742-fold, *hex 70c* upregulated 306-fold, and *hex 110* upregulated 245-fold). According to the expression profile, *hex 70b* may play the most important roles among the four hexamerins before diapause. However, it was reported that the biosynthesis of *hex 70b* was affected by the protein content in food in honeybees (Cunha et al. 2005). Hence, the effect of food on the expression of *hex 70b* in bumble bees should be further demonstrated in the future.

Conclusions

Four hexamerin genes (*hex 70a*, *hex 70b*, *hex 70c*, and *hex 110*) were first cloned and characterized from bumble bees. These genes play important roles in bumble bee metamorphosis and queen diapause. It was demonstrated that *hex 70a*, *hex 70b*, and *hex 70c* are vital to the Pb pupae development stage. Gene *hex 110* contributes strongly to larval development. High expression of four hexamerin genes at queen prediapause status indicated that they may provide supplementary amino acids and energy for diapause. Unfortunately, the functions of four hexamerin genes were not validated in this study, especially their functions during the nonfeeding period.

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

Table S1. Primers used in this study.

Table S2. Amino acid composition of four hexamerin proteins of bumble bee *Bombus terrestris*.

Table S3. Sequences and accession numbers of hexamerins used in molecular phylogeny analysis.

Fig. S1. The alignment of obtained sequences and the predicted sequences.

Fig. S2. The sequence alignment and identity of the exon of *hex 70a*, *hex 70b*, *hex 70c*, and *hex 110*.

Acknowledgments

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