Transcriptomic analysis of the prothoracic gland from two lepidopteran insects, domesticated silkmoth *Bombyx mori* and wild silkmoth *Antheraea pernyi*

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The prothoracic gland (PG) is an important endocrine organ of synthesis and secretion of ecdysteroids that play critical roles in insects. Here, we used a comparative transcriptomic approach to characterize some common features of PGs from two lepidopteran species *Bombyx mori* and *Antheraea pernyi*. Functional and pathway annotations revealed an overall similarity in gene profile between the two PG transcriptomes. As expected, almost all steroid hormone biosynthesis genes and the prothoracicitropic hormone receptor gene (*Torso*) were well represented in the two PGs. Impressively, two ecdysone receptor genes, eleven juvenile hormone related genes, more than 10 chemosensory protein genes, and a set of genes involved in circadian clock were also presented in the two PGs. Quantitative real time-PCR (qRT-PCR) validated the expression of 8 juvenile hormone and 12 clock related genes in *B. mori* PG, and revealed a different expression pattern during development in whole fifth larval instar. This contribution to insect PG transcriptome data will extend our understanding of the function and regulation of this important organ.

The prothoracic gland (PG) of insect is one of the most important endocrine organs that synthesizes and releases ecdysteroid hormone playing critical roles in regulating growth, moulting and metamorphosis. The insect PG is a CPU-like “decision-making center” that integrates a wide range of systemic cues before permitting the production of an ecdysone pulse. In higher Diptera *Drosophila* the PG, together with the corpus cardiacum (CC) and corpus allatum (CA), is fused into a complex endocrine structure, known as ring gland. However, in other insects, including the domesticated silkworm *Bombyx mori* (Lepidoptera: Bombycidae) and wild silkworm *Antheraea pernyi* (Lepidoptera: Saturniidae), these three endocrine glands form separate structures. In *B. mori* and *A. pernyi*, the PGs are a pair of semi-transparent or transparent saccate cell clusters with conjunct theca, respectively, located in the tracheal clusters of the prothorax.

As an important endocrine organ, the insect PG has been considered as a model for steroid hormone biosynthesis and regulation. A recent study has suggested that the local clock is a key driver of steroid hormone production in *Drosophila* PG. An ultrastructural study in *Drosophila* has suggested that the PG cells may be performing other roles beyond endocrine synthesis. Previous studies on genes expressed in the insect PG were initially focused on characterization of individual genes, particularly those involved in steroid hormone biosynthesis and regulation as well as circadian clock mechanism. Although the ecdysteroid in numerous insects had been studied for decades, yet advances in understanding this important organ at the molecular level remains largely unknown. At the start of this work, the basic genomic information is lacking, although a proteomic approach had been utilized to investigate the feature of *B. mori* PG. Very recently, two research teams just released their results for...
B. mori PG by transcriptomic approach\textsuperscript{7,8}. In the two studies, cell membrane receptors and signalling pathways and new players in ecdysozoogenesis of B. mori PG were focused on. However, a better understanding of insect PG requires an expansion of the taxon samplings.

In this paper, we used Illumina sequencing of cDNAs from the larval PGs of two economically important silkmoth species, B. mori and A. pernyi to characterize their common expressed genes that present the basic factors necessary for the function of the PG. The former is the model insect for the order Lepidoptera, and has economically important values for silk production\textsuperscript{9}. The latter is one of the most well-known wild silkmoths used for silk production and as a source of insect food for human consumption. A. pernyi is also a model system in study of insect diapause and endocrine regulation due to its pupal-diapause and large size\textsuperscript{10}. We generated over 24 million high-quality sequence reads that assembled into about 50,000 transcripts. The transcriptome data will contribute to knowledge of the molecular components in the PG of insects. By searching against the transcriptome data, we have identified almost all the steroid hormone biosynthesis genes and the prothoracicitropic hormone receptor gene (torso), several juvenile hormone related genes, two ecdysone receptor genes and a set of clock genes in the PGs of two silkworms. We also identified more than 10 chemosensory protein genes (CSPs) in the two PGs. To our knowledge, this is the first comparative view of the genes transcribed in this unique organ.

Results

Illumina sequencing and transcriptome assembly. Transcriptomic sequence data were generated using two PG cDNA libraries from B. mori and A. pernyi, and Illumina HiSeq 2500 technology. The PGs were collected from a pool of ~30 silkworm larvae of fifth instar. We acquired 28,159,208 and 24,408,498 clean reads from the PG transcriptomes of A. pernyi and B. mori, respectively, after removing adapters, ambiguous nucleotides and low quality sequences. For B. mori, 5.73 Gbp of clean sequence data was generated with a Q30 value of 91.35% and a GC content of 44.55%. The assembly resulted in 49,287 transcripts longer than 200 bp, which were further assembled into 32,302 unigenes, with an N50 of 1,510 and mean length of 790 bp. For A. pernyi, we ultimately obtained 6.68 Gbp of clean sequence data with a Q30 value of 91.73% and a GC content of 45.75%. The Trinity assembly of the clean sequence data of A. pernyi resulted in 64,301 transcripts longer than 200 bp, which were further assembled into 44,067 unigenes, with an N50 of 865 and mean length of 549 bp. For each species, at least 6100 unigenes exceed 1,000 bp and 12,500 bp in length (Additional file: Fig. S1). An overview of the sequencing and assembly process is presented in Additional file: Table S1. The sequence data for B. mori and A. pernyi PGs have been deposited in the NCBI Sequence Read Archive (SRA) database under accessions SRX2434884 and SRX2434884, respectively, and the assembled sequences have been deposited in Transcriptome Shotgun Assembly (TSA) database under accessions GFCX00000000 and GFCY00000000 associated with Bioproject PRJNA357974 and PRJNA357975 for A. pernyi and B. mori PGs, respectively.

Functional annotation revealed an overall similarity in gene profile between the two PGs. For functional annotation, we searched all unigene sequences using Blastx tool against NCBI non-redundant protein database (Nr), with a cut-off E-value of $10^{-5}$. Using this approach, 15,187 (47.02% of all distinct sequences) and 19,035 (43.19%) unigenes for B. mori and A. pernyi returned a Blast hit in the Nr database, respectively, 8,791 and 10,283 unigenes had specific matches in the Swiss-Prot database, and 9,188 and 10,974 unigenes had matches in the Pfam database. Totally, 23,157 (71.69%) and 22,402 (50.84%) unigenes were annotated in at least one database for B. mori and A. pernyi PGs (Additional file: Table S1).

Firstly, we used Blast2GO\textsuperscript{11} to perform functional annotation for the PG transcriptome via gene ontology (Fig. 1). For B. mori, a total of 8,557 unigenes were assigned GO terms, including 6,208 with hits at the Biological Process level, 4,065 at the Cellular Component level and 7,337 at the Molecular Function level. For A. pernyi, 10,621 unigenes were assigned GO terms, including 7,382 at the Biological Process level, 4,662 at the Cellular Component level and 9,170 at the Molecular Function level. Within the Biological Process GO categories, the most abundant transcript for the two PGs were assigned to “metabolic process” (6,218 in A. pernyi and 4,926 in B. mori), “cellular process” (5,406 in A. pernyi and 4,625 in B. mori), and “single-organism process” (4,006 in A. pernyi and 3,342 in B. mori). “Cell part” (3,337 in A. pernyi and 2,905 in B. mori), “cell” (3,323 in A. pernyi and 2,894 in B. mori), and “organelle” (2,439 in A. pernyi and 2,103 in B. mori) were the most represented GO terms for Cellular Components in both PGs. For Molecular Function, “binding” (5,751 in A. pernyi and 4,587 in B. mori), “catalytic activity” (5,385 in A. pernyi and 4,111 in B. mori) were the most prevalent in the two PGs. Overall, the percentage of Blastx hits distributed among GO categories was highly similar in both PGs.

Secondly, unigenes of the two PGs were characterized by KOG classification to enable conceptualization of its transcripts into potential functional groups. In total, 10,404 and 12,837 unigenes for B. mori and A. pernyi were annotated to 25 KOG categories, respectively (Additional file: Fig. S2). The numbers of each KOG category were similar between A. pernyi and B. mori PG transcriptome. The KOG classification indicated that except general function prediction, genes involved in “signal transduction mechanisms” (1,373 unigenes in B. mori and 1,454 in A. pernyi), “post translational modification, protein turnover, chaperones” (790 unigenes in B. mori and 1,052 in A. pernyi), and “translation, ribosome structure and biogenesis” (638 unigenes in B. mori and 618 in A. pernyi) were most abundant.

Lastly, KEGG orthology (KO) assignments\textsuperscript{13} were comparable between the two PGs. The KO assignment analysis resulted in annotation of 179 and 195 pathways, corresponding to 4,665 and 5,820 unigenes in B. mori and A. pernyi, respectively, and the global KO assignments showed similar trends in both PGs (Fig. 2A). The second- and third-tier pathways (Fig. 2B,C) also indicated a common expressed-gene profile between the two PGs. In the KEGG second-tier pathway hierarchy, “translation”, “folding, sorting and degradation” and “transport and catabolism” pathways ranked first to third in the two PGs, respectively.
Expression of ecdysteroidogenesis genes in two PGs. Insect PG is one of the most important endocrine organs that synthesizes and releases ecdysteroid hormone. As expected, almost all known steroid hormone biosynthesis genes (neverland, spook, phantom, disembodied, shadow, shroud, Cyp6u1) and the prothoracitropic hormone receptor gene (torso) are well represented in two PGs (Table 1). Local blast search against the transcriptome data of two silkmoths indicated that no expression of the shade gene is detected, which is consistent with ecdysone being activated to 20-Hydroxyecdysone (20-E) in peripheral tissues and not the PG13. Note that B. mori neverland was obtained by searching Blastn against a recent released PG transcriptome data (SRX1142589)8. The shroud gene in B. mori that encodes a short-chain dehyrogenase/reductase involving in the ecdysteroid biosynthesis pathway is PG- and ovary-specific14; we got the sequences of the shroud gene in both PG transcriptome data. A recent work in D. melanogaster has provided strong evidence that Cyp6u1 may have a role in ecdysteroidogenesis, possibly in the Black Box 15; the homologues were also identified in two silkworm PGs. We also identified two genes encoding ecdysone receptor B and ultraspiracle 2 that are expressed in the PG transcriptomes of two silkworms. These genes were confirmed by comparing them with known genes from D. melanogaster using phylogenetic analysis (Additional file: Fig. S3). The RPKM values of these genes were also evaluated, and spook was the most abundant. The high expression of spook in two silkworm PGs was not consistent with the low expression in D. melanogaster15.

Genes related to juvenile hormone regulation in PGs. Like ecdysteroids, juvenile hormone (JH) is also an important endocrine hormone that determines the nature of molt, and the CA has been considered as the only source of JH in insects16. In this study, we identified 11 juvenile hormone related genes that are represented in the PG transcriptome data from two silkworms (Table 1 and Fig. 3A), including Farnesyl diphosphate phosphatase (FPPP), Aldehyde dehydrogenase (ALDH), juvenile hormone acid methyltransferase (JHAMT), juvenile hormone epoxide hydrolase (JHEH), juvenile hormone esterase (JHE), cytosolic juvenile hormone-binding protein (jHBP), juvenile hormone binding protein (JHP), juvenile hormone esterase binding protein (JHEBP), hexamerin, broad and allatostatin receptor. However, we did not get the sequences of the genes such as NADP+ dependent farnesol dehydrogenase (FOHSDR), methyl farnesoate epoxidase/farnesoxide epoxidase (CYP15A1), juvenile hormone diol kinase (JHDK) and sesquiterpenoid omega-hydroxylase (CYP4C7). All these juvenile hormone related genes were confirmed by phylogenetic analysis (Additional file: Fig. S4).
Genes involved in circadian clock in PGs. Physiological experiments by transplantation have evidenced the presence of a local clock in PG of the saturniid moth *Samia cynthia ricini*. A local clock has also been suggested to play a key driver of steroid hormone production in *Drosophila* PG, and all of genes related to circadian clock were well represented in the ring gland transcriptome. In the present study, a set of genes important for circadian clock mechanism were identified in the PGs of two silkmoths (Table 1 and Fig. 3B), including *cry1*, *cry2*, *cycle*, *clock*, *vrille*, *timeless*, *slimb*, *period*, *double time*, *shaggy*, *PAR-domain protein 1ε*, *casein kinase 2 alpha*, *casein kinase 2 beta*, which further confirmed the presence of a local clock in the PG, although the expression level of each gene was low. Among these genes, the expression of *clock* was the least and *casein kinase 2 beta* was the most abundant. We confirmed these clock related genes by phylogenetic analysis (Additional file: Fig. S5).

Chemosensory protein genes in PGs. The genome of *B. mori* harbours 16 CSP genes (BmCSP1-16); we identified 11 of them and one novel BmCSP17 in the PG transcriptome data (Table 2; Fig. 4A). BmCSP17 exhibited a highest sequence identity with BmCSP16, with a value of 61%. In *A. pernyi* PG, we also identified 10 CSPs. These 10 ApCSPs demonstrated 45–77% amino acid sequence identities with corresponding BmCSPs. Sequence comparison between *A. pernyi* and *B. mori* showed that there are six pairs of homologous (Fig. 4B).

Changes in expression level of juvenile hormone regulation and circadian clock related genes in PG during development in whole fifth larval instar. We further used quantitative real-time PCR (qRT-PCR) to validate and investigate the changes in expression level of 11 juvenile hormone regulation and 13 circadian clock related genes in *B. mori* PG during development in whole fifth larval instar. The qRT-PCR results confirmed the expression of 8 genes out of these 11 juvenile hormone regulation related genes (Fig. 5). Among them, 4 genes (*ALDH*, *JHEH*, *cJHBP* and *JHEBP*) exhibited a similar expression pattern with a trend of rise first...
Table 1. Genes of interest present in two PGs.

| Gene                          | A. pernyi PG                  | B. mori PG                  |
|-------------------------------|------------------------------|----------------------------|
| Unigene ID                    | BLASTx annotation            | Identity (%) | RPKM | Unigene ID | FPKM |
| Neverland                     | ApPG.21305                   | NP_001037626 [Bombyx mori] | 64   | 147.04     | —    | —     |
| Spook                         | ApPG.15723                   | BA947267 [Bombyx mori]     | 73   | 659.32     | 1840.90 |
| Phantom                       | ApPG.17763                   | BAM73853 [Bombyx mori]     | 80   | 254.14     | 331.55 |
| Disembodied                   | ApPG.18472                   | BAM73849 [Bombyx mori]     | 71   | 162.28     | 149.33 |
| Shadow                        | ApPG.19814                   | BAM73862 [Bombyx mori]     | 62   | 187.60     | 620.00 |
| Shroud                        | ApPG.20406                   | NP_001171333 [Bombyx mori] | 67   | 262.63     | 423.82 |
| Cyto61                        | ApPG.21208                   | NP_001296520 [Bombyx mori] | 79   | 7          | 851.44 |
| Torso-like                    | ApPG.20311                   | XP_012546780 [Bombyx mori] | 74   | 22.87      | 263.03 |
| Ecstasy receptor B            | ApPG.17239                   | AB857644 [Bombyx mori]     | 83   | 2.6        | 358.7 |
| Ultraspiracle                 | ApPG.20814                   | NP_001037470 [Bombyx mori] | 86   | 3.89       | 2862.45 |
| Farnesyl diposphosphate phosphatase | ApPG.18455                  | NP_001040333 [Bombyx mori] | 67   | 2.77       | 494.7  |
| Aldehyde dehydrogenase (NAD+) | ApPG.20532                   | XP_004931115 [Bombyx mori] | 75   | 21.38      | 331.4  |
| Juvenile hormone acid methyltransferase | ApPG.39533                 | AEV45620 [Bombyx mori]     | 39   | 0.63       | 253.36 |
| Juvenile hormone epoxide hydrolase | ApPG.21667                   | BA81491 [Bombyx mori]     | 62   | 95.86      | 669.00 |
| Juvenile hormone esterase      | ApPG.8009                    | AAR37335 [Bombyx mori]     | 53   | 1.2        | 284.47 |
| Cytosolic juvenile hormone binding protein | ApPG.16002                 | NP_001037668 [Bombyx mori] | 86   | 159.49     | 641.9  |
| Juvenile hormone binding protein | ApPG.12156                   | BA971100 [Bombyx mori]     | 54   | 22.63      | 710.09 |
| Juvenile hormone esterase binding protein | ApPG.17810                   | AB23690 [Bombyx mori]     | 79   | 37.81      | 692.29 |
| Hexamerin                     | ApPG.199575                  | XP_004931806 [Bombyx mori] | 67   | 19.35      | 233.50 |
| Broad                         | ApPG.15073                   | NP_004931990 [Bombyx mori] | 77   | 2.66       | 3380.11 |
| Allatostatin receptor         | ApPG.9900                    | NP_001127736 [Bombyx mori] | 84   | 57.34      | 1024.23 |

Discussion

Despite PG is an important endocrine organ in insects, little information on it at the global level is already known, especially the features beyond endocrine synthesis. The present study, for the first time, use a comparative view to analyze the gene network in the PG of two silkmoths, B. mori and A. pernyi, by taking a transcriptomic approach. Since the complete genomes can be available, their annotation rates reach 71.7% for B. mori and 57.8% for D. melanogaster ring glands transcriptomes, respectively. The annotation rate of A. pernyi (50.8%) is lower than that of B. mori, but it is comparable to those of other lepidopteran insects such as Helicoverpa armigera (50.8%), H. assulta (54.0%), Spodoptera frugiperda (51.1%) and Athetis lepigone (41.5%). Comparison of A. pernyi and B. mori PG GO terms and KOG categories revealed that both transcriptomes have similar gene expression profiles. The high similarity of percentage of KO assignments across global KEGG pathways between A. pernyi

then fall; JHE and Broad showed a gradual rise tendency with the highest expression at day 10; FPPP generated a fluctuation change with a gradual rise until day 8, then decreased and increased again; JHBP remained a very low expression level, but revealed a distinct expression change with a trend of fall first then rise on the final day.

Out of 13 clock related genes identified in two PGs, 12 were indeed expressed in the larval PG of B. mori by qRT-PCR detection method (Fig. 6). Among them, 7 genes (cryptochrome 1, cryptochrome 2, timeless, double time, slimb, casein kinase 2 alpha and casein kinase 2 beta,) presented a similar expression pattern with a trend of rise then fall first; 4 genes (cycle, clock, shaggy and PAR-domain protein 1ε) showed a gradual rise tendency with the highest expression at day 10; period gave a distinct expression change with a trend of fall first then rise.
and *B. mori* also indicates that the PG transcriptomes share a common expressed-gene profile. This validates the application of RNA-Seq technology for identification of orthologs in non-model organism. Thus, the PG transcriptomes are useful resources to identify gene networks controlling PG ecdysteroidogenesis and understand the novel features of PG beyond endocrine synthesis.

20-E exerts its effect through binding to its receptor, a heterodimer of ecdysone receptor B (EcR) and ultraspiracle (USP). Two genes encoding EcRB and USP2 are expressed in the PG transcriptomes of two silkworms, providing further evidence that 20-E is involved in feedback loops in the PG22,23.

Recent studies have shown that JH plays an important role in regulating PG activity24,25; however, the underlying molecular mechanism is severely limited. Deciphering the mechanism underlying cross-talk between JH and ec dysone is key to understanding the control of insect growth and development. JH is degraded predominantly by hydrolytic enzymes, JHE and JHEH25. JHEBP might function in JHE transportation and degradation when the JH III titer is high26. JHBP serves as a carrier supplying JH to the target tissues. Hexamerin modulates JH availability27. Broad is a transcription factor that mediates the effects of ec dysone and juvenile hormone. Our qRT-PCR results confirmed the expression of 8 genes (ALDH, JHEH, cJHBP, JHEBP, JHE, Broad, FPPP and JHBP) in *B. mori* PG. No detectable expression of 3 genes (JHAMT, hexamerin and allatostatin receptor) by qRT-PCR method was consistent with the RNA-seq results by a very low FPKM value. It has already been shown that in *B. mori*
JHAMT was specifically expressed in the CA, and trace amounts in the PG28. The presence of 8 JH related genes in the PG suggested that through these JH related genes JH cross talk with ecdysteroid hormone. To understand the function of these JH related genes in the PG, it is necessary to investigate whether they are functionally important by performing qRT-PCR with CA and other tissues (for example, fat body) in the future work.

Circadian clock is an important regulator of behavior and physiology in insects. In addition to the central clock in brain, peripheral clocks reside in various organs and tissues29. For examples, the peripheral clocks in the Malpighian tubules, antenna, fat body of *Drosophila* have been well studied. These peripheral clocks are independent of the central clock, and the oscillatory machinery and entrainment mechanism of peripheral clocks vary between different tissues and organs. In *Drosophila*, the eclosion rhythm is set by a local clock residing in the PG that is a key driver of steroid hormone production3. Compared with *Drosophila*, very little attention has been paid to the peripheral clocks in lepidopteran insects17. In the PG transcriptomes of two silkmoths, we also identified 13 genes important for circadian clock mechanism and qRT-PCR results confirmed the expression of 12 genes in the *B. mori* PG, thus extending our understanding of the local peripheral clock residing in the PG of lepidopteran insects17 that may also involve in steroid hormone production. To address this issue, we will investigate the fluctuations in expression levels of circadian clock related genes of night and day PG samples in the future.

Insect CSPs are small soluble acidic proteins that are believed to be involved in chemical communication, including perception, identification, transport and transduction of semiochemicals from environment (olfaction, taste and others) and may be associated with regulation of circadian rhythms and maturation of tissue or appendage30. CSPs are expressed not only in insect sensory organs, but also in other tissues that lack gustatory and

| Gene Name | Unigene ID | ORF (aa) | Complete ORF | Blastx annotation | Blastx acc. no. | Blastx species | Identity (%) | FPKM |
|-----------|------------|----------|--------------|-------------------|----------------|----------------|--------------|------|
| A. pernyi PG | ApCSP1 | ApPG_15810 | 128 | Yes | chemosensory protein 1 | AAV34688 | Bombyx mori | 68 | 278.20 |
| | ApCSP2 | ApPG_8092 | 128 | Yes | chemosensory protein 5 | NP_001037062 | Bombyx mori | 60 | 34.10 |
| | ApCSP3 | ApPG_18212 | 121 | Yes | chemosensory protein 6 | NP_001037400 | Bombyx mori | 71 | 17.37 |
| | ApCSP4 | ApPG_12457 | 122 | Yes | chemosensory protein 7 | NP_001037068 | Bombyx mori | 66 | 500.23 |
| | ApCSP5 | ApPG_30646 | 69 | Not | chemosensory protein 8 | NP_001037067 | Bombyx mori | 68 | 1.16 |
| | ApCSP6 | ApPG_5694 | 124 | Yes | chemosensory protein 9 | NP_001037066 | Bombyx mori | 49 | 72.76 |
| | ApCSP7 | ApPG_10897 | 122 | Yes | chemosensory protein 9 | NP_001037066 | Bombyx mori | 45 | 207.57 |
| | ApCSP8 | ApPG_15471 | 122 | Yes | chemosensory protein 11 | NP_001091779 | Bombyx mori | 64 | 18.55 |
| | ApCSP9 | ApPG_12919 | 107 | Yes | chemosensory protein 16 | NP_001091782 | Bombyx mori | 77 | 2.54 |
| | ApCSP10 | ApPG_18214 | 106 | Yes | chemosensory protein 16 | NP_001091782 | Bombyx mori | 65 | 1.37 |
| B. mori PG | BmCSP1 | BmPG_8551 | 123 | Yes | chemosensory protein 1 | NP_001037065 | Bombyx mori | 100 | 3.81 |
| | BmCSP3 | BmPG_7145 | 127 | Yes | chemosensory protein 3 | NP_001037063 | Bombyx mori | 100 | 29.68 |
| | BmCSP4 | BmPG_7684 | 127 | Yes | chemosensory protein 4 | NP_001037052 | Bombyx mori | 100 | 7.85 |
| | BmCSP5 | BmPG_26685 | 125 | Yes | chemosensory protein 5 | NP_001037062 | Bombyx mori | 100 | 52.83 |
| | BmCSP7 | BmPG_7391 | 122 | Yes | chemosensory protein 7 | NP_001037068 | Bombyx mori | 100 | 14.46 |
| | BmCSP8 | BmPG_28989 | 124 | Yes | chemosensory protein 8 | NP_001037067 | Bombyx mori | 100 | 2.36 |
| | BmCSP9 | BmPG_7078 | 127 | Yes | chemosensory protein 9 | NP_001037066 | Bombyx mori | 100 | 39.41 |
| | BmCSP10 | BmPG_11310 | 122 | Yes | chemosensory protein 10 | ABH88203 | Bombyx mori | 100 | 1.27 |
| | BmCSP11 | BmPG_11636 | 121 | Yes | chemosensory protein 11 | NP_001091779 | Bombyx mori | 100 | 1.35 |
| | BmCSP15 | BmPG_11195 | 103 | Not | chemosensory protein 15 | NP_001091781 | Bombyx mori | 100 | 2.10 |
| | BmCSP16 | BmPG_7447 | 106 | Yes | chemosensory protein 16 | NP_001091782 | Bombyx mori | 100 | 12.32 |
| | BmCSP17 | BmPG_26561 | 104 | Yes | chemosensory protein 16 | NP_001091782 | Bombyx mori | 61 | 14.88 |

Table 2. Chemosensory protein genes present in two PGs.

JHAMT was specifically expressed in the CA, and trace amounts in the PG28. The presence of 8 JH related genes in the PG suggested that through these JH related genes JH cross talk with ecdysteroid hormone. To understand the function of these JH related genes in the PG, it is necessary to investigate whether they are functionally important by performing qRT-PCR with CA and other tissues (for example, fat body) in the future work.

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Figure 4. Comparison of chemosensory proteins (CSPs) identified in the PG transcriptomes of two silkworms, *A. pernyi* and *B. mori*. (A) Sequence alignment. (B) Neighbor-joining tree. Bootstrap values are calculated by 1000 replicates and those larger than 50% are marked on the nodes. The accession numbers of sequences are available in Additional file.

Figure 5. qRT-PCR results of expression of the JH related genes. The expression patterns of 8 genes related to JH in *B. mori* PG during development in whole fifth larval instar. The relative expression levels were normalized to the *Bmrp49* levels. The values are the mean ± SEM (n = 3) of three repeat experiments using qRT-PCR.

olfactory neurons. These non-chemosensory tissues included cuticle, legs, labial palp, pheromone gland, tarsi, proboscis, wings, testes, ovaries, compound eyes, hemolymph and ejaculatory ducts. The PG transcriptome data of two silkworms offered us an opportunity to investigate gene expression profiles of CSP genes on a large-scale in
PGs. Our study evidenced the presence of 12 and 10 CSPs in *B. mori* and *A. pernyi* PG, suggesting that there have a link between CSPs and ecdysteroids.

Earlier study in *B. mori* demonstrated that KK-42 can reduce the incidence of embryonic diapause when administered to the mother during her final larval instar. By contrast, our recent results showed that KK-42 can delay termination of the pupal diapauses in *A. pernyi* and *Helicoverpa zea*, and boost pupal diapause incidence when administered to larvae of *H. zea*. The mechanism is that KK-42 appears to act by inhibiting ecdysteroid biosynthesis within the PG, without killing the PG cells. Previous studies suggested that a KK-42 binding protein might be a receptor of an endogenous signaling compound; the expression of this gene could be detectable in many organs of *A. pernyi*. However, we did not detect the expression of this gene in the two PGs, indicating that KK-42 inhibits ecdysteroid biosynthesis within the PG not by the KK-42 binding protein. How does KK-42 influence ecdysteroid biosynthesis within the PG? To address this issue, comparison of KK-42-treated and untreated PGs would provide valuable clues in the near future.

In conclusion, this present work provided a comparative analysis of the PG transcriptomes of two silkmoths, whose associated expressed-gene profile were highly similar. Our results uncovered the presence of at least 8 juvenile hormone related genes, 12 circadian clock genes, and 10 chemosensory protein genes in both PGs. This contribution to insect PG transcriptome data will extend our understanding of the function and regulation of this important organ.

**Materials and Methods**

**Insect materials and samples collection.** Samples were derived from strain *Shenhua no. 2* of *A. pernyi* and *Dazao of B. mori* maintained at the Department of Sericulture, Shenyang Agricultural University in Shenyang. Larvae of *A. pernyi* strain were reared on oak trees in the field until the fifth larval stage, and then reared at room (25°C, natural humidity) using oak branches with leaves. Larvae of *B. mori* were reared using mulberry leaves during the whole larval stage at 25°C and 50–70% relative humidity in natural light. To generate samples, ~30 worms of *B. mori* and *A. pernyi* were collected in the third day and the fifth day of the fifth instar,
respectively. The PGs were carefully removed from the worms in insect Ringer physiological saline buffer under dissecting microscope, then immediately placed in 300μl TRIzol reagent (Beijing Sinogene, China) and stored at −80°C. Frozen tissues in TRIzol were shipped to Biomarker Technologies in Beijing, China for RNA extraction, library preparation and DNA sequencing.

RNA extraction and sequencing. Total RNAs were extracted from frozen PGs with TRIzol. RNA integrity and concentration were assessed with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Sequencing libraries were prepared using NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, E7530) and NEBNext Multiplex Oligos for Illumina (NEB, E7500). Two RNA samples were sequenced on the Illumina HiSeq™ 2500 sequencing platform (paired-end, 125 bp reads).

Transcriptome assembly. High-quality clean reads were obtained by removing the adaptor sequences, duplicated sequences, ambiguous reads (‘N’), and low-quality reads. For transcriptome data of *A. pernyi* that has no complete genomic data available, the clean reads were pooled for assembly using Trinity (http://trinityrnaseq.sourceforge.net) 34, and the related contigs were then clustered using the TGICL software 39 to yield unigenes (without N) that cannot be extended on either end, and redundancies were removed to acquire non-redundant unigenes. For transcriptome data of *B. mori* that has complete genomic data available, the clean reads were mapped to genome using Tophat2 software 40. Transcript expression levels were estimated with FPKM values (fragments per kilobase of exon per million fragments mapped) by the Cufflinks software 41. N50 and mean lengths of the transcripts associated with each sample were calculated. The values for N50 length and mean length indicated high quality samples, sequences and assemblies for the PGs of two silkmoths.

Annotation. The unigenes of the two silkmoths were compared against public databases, including NR (non-redundant), GO (gene ontology), KOG (eukaryotic ortholog groups), KEGG (Kyoto Encyclopedia of Genes and Genomes), Swiss-Prot and TrEMBL databases using BLASTx with an E-value cutoff at 10−5 to retrieve protein functional annotations with the highest sequence similarity. High-priority databases (followed by Nr, Swiss-Prot, and KEGG) were selected to determine the direction of the unigene sequences. The best aligning results were used to predict the coding region sequences from unigenes, and the coding sequences were translated into amino sequences using the standard codon table.

Phylogenetic analysis. The accession numbers of sequences used for phylogenetic analysis are listed in Additional file: Table S2. Amino acid sequences were aligned with ClustalX 1.83 42 and unrooted trees were constructed with MEGA6.0 33 using the neighbour-joining method, with Poisson correction of distances and bootstrap replications set at 1000.

Quantitative real time-PCR (qRT-PCR). Two PGs per larva were used as one sample to extract the total RNA from 1-day-old fifth instar larva to 10-day-old fifth instar larva (matured silkworm) in the present study. All the extracted RNA (at least 2.6μg/per fifth instar larva) using TRIPure (Beijing Aidlab biotechnologies Co. Ltd.) was converted into cDNA using the oligo(dT)18 primer with the PrimeScript RT reagent Kit with gDNA Eraser (Takara Biotechnology Dalian Co. Ltd.). The total volume of qRT-PCR reactions was 10μl, containing 3.6μl of specific primers (10μM), 1μl of cDNA and 5μl of ddH2O. qRT-PCR was performed with a BIO-RAD CFX Connect Real-Time System, and the conditions were as follows: 95 °C for 30 s followed by 40 cycles in 95 °C for 5 s and 60 °C for 30 s. Gene-specific primers used for qRT-PCR analysis are in Additional file: Table S3. The mRNA expression levels of the genes of interest were calculated with the 2−ΔΔCt method and normalized to the abundance of a house-keeping gene, ribosome protein 49 (rp49). The relative mRNA levels of each gene were represented as folds over the expression levels of *rp49*.

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Y.Q.L. and L.Q. conceived and designed the experiments; H.X.B., D.B.C., X.X.Z. and H.F.M. performed the experiments; X.X.Z., H.X.B., H.F.M. and Q.L. analyzed the data; Y.Q.L. evaluated the conclusions; H.W. and
Acknowledgements
This research was supported by the National Natural Science Foundation of China (Nos. 31672493 and 31372372) and the China Agriculture Research System (CARS-18). The authors thank Xue Yin (Beijing Biomarker Technologies, China) for sequencing services.
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Y.Q.L. and L.Q. conceived and designed the experiments; H.X.B., D.B.C., X.X.Z. and H.F.M. performed the experiments; X.X.Z., H.X.B., H.F.M. and Q.L. analyzed the data; Y.Q.L. evaluated the conclusions; H.W. and
R.X.X. reared the silkworms; Y.R.J. and Y.Q.L. contributed to the revision; H.X.B., Y.P.L. and Y.Q.L. wrote the paper. All authors read and approved the final manuscript.
Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-019-41864-0.
Competing Interests: The authors declare no competing interests.

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