Identification of sex-biased and neurodevelopment genes via brain transcriptome in *Ostrinia furnacalis*

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Insect brains play important roles in the regulation of sex-biased behaviors such as mating and oviposition. The neural structure and function of brain differences between males and females have been identified, in which the antenna lobes (AL) showed the most discrepancy, however, the whole repertoire of the genes expressed in the brains and the molecular mechanism of neural signaling and structural development are still unclear. In this study, high-throughput transcriptome analysis of male and female brains was carried on in the Asia corn borer, *Ostrinia furnacalis*, and a total of 39.23 Gb data and 34,092 unigenes were obtained. Among them, 276 genes displayed sex-biased expression by DEG analysis, of which 125 genes were highly expressed in the males and 151 genes were highly expressed in the females. Besides, by homology analysis against genes that have been confirmed to be related to brain neurodevelopment, a total of 24 candidate genes were identified in *O. furnacalis*. In addition, to further screen the core genes that may be important for sex-biased nerve signaling and neurodevelopment, protein-protein interaction networks were constructed for the sex-biased genes and neurodevelopment genes. We identified 10 (Mhc, Mlc1, Mlc2, Prm, Mf, wupA, TpnC25D, fln, I(2)efl, and Act5C), 11 (POQ2, GNBP3, Spn77Ba, Ppn, yellow-d2, PGRP-LB, PGRP-SD, PGRP-SC2, Hml, Cg25C, and vkg) and 8 (dac, wg, hh, ci, run, Lim1, Rbp9, and Bx) core hub genes that may be related to brain neural development from male-biased, female-biased, and neurodevelopment gene groups. Our results provide a reference for further analysis of the dimorphism of male and female brain structures in agricultural pests.

KEYWORDS
PPI network, sex-biased genes, transcriptome analysis, *Ostrinia furnacalis*, brain neurodevelopmental genes
Introduction

Insects display different behaviors between males and females, and these differences are probably caused by the dimorphism of brain structure and function (Cachero et al., 2010). For example, in Lepidopteran insects, females release sex pheromones and males could detect the pheromones through a sensitive olfactory system (Berg et al., 1995; Hansson et al., 1995; Wang et al., 2018). In the olfactory central nervous system of the brains, the antennal lobe (AL) of males contains a macroglomerular complex (MGC) structure with the main function of sex pheromone recognition (Anton and Homberg, 1999; Zhao and Berg, 2010; Dong et al., 2020). However, the brain structure is quite different between males and females. The female brains have a larger female glomerulus (LFG) instead of MGC, which may function to perceive information related to female-specific behaviors (Rospars and Hildebrand, 2000; Berg et al., 2002; Skiri et al., 2005). The dimorphism of brain structures and functions may be specifically regulated by some genes expressed in the brains, but only a few of them were identified and functionally characterized in insects, especially in agricultural pests.

Sex-biased genes in the brains might be involved in the different neural signaling and structural development. The sex-determining genes doublesex (dsx) and fruitless (fru) regulate the Drosophila takeout gene and affect male courtship behavior (Dauwalder et al., 2002). In addition, some genes displayed similar expression patterns between males and females and they exhibited complex functions in neural development. For example, dachshund (dac) encoded a new nuclear protein that was necessary for the development of normal eye and mushroom bodies (Mardon et al., 1994; Kurusu et al., 2000). The scratch (scrt) was expressed in neuronal precursor cells and encoded a predicted zinc finger transcription factor and it was confirmed to be involved in neuronal development (Roark et al., 1995). However, most of these studies were sporadic and focused on one or two specific genes, while a whole organism view of genes expressed in the brains is still needed (Kasai et al., 1998; Janssens et al., 2010).

The development of sequencing technology has allowed for the collection of whole repertoires of genes expressed in the brains and is beneficial to our understanding of the exhaustive molecular mechanism of neural signaling and structural development of the brains. Transcriptome analyses have been reported in many insects including fruit flies (Zhan et al., 2007; Hughes et al., 2012), bees (Vleurinck et al., 2016; Li et al., 2019; Steffen and Rehan, 2020), ants (Calkins et al., 2018; Romain et al., 2018; Wang et al., 2020), wasps (Berens et al., 2017), butterflies (Zhu et al., 2008; Lugena et al., 2019), silkworm (Wang et al., 2015), and two species of Noctuidae moths (Walker III et al., 2019; Cinel and Taylor, 2019). Candidate genes that regulate job differentiation in social insects such as bees and ants and genes related to age and clock in fruit flies have been identified. In the case of Drosophila melanogaster, circadian transcriptome analysis of the brain demonstrates that extensive circadian rhythm control of noncoding RNAs (ncRNAs) was involved in circadian rhythm control (Hughes et al., 2012).

Transcriptome data generally contains thousands of genes, and these data can be narrowed down by a determination of the protein-protein interaction (PPI) network which is used to screen and pick up core genes. The PPI network intuitively displays the main characteristics of the interaction and functional characteristics of proteins in birth objects and reflects the unique and essential proteins, which have been widely used in gene function annotation and prediction (Li et al., 2013; Zhang and Zhang, 2020; Xin and Zhang, 2021). STRING (https://STRING-db.org/) is a database that searches for known and predicted interactions between proteins, built through PPI networks to better understand complex regulatory networks in organisms (von Mering et al., 2005).
Szklarczyk et al., 2019). In insects, PPI networks are used only in *D. melanogaster* or *Bombyx mori* for screening key proteins related to insecticide resistance, olfactory systems, and detoxification enzymes (Zhang and Zhang, 2019 and 2020; Xin and Zhang, 2020 and 2021).

In the *Ostrinia* genus (Lepidoptera: Crambidae), 21 species have been characterized including serious agricultural pests of maize, the Asian corn borer *Ostrinia furnacalis*, and the European corn borer *Ostrinia nubilalis* (Mutuura and Munroe, 1970; Huang et al., 1998). Olfactory system genes involved in pheromone perception have been identified and functionally analyzed in this genus, indicating that the peripheral nervous system was different between males and females (Ishikawa et al., 1999; Miura et al., 2010; Yang et al., 2015; Liu et al., 2018). As the main olfactory center of the insect brain, AL contains a large number of glomeruli, similar to the olfactory bulb in the brain of vertebrates (Zhao and Berg, 2010). In addition, studies on the brain structure showed that males of *O. nubilalis* have similar large glomerular complexes in the AL, but these large complexes were not found in the females (Karpati et al., 2008). The regulatory gene differences may be related to sex-biased genes or neurodevelopmental genes, but the related candidate genes are completely unknown in this genus.

In this study, high-throughput transcriptome analysis was used to identify the whole repertoire of the genes expressed in the brains of males and females in *O. furnacalis*. Among them, genes with sex-biased expressions were identified by DEG analysis. Neural development genes that were equally expressed between males and females were identified using homology analysis. In addition, PPI network analysis was carried on to further obtain the core hub genes for neural signaling and structural development in the brains. Our results provide a basis for functional studies of the central nervous system of agricultural pests and will help to develop new target genes for pest control in agriculture.

**Materials and methods**

**Insect rearing and tissues collection**

*O. furnacalis* were maintained in the Chinese Academy of Agricultural Sciences, Beijing, China, under laboratory conditions with an artificial diet at 27 ± 1°C, 16:8 (L:D), and 70% relative humidity. Adults were fed with a 10% sugar solution. Brains from 3-day-old males and females were dissected out in fresh Ringer’s solution (in mM; 150 NaCl, 3 CaCl₂, 3 KCl, 25 sucrose, and 10 N-Tris [hydroxy-methyl]-methyl-2-amino-ethanesulfonic acid, pH 6.9) on ice, frozen in liquid nitrogen, and stored at −80°C for the following experiments.

**RNA extraction and transcriptome sequencing**

Total RNA was isolated from 30 brains of males (or females) using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer’s instructions. The RNA was dissolved in RNase-free water, and the quality was assessed by gel electrophoresis. The concentration and purity of RNA were determined on a NanoDrop ND-2000 spectrophotometer (NanoDrop products, Wilmington, DE, United States). A total amount of 1 µg RNA per sample was used as input material for RNA sample preparations. Sequencing libraries were generated using NEBNext Ultra™ RNA Library Prep Kit for Illumina® (NEB, United States) following the manufacturer’s recommendations and sequenced on an Illumina Hiseq 2000 platform. Three repeats of each sample were used for the sequencing.

**Transcriptome assembly and gene functional annotation**

Raw reads were filtered and their qualities were calculated by Q30. Read number, base number, and GC-content were calculated from the filtered clean reads. Trinity was used for the *de novo* assembly (Grabherr et al., 2011). The obtained unigenes were annotated using different databases including NR (Deng et al., 2006), Swiss-Prot (Apweiler et al., 2004), GO (Ashburner et al., 2000), COG (Tatusov et al., 2000), KOG (Koonin et al., 2004), eggNOG (Huerta-Cepas et al., 2016), and KEGG (Kanehisa et al., 2004). The HMMER (Eddy, 1998) software was used to compare with the Pfam (Finn et al., 2014) database to obtain the annotation information of unigenes. BLAST parameter e-values were not greater than 1−10, and the HMMER parameter e-value was not greater than 1−10.

**Differentially expressed gene analysis**

Reads were mapped to the unigenes using Bowtie (Langmead et al., 2009), and the expression level of each unigene was estimated with RSEM (Li and Dewey, 2011). The different expression patterns of unigenes between males and females were calculated with FPKM values by DESeq2 (Trapnell et al., 2010). The generally accepted and effective Benjamini–Hochberg method was used to correct the significant *p*-values obtained by the original hypothesis test. Then, the differentially expressed genes among the sample groups annotated to the GO database were enriched and analyzed by topGO (Alexa and Rahnenfuehrer, 2010) software. In addition, the differentially expressed genes were classified by COG and eggNOG, as well as KEGG annotation and pathway enrichment analysis.

**Identification of developmental genes**

Through a literature review, we downloaded the protein sequences of developmental genes from previous studies from NCBI and used them as a query to screen the candidate neural
development genes against the unigenes expressed in male and female brains in *O. furnacalis*. By best hit, only the best and longest sequence results were used. The obtained protein sequences were subsequently verified by BLAST orientation in the NCBI database to remove low confidence genes in order to finally obtained the candidate neural development genes in *O. furnacalis*.

### Construction and analysis of PPI network

PPI networks were constructed using STRING 11.5 ([https://cn.STRING-db.org](https://cn.STRING-db.org)) (von Mering et al., 2005). We chose *D. melanogaster* as the organism. The PPI network was optimized and analyzed by Cytoscape 3.7.1 (Shannon et al., 2003). The core modules in the PPI network (Node Score cutoff: 0.2, K-score: 2) were screened by the Molecular Complex Detection (MCODE) plug-in (Bader and Hogue, 2003). The CytoHubba algorithm plug-in was used to find the key targets and subnetworks of PPI networks and determine the hub genes. Finally, the degree and intermediary centrality of each node was calculated by the Network Analyzer plug-in (Brandes, 2008).

### Results

#### Sequencing and annotation

By sequencing three cDNA libraries of each sex, more than 131 million reads were obtained, of which 67,102,198 reads and 64,226,235 reads were obtained from female and male brains, respectively (Table 1). The sequencing raw data was uploaded to the NCBI Sequence Read Archive database (Accession number: PRJNA818099). The Q30 values were 94.51%–94.93%, and the overall GC percentages and mapping ratio were 45.01%–46.72% and 82.88%–85.15%, respectively. The assembly results obtained by Trinity are summarized in Table 2. A total of 34,092 unigenes were obtained, with a total length of 42,252,036 bp. The average

| Sample | Read number | Base number | GC content (%) | %≥Q30 | Mapped reads | Mapped ratio (%) |
|--------|-------------|-------------|----------------|-------|--------------|-----------------|
| Female-1 | 19,943,263 | 5,964,443,252 | 45.01 | 94.64 | 16,934,654 | 84.91 |
| Female-2 | 22,999,277 | 6,882,726,768 | 45.22 | 94.51 | 19,533,622 | 84.93 |
| Female-3 | 24,159,658 | 7,228,792,424 | 45.43 | 94.93 | 20,572,772 | 85.15 |
| Male-1 | 22,095,932 | 6,577,971,876 | 45.92 | 94.80 | 18,132,073 | 82.88 |
| Male-2 | 21,380,928 | 6,389,253,286 | 45.46 | 94.58 | 18,160,905 | 84.94 |
| Male-3 | 20,749,375 | 6,190,427,112 | 46.72 | 94.62 | 17,350,250 | 83.62 |

| Length range | Transcript | Unigene |
|--------------|------------|---------|
| 300–500      | 21,856 (20.88%) | 14,413 (42.28%) |
| 500–1000     | 20,539 (19.62%) | 8,171 (23.97%) |
| 1000–2000    | 22,716 (21.70%) | 5,311 (15.58%) |
| 2000+        | 39,564 (37.80%) | 6,197 (18.18%) |
| Total Number | 104,675 | 34,092 |
| Total Length | 223,008,365 | 42,252,036 |
| N50 Length   | 3,644 | 2,352 |
| Mean Length  | 2130.48 | 1239.35 |

| Anno_Database   | Annotated_Number | 300≤length≤1000 | Length≥1000 | DEG number |
|-----------------|------------------|-----------------|-------------|------------|
| COG_Annotation  | 4,542            | 1,482           | 3,060       | 79         |
| GO_Annotation   | 7,508            | 2,803           | 4,705       | 100        |
| KEGG_Annotation | 6,750            | 2,199           | 4,551       | 82         |
| KOG_Annotation  | 9,609            | 3,096           | 6,513       | 123        |
| Pfam_Annotation | 11,236           | 3,780           | 7,456       | 169        |
| Swissprot_Annotation | 8,565 | 2,595         | 5,970       | 147        |
| eggNOG_Annotation| 14,293           | 5,540           | 8,753       | 199        |
| nr_Annotation   | 15,752           | 6,689           | 9,063       | 214        |
| All_Annotated   | 16,174           | 7,085           | 9,089       | 220        |
length and the N50 length of unigenes were 1239.35 bp and 2,352 bp, respectively. According to different annotation methods, 16,174 unigenes were annotated, in which 7,085 unigenes ranged from 300 to 1,000 bp in length, and 9,089 unigenes were over 1,000 bp in length (Table 3).

**Analysis of differentially expressed gene**

By DEG analysis, 276 genes displayed sex-biased expression, with 125 and 151 highly expressed genes found in the male and female brains, respectively (Figure 1A, Supplementary Table S1). Among them, 6 genes were specifically expressed in females and 15 genes were specifically expressed in males (Table 4), while 18 genes were 50 times different between males and females (Table 5). Through hierarchical clustering analysis, the clustering results of the DEGs among sample groups were obtained (Figure 1B), which again demonstrated the obvious difference between females and males. In addition, 220 of the 276 sex-biased genes were annotated and their functions were analyzed against different databases (Table 3). By GO annotation, 100 genes were clustered into different functional groups including cellular components, molecular function, and biological process, in which cellular components contained more male-biased genes and biological processes contained more female-biased genes (Figure 2A). By COG annotation, 79 genes were clustered into 19 functional groups. Female and male DEGs corresponded to 17 and 16 functional groups, which accounted for the largest proportion in carbohydrate transport and metabolism categories, respectively. Meanwhile, the second-largest category of female DEGs included 6 genes, which specifically corresponded to the cell wall, membrane, or envelope biogenesis (Figure 2B). According to KEGG enrichment analysis, the longevity regulating pathway in males and the AGE-RAGE signaling pathway in diabetic complications in females corresponded to the largest number of genes, and the enrichment results were the most reliable (Figures 2C,D).
## TABLE 4 Summary of genes that displayed sex-specific expressions in the brains of *Ostrinia furnacalis*.

| Number | Gene id   | Female-FPKM (average) | Male-FPKM (average) | Fdr       | log2 (FC) | Annotation                                      |
|--------|-----------|------------------------|---------------------|-----------|-----------|------------------------------------------------|
| 1      | c34079    | 157.49                 | 0                   | 1.08E-138 | -5.5882   | Cecropin family                                |
| 2      | c25808    | 9.40                   | 0                   | 4.84E-15  | -2.2211   | Transcription activator MBF2                   |
| 3      | c27820    | 8.42                   | 0                   | 1.76E-28  | -2.9946   | Peptidoglycan recognition protein              |
| 4      | c27992    | 5.51                   | 0                   | 9.83E-06  | -1.3075   | Uncharacterized protein                         |
| 5      | c47702    | 1.66                   | 0                   | 3.59E-17  | -2.3693   | Endonuclease-reverse transcriptase              |
| 6      | c42189    | 1.49                   | 0                   | 3.57E-06  | -1.3667   | —                                              |
| 7      | c34354    | 0                      | 0.86                | 0.00024709 | 1.0841    | Myosin heavy chain                             |
| 8      | c41665    | 0                      | 0.86                | 0.0006771 | 1.0202    | —                                              |
| 9      | c32331    | 0                      | 0.95                | 6.33E-06  | 1.3328    | —                                              |
| 10     | c38932    | 0                      | 1.16                | 1.92E-06  | 1.4035    | Posttranslational modification, protein turnover, chaperones |
| 11     | c35757    | 0                      | 2.05                | 2.97E-05  | 1.2355    | —                                              |
| 12     | c45967    | 0                      | 2.41                | 1.21E-05  | 1.2951    | Protease inhibitor/seed storage/LTP family     |
| 13     | c37529    | 0                      | 3.38                | 0.00046178| 1.0390    | Energy production and conversion; Biological Process: electron transport chain; Cytochrome C oxidase subunit II |
| 14     | c40218    | 0                      | 3.71                | 3.60E-05  | 1.2113    | Cytochrome C oxidase subunit 1                 |
| 15     | c25751    | 0                      | 4.06                | 9.50E-11  | 1.8680    | Lectin C-type domain                           |
| 16     | c45629    | 0                      | 4.85                | 1.23E-05  | 1.2931    | Cys-rich Gliadin N-terminal; Protease inhibitor/seed storage/LTP family |
| 17     | c44994    | 0                      | 5.01                | 2.42E-10  | 1.8300    | Cys-rich Gliadin N-terminal; Protease inhibitor/seed storage/LTP family |
| 18     | c44355    | 0                      | 5.19                | 1.64E-08  | 1.6315    | PREDICTED: uncharacterized protein             |
| 19     | c41757    | 0                      | 5.83                | 1.10E-16  | 2.3368    | —                                              |
| 20     | c41828    | 0                      | 8.16                | 1.35E-14  | 2.1868    | Carbohydrate transport and metabolism, Ribulose bisphosphate carboxylase, a small chain |
| 21     | c34491    | 0                      | 9.39                | 2.98E-22  | 2.6809    | —                                              |

## TABLE 5 Summary of genes that displayed sex-biased expressions (more than 50 times) in the brains of *Ostrinia furnacalis*.

| Number | Gene id   | Female-FPKM (average) | Male-FPKM (average) | f/m       | m/f       | Fdr       | log2 (FC) | Annotation                                      |
|--------|-----------|------------------------|---------------------|-----------|-----------|-----------|-----------|------------------------------------------------|
| 1      | c33902    | 457.81                 | 3.44                | 132.96    | 0.01      | 6.56E-40  | -3.5166   | —                                              |
| 2      | c42325    | 412.00                 | 6.37                | 64.71     | 0.02      | 0         | -6.1934   | Gloverin-like protein                           |
| 3      | c36790    | 305.16                 | 0.89                | 344.17    | 0.00      | 2.98E-205 | -6.0853   | Antimicrobial peptide cecropin                  |
| 4      | c48756    | 178.46                 | 0.64                | 277.40    | 0.00      | 0         | -7.7166   | Vitellogenin                                    |
| 5      | c43880    | 167.88                 | 2.23                | 75.28     | 0.01      | 0         | -5.6779   | Proline-rich protein                            |
| 6      | c25520    | 115.43                 | 0.17                | 665.94    | 0.00      | 8.79E-192 | -6.1372   | Peptidoglycan recognition protein              |
| 7      | c44151    | 96.21                  | 0.49                | 197.69    | 0.01      | 1.62E-232 | -6.0821   | Peptidoglycan recognition protein              |
| 8      | c42816    | 89.50                  | 0.51                | 176.64    | 0.01      | 2.39E-292 | -6.3075   | Tenascin-like                                   |
| 9      | c38646    | 44.18                  | 0.21                | 213.79    | 0.00      | 3.40E-136 | -5.3300   | Trypsin Inhibitor                              |
| 10     | c42870    | 26.73                  | 0.23                | 117.91    | 0.01      | 8.10E-122 | -4.9811   | —                                              |
| 11     | c38659    | 23.37                  | 0.10                | 241.76    | 0.00      | 1.03E-61  | -4.0577   | —                                              |
| 12     | c49826    | 21.64                  | 0.41                | 52.79     | 0.02      | 9.98E-19  | -2.4514   | —                                              |
| 13     | c42325    | 15.13                  | 0.07                | 226.90    | 0.00      | 2.43E-55  | -3.9040   | PREDICTED: gloverin-like                       |
| 14     | c42964    | 5.87                   | 0.03                | 220.00    | 0.00      | 4.23E-20  | -2.5502   | —                                              |
| 15     | c40593    | 4.18                   | 0.02                | 179.29    | 0.01      | 2.52E-14  | -2.1677   | —                                              |
| 16     | c42506    | 1.77                   | 0.03                | 66.25     | 0.02      | 8.84E-10  | -1.7864   | Immunoglobulin domain                          |
| 17     | c27864    | 0.10                   | 5.35                | 0.02      | 55.38     | 3.40E-12  | 2.0014    | Flightin OS                                    |
| 18     | c50115    | 0.02                   | 1.32                | 0.02      | 65.83     | 0.0006859 | 1.0338    | —                                              |
Identification of candidate neurodevelopment genes in *O. furnacalis*

We obtained the protein sequences of 343 developmental genes expressed in the brains of 163 different species such as *Apis mellifera* (Li et al., 2019), *D. melanogaster* (Dahl et al., 1997; Posnien et al., 2011; Sinigaglia et al., 2013), *Tribeolum castaneum* (Choe and Brown, 2007), *Bombyx mori* (Ferguson et al., 2014), and *Bemisia tabaci* (He et al., 2020) from NCBI (Supplementary Table S2). By local BLAST analysis of the sequencing data against the query, 40,285 candidates were obtained from the homology analysis of developmental genes. Subsequently, 2,365 candidates were obtained through the best hit method. In addition, candidates that only had a few matches compared to the query genes were deleted, and the remaining 108 candidates were re-verified by BLAST in the NCBI database. Finally, 24 candidate genes were obtained that could be the neural development genes in *O. furnacalis* (Table 6, Supplementary Table S3).

PPI network analysis for sex-biased genes

Two PPI networks were constructed from 125 male DEGs and 151 female DEGs using the STRING database. Among them, the male network matched 56 nodes and 52 edges (enrichment p-value: <1.0 × 10^-16, average node degree: 1.86) (Figure 3A). The female network matched 50 nodes and 25 edges (enrichment p-value: <2.15 × 10^-10, average node degree: 1.00) (Figure 4A). Through the MCODE algorithm of Cytoscape, the core interaction network modules that played an important role in the stability of the entire protein interaction network were selected. The core network of males contained 9 hub genes (*Mhc, Mlc1, Mlc2, Prm, Mf, wupA, Tpm25D, fln*, and *Act5C*) and...
PPI network analysis for neurodevelopment candidate genes

A protein interaction network with 24 nodes and 15 edges (enrichment p-value: <1.27\(^{-13}\), average node degree: 1.36) was obtained by matching 24 candidate genes for brain neural development with the STRING database (Figure 5A). Cytoscape’s MCODE algorithm was used to select one core network module with close relationships and important roles in the stability of the whole protein interaction network, involving 4 hub genes (ci, wg, hh, and dac) and 6 interaction relationships (score = 4.000) (Figure 5B). Furthermore, the CytoHubba plug-in was used to extract 8 hub genes (dac, wg, hh, ci, run, Lim1, Rbp9, and Bx) from the PPI network by degree (Figure 5C), and compared with the results of MCODE, the 4 highest hub genes were consistent. The two algorithms obtained a total of 8 core hub genes, which were mostly related to neurodevelopmental functions in STRING (Table 7). Using the NetworkAnalyzer plug-in, the highest degree and betweenness centrality was obtained for wg, dac, Lim1, Rbp9, and hh, which were all included in the 8 hub genes identified in this work (Figure 5D, Supplementary Table S5).

### TABLE 6 List of 24 brain neurodevelopment candidate genes identified in *Ostrinia furnacalis*. The citation of Table 6 is missing and must be cited. Please note that Figures and Tables must be cited sequentially.

| Number | Ofur   | ORF (aa) | Identity (%) | Gene Name       | Function                      | References                  |
|--------|--------|----------|--------------|-----------------|-------------------------------|-----------------------------|
| 1      | c47037 | 354      | 64           | hwu/elav        | nervous system                | Satô et al., 2001; Lowe et al., 2003 |
| 2      | c48600 | 362      | 64           | dac             | eye, mushroom bodies          | Mardon et al., 1994; Kurusu et al., 2000 |
| 3      | c47752 | 694      | 67           | lmi1            | brain; eye                    | Fujii et al., 1994; Roignant et al., 2010 |
| 4      | c46011 | 446      | 53           | tyrosine aminotransferase | CAMP-dependant protein kinase | long-term memory Müller, 2000 |
| 5      | c47353 | 393      | 46           |                |                               |                             |
| 6      | c31427 | 268      | 42           |                |                               |                             |
| 7      | c29331 | 229      | 95           | Wnt             | signals of nerve axis         | Janssens et al., 2010      |
| 8      | c34286 | 325      | 64           | ftxQ2           | central brain                  | Kitzmann et al., 2017      |
| 9      | c49243 | 742      | 55           | Dorsal          | dorsoventral axis              | Zeinlinger et al., 2007; Fonseca et al., 2008 |
| 10     | c43582 | 421      | 87           | runt            | nervous system                 | Butler et al., 1992        |
| 11     | c42641 | 337      | 46           | Phm             | mesoderm                      | Zeinlinger et al., 2007     |
| 12     | c49033 | 1385     | 49           | ci              | regulating the Hedgehog       | Hepler et al., 1997; Amin et al., 1999 |
| 13     | c49331 | 548      | 73           | sim             | central nervous system         | Kasai et al., 1998         |
| 14     | c46786 | 294      | 82           | sox1/2/3        | nervous system                 | Lowe et al., 2003          |
| 15     | c41255 | 208      | 82           |                |                               |                             |
| 16     | c49059 | 363      | 71           | cAMP-dependent protein kinase | long-term memory Müller, 2000 |
| 17     | c44784 | 460      | 87           | fce1            | head                          | Possien et al., 2011       |
| 18     | c44001 | 383      | 53           | hh              | head                          | Amin et al., 1999; Possien et al., 2011 |
| 19     | c44769 | 260      | 48           | AQP4            | central nervous system         | Scharffman and Binder, 2013 |
| 20     | c46478 | 270      | 46           |                |                               |                             |
| 21     | c48478 | 618      | 73           | trp              | visual system                 | Gutovov et al., 2022       |
| 22     | c32815 | 445      | 57           | tyrosine aminotransferase     | immune                        | Li et al., 2019             |
| 23     | c37696 | 435      | 54           |                |                               |                             |
| 24     | c47271 | 685      | 56           | phenoloxidase subunit A3      | immune                        | Li et al., 2019             |

64 interactions (score = 8.000) (Figure 3B). The core network of females also contained 9 hub genes (PPO2, GNBP3, Spn77Ba, Ppn, yellow-d2, PGRP-LB, PGRP-SD, Cg25C, and vkg) and 22 interaction relationships (score = 2.750) (Figure 4B). The CytoHubba plug-in was used again to extract the hub genes (male: Mhc, Mlc1, Mlc2, Prm, Mf, wipA, TpnC25D, fln, l(2)efl, and Act5C) (Figure 3C) and (female: PPO2, GNBP3, Spn77Ba, Ppn, yellow-d2, PGRP-LB, Cg25C, PGRP-SC2, and Hml) (Figure 4C). Combining the two algorithms, 125 male DEGs obtained a total of 10 core hub genes, which were mostly described in STRING as related to development and reproduction. 151 female DEGs obtained a total of 11 core hub genes, which were mostly related to immune function (Table 7). When using the NetworkAnalyzer plug-in to calculate the degree and betweenness centrality of each node, the genes with higher degree and betweenness centrality values were basically consistent with the core hub genes we found. In the male network, the degree of Mlc1 was the highest, and the betweenness centrality of Act5C was the highest (Figure 3D, Supplementary Table S4). The degree and the betweenness centrality of PPO2 were the highest in the female network (Figure 4D, Supplementary Table S5).
Discussion

In this study, we identified 276 sex-biased genes and 24 neurodevelopmental candidate genes, of which 29 core genes were screened by PPI network analysis. Most of the 24 neurodevelopmental candidate genes homologous to the functionally known genes were equally expressed between males and females. Interestingly, sex-biased genes displayed significant differences in gene functions between sexes. In the males, sex-biased genes were identified to be associated with development, but female-biased genes were identified to be associated with immunity. One hypothesis to explain this phenomenon is that the normal neurodevelopmental candidate genes might be involved in the basic progress of neurodevelopment so that they were equally expressed in the male and female brains, while the newly identified male-biased genes might be specifically involved in the regulation of development of male-specific MGC structure; this needs to be further confirmed by gene knock-out or knock-down studies.

In the male brains, 125 genes displayed male-specific or biased expressions, of which 11 were identified to be the core genes in the PPI network analysis, and most were functionally associated with development and reproduction (Table 7). The c26838 gene corresponded to myosin alkali light chain 1 (Mlc1), which had the highest degree in the PPI network analysis, and was shown to be involved in mesodermal development (Picchio et al., 2018). The c38747 gene corresponds to Actin-5C (Act5C), which was one of the core hub genes highly expressed in the developing cells, with various functions related to development and reproduction, and it plays important roles in cytokinesis and spermatogenesis (Wagner et al., 2002; Noguchi and Miller, 2003; Wilson et al., 2008; Edwards et al., 2009; Guan et al., 2019). The c40474 gene corresponds to wings up A (wupA) and, encodes Troponin I, with involvement in muscle and nervous system development and maintenance (Fishilevich et al., 2019). The c45921 gene corresponds to myosin heavy chain (Mhc) and is known to be involved in muscle cell differentiation, cell component movement, and flight (Picchio et al., 2018). These
results indicated that the core genes with male-specific or biased expressions might play important roles in the brain neural development and might be related to the development of MGC structure in the male brains of *O. furnacalis*.

In the female brains, 151 genes displayed female-specific or biased expressions, of which 10 were identified to be the core genes in the PPI network analysis, and most were functionally associated with immunity (Table 7). Among them, the c47271 gene corresponds to *Prophenoloxidase 2* (*PPO2*), which plays an important role in both melanin formation and immunity (Banerjee et al., 2019; Schmid et al., 2019). The c46696 gene corresponds to *serine protease inhibitor 77Ba* (*Spn77Ba*), which was identified to be involved in the regulation of immune responses by inducing systemic expression of the antifungal peptide drosomycin through the Toll pathway and disruption of tracheal melanosis (Tang et al., 2008). GNBP3 encodes a hemolymphatic protein, while PGRP-SD, PGRP-SC2, and PGRP-LB encode peptidoglycan-recognition proteins, which are a family of pattern recognition molecules identified to be involved in the regulation of the Toll and Imd signaling pathway. These pathways are related to immune response and development, and correspond corresponding to the c25523, c25520, c42129, and c45285 genes (Tanji et al., 2007; Royet et al., 2011; Iatsenko et al., 2016; Zhu et al., 2017; Yang et al., 2018; Orlans et al., 2021). Immune genes may be partly related to microglia, which are the resident immune cells in the brain (Hammond et al., 2019). Interestingly, the female-biased genes were mostly related to immunity, but the exact reason for this accumulation of immunity-related genes remains unknown and requires further study. In addition, several peptides (c25808, c34079, c36790, c42325) and peptidase (c33646) were identified as highly expressed in the female brains, which might be involved in female-specific signaling or the development of the LFG structure in *O. furnacalis*.

Additionally, 24 candidate genes for brain neurodevelopment were identified by homology analysis, and eight of them were
| Number | Gene       | Node name | Numbers of description | Description                                                                 |
|--------|------------|-----------|------------------------|----------------------------------------------------------------------------|
| 1      | c26838     | Mlc1      | 5                      | cell development; tissue development; anatomical structure development; calcium ion binding; supramolecular complex |
| 2      | c38747     | Act5C     | 5                      | cell development; cell differentiation; anatomical structure morphogenesis; multicellular organism development; anatomical structure development |
| 3      | c40111     | Prm       | 8                      | muscle structure development; cell development; tissue development; cell differentiation; anatomical structure morphogenesis; multicellular organism development; anatomical structure development; supramolecular complex |
| 4      | c45921     | Mhc       | 8                      | muscle structure development; cell development; tissue development; cell differentiation; anatomical structure morphogenesis; multicellular organism development; anatomical structure development; supramolecular complex |
| 5      | c34456     | Mlc2      | 6                      | muscle structure development; cell development; cell differentiation; anatomical structure morphogenesis; anatomical structure development; calcium ion binding; supramolecular complex |
| 6      | c40474     | wupA      | 8                      | muscle structure development; cell development; tissue development; cell differentiation; anatomical structure morphogenesis; multicellular organism development; anatomical structure development; supramolecular complex |
| 7      | c39951     | Mf        | 1                      | supramolecular complex                                                      |
| 8      | c27864     | Fln       | 6                      | muscle structure development; cell development; cell differentiation; anatomical structure morphogenesis; multicellular organism development; supramolecular complex |
| 9      | c39641     | TpnC25D   | 2                      | calcium ion binding; supramolecular complex                                   |
| 10     | c49494     | 1 (2)el   | 6                      | muscle structure development; cell development; cell differentiation; anatomical structure morphogenesis; anatomical structure development; supramolecular complex |
| 1      | c47271     | PPO2      | 6                      | innate immune response; immune response; response to an external stimulus; catalytic activity; extracellular space; extracellular region |
| 2      | c25523     | GNBP3     | 5                      | immune response; immune response; response to an external stimulus; Toll and Imd signaling pathway; extracellular region |
| 3      | c46696     | Spn77Ba   | 3                      | extracellular space; extracellular space; extracellular region              |
| 4      | c48631     | Ppn       | 2                      | catalytic activity; extracellular region                                     |
| 5      | c41866     | yellow-d2 | 1                      | extracellular region                                                        |
| 6      | c45285     | PGRP-LB   | 6                      | innate immune response; immune response; response to an external stimulus; catalytic activity; Toll and Imd signaling pathway; extracellular region |
| 7      | c25520     | PGRP-SD   | 6                      | innate immune response; immune response; response to an external stimulus; Toll and Imd signaling pathway; extracellular space; extracellular region |
| 8      | c42129     | PGRP-SC2  | 6                      | innate immune response; immune response; response to external stimulus; catalytic activity; Toll and Imd signaling pathway; extracellular region |
| 9      | c49487     | Hml       | 5                      | innate immune response; immune response; response to external stimulus; ECM-receptor interaction; extracellular region |
| 10     | c47432     | Cg25C     | 3                      | ECM-receptor interaction; extracellular space; extracellular region         |
| 11     | c46305     | Vkg       | 3                      | ECM-receptor interaction; extracellular space; extracellular region         |
| 1      | c47752     | Dac       | 7                      | genital disc sexually dimorphic development; central nervous system development; sensory organ development; axonogenesis; neuron differentiation; neuron development; nervous system development |
| 2      | c29331     | Wg        | 6                      | genital disc sexually dimorphic development; ectoderm development; central nervous system development; sensory organ development; neuron differentiation; nervous system development |
| 3      | c44001     | Hh        | 5                      | central nervous system development; immune response; sensory organ development; neuron differentiation; nervous system development |
| 4      | c49013     | Ci        | 5                      | immune response; sensory organ development; neuron differentiation; neuron development; nervous system development |
| 5      | c43582     | Run       | 6                      | central nervous system development; sensory organ development; axonogenesis; neuron differentiation; neuron development; nervous system development |
| 6      | c46011     | Lim1      | 3                      | sensory organ development; neuron differentiation; nervous system development |
| 7      | c48600     | Rbp9      | 2                      | central nervous system development; nervous system development              |
| 8      | c34127     | Bx        | 1                      | sensory organ development                                                   |
identified to be core genes by PPI network analysis. These genes might be associated with neuronal differentiation, nervous system development, cell differentiation, and anatomical morphogenesis in the brain. Among them, only one gene (*Phenoloxidase subunit A3*) displayed female-biased expression and the others were equally expressed between male and female brains. Phenoloxidase is a polycopper oxidase that plays an important role in melanin synthesis, cuticle pigmentation, wound healing, and defense against microbial and parasitic invasion (Elsik et al., 2014). The key core gene *dachshund* (*dac, c47752*) was identified being involved in the development of eyes and mushroom bodies (Mardon et al., 1994; Kurusu et al., 2000). *Hedgehogs* (*hh, c44001*) play an important role in the development of head segment polarity (Amin et al., 1999; Ntini and Wimmer, 2011; Possnien et al., 2011), as well as regulation of eye size and pattern (Míguez et al., 2020). In addition, *hh* and *cubitus interruptus* (*ci, c49013*) were identified that being involved in regulating the Hedgehog signaling pathway, which is critical in embryonic development and adult tissue homeostasis (Joulia et al., 2005).

**Data availability statement**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA818099.

**Author contributions**

GW, XZ, and BY designed the experiment. YC, YZ, CD, and LL carried out the experiments. YC and BY wrote the manuscript. YC and BY analyzed the experimental results.

**Funding**

Projects are subsidized by the National Natural Science Foundation of China (31861133019 and 32172501).

**Acknowledgments**

We thank our colleague Q-YW for the help with insect rearing.
Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.953538/full#supplementary-material

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