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Comparative transcriptomics analyses of chemosensory genes of antenna in male red swamp crayfish *Procambarus clarkii*

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The red swamp crayfish, *Procambarus clarkii*, is a globally invasive species and has caused huge damage to aquaculture, biodiversity, and ecology worldwide. Antenna-expressed receptors are important for *P. clarkii* to detect chemosensory cues for mate attraction. In this study, we tested the behavior of male *P. clarkii* to the conditioned water from female *P. clarkii* during the mating and non-mating periods, and performed RNA sequencing to investigate the chemosensory-related genes of the antenna of male *P. clarkii*. The results of the behavioral assay have shown that for the female-conditioned water, male *P. clarkii* within the mating period can be significantly attracted, but not during the non-mating period. This suggested that the expressions of chemosensory-related genes in the antenna of male *P. clarkii* may change significantly with mating seasonal variation. Antenna transcriptomes found that a total of 59,218 unigenes with an average length of 1,056.41 bp, and 4,889 differentially expressed unigenes (DEGs), among which 2,128 were upregulated, while 2,761 were downregulated were obtained. A total of 12 upregulated and nine downregulated DEGs were associated with chemical reception, including four ionotropic receptors (IRs) or ionotropic glutamate receptors (iGluRs), eight G-protein-coupled receptors (GPCRs), five transient receptor potential channels (TRP channels), one sodium–calcium exchanger, one isomerase, and two uncharacterized proteins (chemosensory proteins-like, CSPs). CSPs were preliminarily classified as pheromone receptors in the antenna of male *P. clarkii*. Furthermore, the calcium transduction-related pathways may play an important role in the sex pheromone reception of the male *P. clarkii*'s antenna. The results of quantitative real-time reverse transcriptase PCR (RT-qPCR) showed that

the trends of expression of eight selected unigenes were consistent with RNA-Seq results. Our results provide more comprehensive data for chemical communication mechanisms after *P. clarkii* enter the mating period and eventually would develop better control strategies in further.

KEYWORDS

***Procambarus clarkii*, antenna, transcriptome, RNA-Seq, chemosensory, chemical communication**

Introduction

Chemoreception is the dominant sensory modality for most crustaceans due to the complex aquatic environment (Harzsch and Krieger, 2018), which is the base of crustaceans for their daily activities, such as searching for food, reproduction, and communication (Blinova and Cherkashin, 2012). Previous studies suggest that the antenna is one of the predominant chemosensory organs of most crustaceans (Harzsch et al., 2011; Waldrop, 2013; Urbschat and Scholtz, 2019). Crustaceans receive chemical signals through several parallel channels, which can be summarized into two modes of "olfaction" and "distributed chemoreception" (Schmidt and Mellon, 2011). "Olfaction" is mediated by unimodal olfactory sensilla called "aesthetasc," which are only present on the first antenna of the crustaceans (Harzsch and Krieger, 2018). Aesthetascs are innervated by olfactory sensory neurons (OSNs) (Derby et al., 2016). "Distributed chemoreception" is mediated by various bimodal sensillae in the first antenna (Solari et al., 2017), mouthparts (Garm et al., 2003; Garm et al., 2004), chelipeds, and walking appendages (Altner et al., 1983; Schmidt and Gnatzy, 1984). It is innervated by both chemoreceptor neurons (CRNs) and mechanoreceptor neurons (MRNs). However, there is a gap in the data for chemoreceptors of crustaceans. So far, the predicted chemosensory-related proteins in crustaceans include ionotropic receptors (IRs), ionotropic glutamate receptors (iGluRs), G-protein-coupled receptors (GPCRs), transient receptor potential channel (TRP channel), chemosensory proteins (CSPs), and gustatory receptor (GRs) (Eyun et al., 2017; Kozma et al., 2020a,b). Identification of candidate chemosensory-related genes helps to better understand the molecular basis of chemoreception in pests and eventually develop better control strategies (Wang et al., 2014; Gonzalez et al., 2021).

The red swamp crayfish, *Procambarus clarkii* (Girard, 1852), has its origins in North-eastern Mexico and South America (Shen et al., 2014), and was introduced to China from Japan during the 1930s (Shen et al., 2020). At present, it is an economically important animal (Liu et al., 2021). However, invasive crayfish *P. clarkii* into the natural ecosystems of China has caused huge losses to agriculture, biodiversity, and aquaculture. Moreover, *P. clarkii* is a carrier of the white spot syndrome virus (WSSV) and parasites, whereby it may cause infection and death of other commercial shrimps in case of spread without effective control (Zhu et al., 2009). Therefore, effectively and specifically controlling *P. clarkii* requires more attention. In recent years, control of invasive species using sex pheromone has been adopted for other species of insects (Gherardi et al., 2011; Keller-Costa et al., 2014; Johnson et al., 2015). Previous studies confirm that males can detect sex pheromones through their chemosensory organ uniaxially, and then search for the signal source or perform courtship behaviors (Oyama et al., 2020). In addition, the suitable reproduction temperatures of *P. clarkii* are ranging from 21 to 25°C, and it reduced mating behavior below 15°C (Jin et al., 2019; Dong et al., 2020). The male *P. clarkii* recognizes female mating receptivity by detecting the urinary components of females (Kubec et al., 2019). However, little is known about the chemosensory mechanism of action of these essential chemicals (such as sex pheromone components) in male *P. clarkii*.

Like other crustaceans, the *P. clarkii*'s antenna is a critical chemosensory organ that perceives and locates chemical signals or pheromones released by their mates (Breithaupt, 2011). Although the candidate chemosensory genes of the lateral flagellum of the antennule in *P. clarkii* were reported (Kozma et al., 2020a), the chemosensory genes in the antenna are still unknown. Therefore, to further understand the chemical communication mechanism through chemosensation in male *P. clarkii*, we performed the behavioral assays of male *P. clarkii* on the crude conditioned water from female *P. clarkii* (in the mating period, MP) during the mating (MP) and non-mating period (NMP), and collected the antenna of the male *P. clarkii* in the MP as the experimental group and the NMP as the control group. Subsequently, next-generation sequencing (NGS) and assembly were used to obtain a male antenna transcriptome of *P. clarkii* in MP vs. NMP. Our findings may help better understand the mechanisms of chemosensory responses in *P. clarkii*, and provide a solid foundation for further studies on the relationship between chemosensory-related genes and semiochemicals.

Materials and methods

Animal collection and preparation

Procambarus clarkii (400 males and 20 females) was purchased from Guilin city, Guangxi province, China in

August 2020. The average weight of the mature *P. clarkii* was 16.22 ± 1.82 g. The length of the antenna was longer than 5 cm. A total of 20 *P. clarkii* were cultured in one water tank and fed two times a day on artificial food throughout the experimental period (Li et al., 2012). A total of 20 mature female *P. clarkii* were fasted for 48 h and placed in a water tank with 1 L of ultrapure water (for crude conditioned water collections) for further cultivation for 24 h. The feeding temperature of female *P. clarkii* was between 23 and 25°C. The conditioned water of female *P. clarkii* was centrifuged for 20 min (8000 rpm, 4°C), and removed the insoluble substances to obtain the crude conditioned water (collected in the mating period, for behavior assay). The crude conditioned water samples were stored at −80°C until further behavior assay experiment.

All male *P. clarkii* had no contact with female crayfish or pheromones during the feeding process. We randomly divided male *P. clarkii* into two following groups: 200 individuals were fed until January 2021 (NMP, water temperature <15°C) and another 200 individuals were fed until April 2021 (the initial stage of the MP, water temperatures >22°C).

The behavioral assay of male *Procambarus clarkii* during the non-mating period and mating period

The behavioral assays were performed using the Y-maze (self-made with glass) to determine the sensitivity of male *P. clarkii* to the crude conditioned water (**Figure 1**). There was a drain hole at the start arm of the Y-maze device to keep the water depth at 5 cm. Fifty male *P. clarkii* were tested for behavioral assays in NMP (in January) and MP (in April), respectively. The procedures of behavioral assays were performed as follows: First, 500 ml of ultrapure water was poured into Y-maze and regulated the water flow rate (20 ml/min) of infusion tubes to the same velocity by flow meters. Thereafter, cylindrical sponge blocks with stimulus (1 ml of crude conditioned water, experimental group) and ultrapure water (1 ml, control group) were placed in the end arm, respectively. Finally, the male *P. clarkii* was put into the start arm and observed the arm entry by *P. clarkii*. Each animal was tested for the crude conditioned water only once. At the end of each operation, the Y-maze would be emptied and wiped with alcohol on the surface to prevent leftover interference from the last operation. Each assay lasted 5 min. If no choice was made for more than 5 min, the male *P. clarkii* would be counted as unresponsive individuals, which would be excluded from the analysis. After every five *P. clarkii* were tested, the positions of the experimental group (crude conditioned water) and the control group (ultrapure water) were switched to prevent directional bias. The data were calculated using the Chi-squared test (Barnard et al., 2016) in SPSS v 25.0 at a significant level of $P < 0.05$.

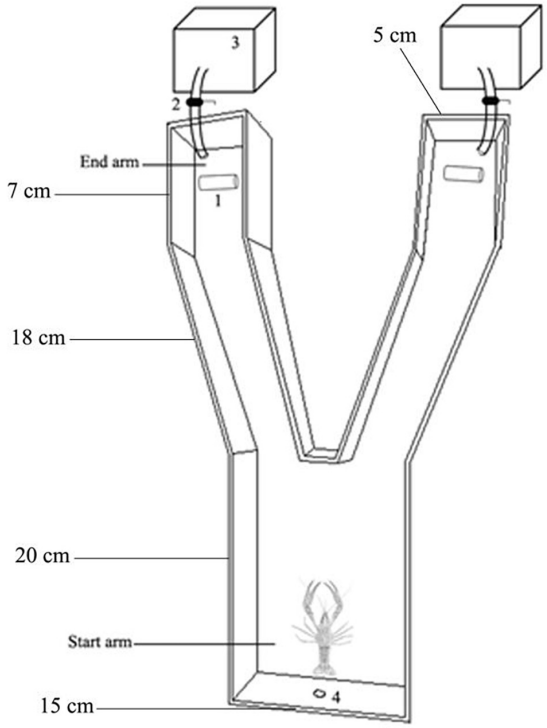

FIGURE 1
The behavioral device: Y-maze. The height of Y-maze is all 5 cm. The Y-maze was symmetrical on both sides. 1: cylindrical sponge block, 2: flow meter, 3: water box, and 4: drain hole.

Total ribonucleic acid extraction and detection

To reveal the difference in male *P. clarkii* responses to female-conditioned water, the antenna transcriptomics of male *P. clarkii* was performed in NMP and MP groups using RNA-Seq technology. When the behavior assay was done, the whole antenna with the hard cuticle of male *P. clarkii* (150 individuals in NMP and MP, respectively) was collected and transferred into sterile 15 ml centrifuge tubes without RNase and DNase (Corning, United States). In order to obtain enough RNA, 50 pairs of male antennae were combined into one sample. NMP and MP groups had three replicate samples, respectively. The samples of NMP and MP groups were frozen in liquid nitrogen and stored at −80°C until the extraction of total RNA. The library construction and sequencing of transcriptomes were performed by Major Technology Co. Ltd. (Shanghai, China). Total RNA extraction was performed using the TRIzol reagent (Invitrogen, Shanghai, China). Both concentration and purity were measured using a Nanodrop 2000 spectrophotometer (Invitrogen, MA, United States). The degradation of extracted RNA was detected on 1% agarose gels and RNA integrity number (RIN) was assessed using Agilent Bioanalyzer 2100 (Agilent Technologies, United States).

Library construction and sequencing

mRNAs were enriched using magnetic beads with Oligo (dT) and randomly fragmented using fragmentation buffer. Under the action of reverse transcriptase, the fragmented mRNAs were used as templates for first-strand cDNA synthesis using random hexamer primers. Subsequently, second-strand cDNA was synthesized using DNA polymerase I and RNase H. We added an end-repair mix (including the end-repair enzyme mix and end-repair buffer) to patch the cohesive ends of the double-strand cDNA, followed by the addition of tail and sequencing adapters. Subsequently, cDNA was amplified by PCR. A total of six cDNA libraries were obtained after purifying the amplification products with AMPure XP beads. Thereafter, QuantiFluor dsDNA System and Quantus^{TM} Fluorometer (Promega, Madison, WI, United States) were used to detect the concentration and inter size of the libraries, respectively. The effective concentration of the libraries was accurately quantified qPCR (quantitative polymerase chain reaction, effective concentration >2 nM) using the Qubit RNA Assay Kit in the Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, United States). Finally, the constructed paired-end libraries were sequenced on the Illumina HiSeqXten/NovaSeq 6000 Sequencing Platform.

de novo assembly and annotation

Using SeqPrep[1] and Sickle,[2] all clean reads from NMP and MP groups were obtained by filtering the sequencing adapters, poly-N, and the low-quality sequences (quality value, QV <20) from raw reads. The assembly of two sets of antenna was combined together and performed by using RNA-Seq *de novo* programs Trinity software (v2.8.5) (Liu et al., 2021). The completeness of the assembly was evaluated using the BUSCO software (v5.3.2). The assembled *de novo* unigenes were obtained, which were the encoding sequences. The redundancy was filtered using the Cd-hit-EST (Foquet et al., 2021). The Transrate (v1.0.3) was used to map reads to contigs and inspect the alignments (Marx et al., 2021).

The unigene sequences were compared using six databases, including the National Center for Biotechnology Information (NCBI) Non-Redundant Protein Sequence Database (Nr),[3] Protein family (Pfam),[4] Gene Ontology (GO),[5] Swiss-Prot,[6] Cluster of Orthologous Groups of proteins (COG),[7] and Kyoto Encyclopedia of Genes and Genomes (KEGG).[8] Functional annotation was processed using the Blast2GO software (Conesa et al., 2005).

Differentially expressed unigene enrichment analysis

The Bowtie software (Langmead et al., 2009) was used to compare clean reads with the unigene library and on combining with RSEM (Li and Dewey, 2011), the expression levels were estimated. The expression of unigenes was calculated using FPKM (fragments per kilobase per million) (Mortazavi et al., 2008) between six libraries. Then, the differentially expressed unigenes (DEGs) were analyzed using the DESeq2 software (v1.24.0) based on the false discovery rate (FDR) correction with Benjamini and Hochberg (BH) method (Varet et al., 2016). The resulting *P*-values were adjusted using the FDR. The DEGs were filtered using the threshold of FDR <0.05 and | log2 (fold change) | ≥1 (Ma et al., 2020). Subsequently, GO and KEGG pathway enrichment analyses were conducted based on Fisher's precision probability test (BH method, *P*-adjust <0.05) using the Goatools software (Klopfenstein et al., 2018). All DEGs were mapped to terms in the KEGG and GO databases using the BLAST algorithm with an *E*-value of ≤1E-5, and the significantly ($P < 0.05$) enriched KEGG and GO terms in DEGs were identified compared with the transcriptome background. The data of pathways were analyzed on the online tool of Majorbio Cloud Platform (https://cloud.majorbio.com/page/tools/) (Ren et al., 2022).

Real-time quantitative polymerase chain reaction

To verify the results of our sequencing analyses, we selected eight sensory-related DEGs with high expressions in MP or NMP groups (**Supplementary Table 1**) from the male antenna of *P. clarkii* for quantitative real-time reverse transcriptase PCR (RT-qPCR) analysis. The total RNA was reversely transcribed into first-strand cDNA using the PrimeScript^{TM} first-strand cDNA Synthesis Kit (TaKaRa, Shanghai, China), following which the newly synthesized cDNA was used as the template for RT-qPCR. Specific primers were designed using the Primer 5.0 software with a concentration of 10 μmol/L. The primer sequences of eight DEGs used for RT-qPCR are shown in **Supplementary Table 1**. The RT-qPCR was performed on an MA-6000 real-time fluorescence quantitative PCR instrument (Molarray, Jiangsu, China). The RT-qPCR reactions (20 μl) contained 1 μl of cDNA, 0.4 μl of each primer (0.2 μmol/L

1 https://github.com/jstjohn/SeqPrep
2 https://github.com/najoshi/sickle
3 www.ncbi.nlm.nih.gov/
4 http://pfam.xfam.org/
5 http://www.geneontology.org
6 http://web.expasy.org/docs/swiss-prot_guideline.html
7 http://www.ncbi.nlm.nih.gov/COG/
8 http://www.genome.jp/kegg/

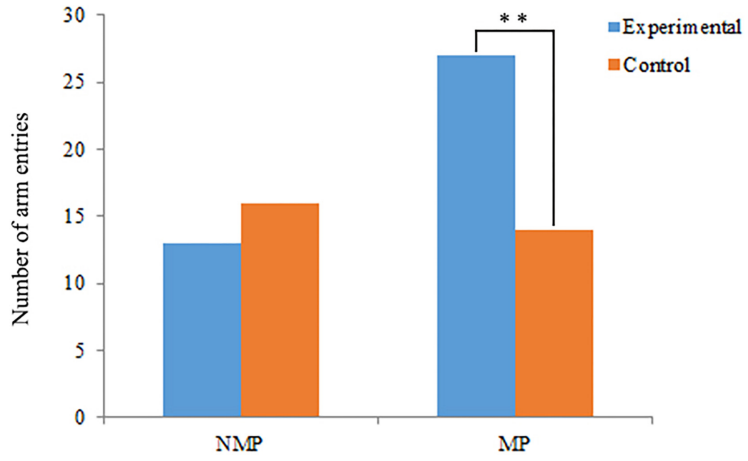

FIGURE 2
Behavioral assays of male *Procambarus clarkii* during the non-mating period (NMP) and mating period (MP). *Represents the significant differences between experimental and control groups ($P < 0.05$, $n = 50$). The numbers of no choice were excluded from the statistical analysis.

in this reaction system), 10 μl of 2 × SYBR real-time PCR premixture (dNTP and buffer), and added the RNase free sterilized ultrapure water. The reaction procedure was as follows: 95°C for 5 min, followed by 40 cycles with 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. To confirm reproducibility, the RT-qPCR reaction for each sample was performed in three technical replicates and three biological replicates based on the MP (>22°C) and NMP (<15°C) groups. The melt curves were used to evaluate the specificity of primers and amplification. All melt curves have a single peak, and PCR efficiency for each primer pair was ranging from 91 to 103%. The level of expression of selected genes was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), and the expression of selected genes was normalized against β-actin expression levels (Yu et al., 2022). Comparative analyses for each target gene among different samples were analyzed using the one-way ANOVA (SPSS, version 25.0).

Results

Behavioral tests of male *Procambarus clarkii* during the non-mating period and mating period

The results of behavioral assays have shown that the conditioned water from female *P. clarkii* can significantly attract the male individuals during the MP ($\chi^2 = 4.12$, $P = 0.04$), but cannot significantly attract the males in NMP ($\chi^2 = 0.31$, $P = 0.58$) (**Figure 2** and **Supplementary Table 2**).

Assembly and splicing

A total of 258.48 million raw reads and 252.98 million clean reads were obtained from six libraries (**Supplementary Table 3**). The Q20 and Q30 were greater than 97 and 92%, respectively. The GC contents were ranging from 44.38 to 49.05% (**Supplementary Table 3**). Moreover, all clean reads from NMP and MP groups were combined to *de novo* assemble the unigenes (**Table 1** and **Supplementary Figure 1**). The BUSCO score showed that the transcriptome was with high completeness (single-copy: 91.8%; duplicated: 4.2%). The raw data files have been uploaded to the NCBI sequence read archive (BioProject accession number: PRJNA839237).

Functional annotation and classification of unigenes

To investigate information on gene functions more comprehensively, a total of 57,812 unigenes were annotated using six databases including Nr, Pfam, Swiss-Prot, COG, GO, and KEGG. The results of annotation of the unigenes are as follows: 18,388 (31.81%) unigenes were annotated in Nr; 14,039 (24.28%) in Pfam; 11,986 (20.73%) in Swiss-Prot; 14,479 (25.04%) in COG; 14,129 (24.44%) in GO; and 9,176 (15.87%) in KEGG (**Supplementary Figure 3** and **Supplementary Table 4**). The annotations of unigenes to different databases are shown in

TABLE 1 The length distribution of assembled transcripts and unigenes.

Length (bp)	Transcripts	Unigenes
Total number	78,138	59,218
Max length	34,133	34,133
Min length	201	201
Mean length	1,147.85	1,059.41
N50 length	2,379	2,229
BUSCO score (%)	C: 96 [S: 91.8; D: 4.2]	C: 96 [S: 91.8; D: 4.2]

The "C," "S," and "D" in BUSCO score represent the "complete," "single-copy," and "duplicate," respectively.

Supplementary Figure 4. Moreover, a total of 111 unigenes were annotated to chemosensory-related functions, including IRs or iGluRs (43 unigenes annotated), TRP channels (30), CSPs-like (2), GPCRs (17), GR (1), and other olfactory-related proteins (18) (Supplementary Excel Table 1).

Identification and annotation analysis of differentially expressed unigenes

Based on the DESeq2 software, a total of 4,889 DEGs with the FDR <0.05 and | log2 (fold change) | ≥1 were identified between MP and NMP groups, including 2,128 upregulated and 2,761 downregulated unigenes (Supplementary Figure 4). The fold change (FC) and annotations of all DEGs are shown in Supplementary Excel Table 2. Furthermore, the annotations from the GO analysis of DEGs were classified into 44 GO categories, including BP (18), CC (14), and MF (12). The top three GO terms in CC were cell part (GO:0044464), membrane part (GO:0044425), and protein-containing complex (GO:0032991); in BP were, cellular process (GO:0009987), metabolic process (GO:0008152), and biological regulation (GO:0065007); in MF were, binding (GO:0005488), catalytic activity (GO:0003824), and structural molecule activity (GO:0005198) (Supplementary Figure 5A). Moreover, the KEGG enrichments have shown that the "Ribosome (*P*-value = 5.067E-31, 156 upregulated and 39 downregulated DEGs)" is the most enriched pathway, followed by "Amoebiasis" and "Complement and coagulation cascades" (Supplementary Figure 5B).

Chemosensory-related differentially expressed unigenes and pathway

As shown in Table 2, 21 identified DEGs were related to chemosensory functions. On comparing the MP and NMP groups, nine DEGs were found to be downregulated, while 13 were upregulated, including four IRs or iGluRs, eight G-protein-coupled receptors, five TRP channels, one sodium–calcium exchanger, one isomerase, and two uncharacterized protein (CSP-like). The sequences of these DEGs are shown in Supplementary Excel Table 3. Among these 21 DEGs, the DEG related to sodium–calcium exchanger (solute carrier family, NCX) was involved in the olfactory transduction and calcium signaling pathway (Figure 3).

TABLE 2 Chemosensory or –like regulation-related differentially expressed unigenes (DEGs).

Gene ID	Description	Categories	log2FC	*P*-value
TRINITY_DN21918_c0_g1	Olfactory ionotropic receptor IR93a	IR	−2.306	0.017
TRINITY_DN25415_c0_g2	Ionotropic glutamate receptor subunit	IR	−3.724	4.187E-5
TRINITY_DN20582_c0_g1	Variant ionotropic glutamate receptor	IR	−3.312	3.370E-5
TRINITY_DN21176_c0_g4	Peroxisomal acyl-coenzyme A oxidase 1-like	GPCR	−6.572	0.021
TRINITY_DN521_c1_g2	Metal ion binding*	GPCR	−1.636	2.049E-4
TRINITY_DN1785_c0_g1	Threonine-protein kinase 26-like*	GPCR	−1.247	6.946E-4
TRINITY_DN3158_c0_g2	Crustacean cardioactive peptide receptor, partial	GPCR	−2.250	1.191E-5
TRINITY_DN10908_c0_g3	G-protein coupled receptor GRL101-like	GPCR	−1.762	0.005
TRINITY_DN28245_c0_g1	Transient receptor potential cation channel subfamily A member 1-like isoform X3	TRP channel	−4.519	0.034
TRINITY_DN518_c0_g1	Uncharacterized protein	CSP-like	8.280	6.233E-5
TRINITY_DN7269_c0_g2	Uncharacterized protein*	CSP-like	7.496	1.077E-6
TRINITY_DN16621_c0_g1	Urotensin-2 receptor-like*	GPCR	1.092	0.033
TRINITY_DN22772_c0_g1	G-protein coupled receptor activity	GPCR	1.039	0.010
TRINITY_DN22075_c0_g1	G-protein coupled receptor moody*	GPCR	1.153	0.002
TRINITY_DN14467_c0_g4	Transient receptor potential channel pyrexia OS	TRP channel	3.075	4.122E-4
TRINITY_DN24541_c0_g1	Putative transient receptor potential cation channel subfamily A member 1 isoform X1	TRP channel	1.660	1.279E-4
TRINITY_DN4439_c0_g1	Ankyrin-11-like*	TRP channel	1.605	1.471E-5
TRINITY_DN2621_c1_g1	Transient receptor potential channel pyrexia-like	TRP channel	1.470	3.018E-4
TRINITY_DN5073_c0_g3	Transient receptor potential cation channel pyrexia	TRP channel	2.021	0.01
TRINITY_DN32840_c0_g1	Sodium-calcium exchanger 1*	sodium-calcium exchanger	1.963	2.972E-4
TRINITY_DN4991_c0_g1	Putative E3 ubiquitin-protein ligase TRIM32*	isomerase	2.52	2.847E-6

*Represents the selected DEGs for RT-qPCR. The log2FC < 0 represents the downregulated DEGs, while the log2FC >0 represents the upregulated DEGs.

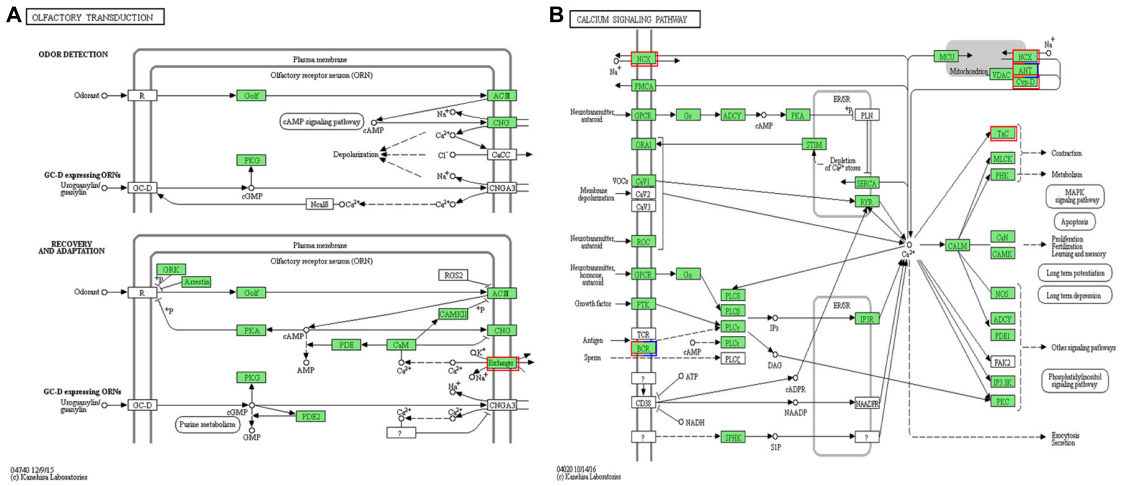

FIGURE 3
The calcium-related differentially expressed unigenes (DEGs) were enriched in **(A)** the olfactory transduction pathway and **(B)** the calcium signaling pathway. The red box represents the upregulated DEGs in mating period (MP) groups. The blue box represents the downregulated DEGs in MP groups, and the box with both red and blue represents that there are both up and downregulated DEGs in MP groups. The green box represents the genes annotated in the pathway without significant difference (Image credits: https://www.kegg.jp/pathway/map=map04740&keyword=olfactory%20transduction and https://www.kegg.jp/pathway/map=map04020&keyword=calcium).

Validation by RT-qPCR analysis

To verify the accuracy of the RNA-Seq data, eight sensory-involved genes were selected from among the DEGs and validated by RT-qPCR analysis. The expression levels of six DEGs were significantly upregulated (GPCR moody, TRIM 32, CSP-like, ankyrin-1, NCX 1, and SSTR 2) in MP groups and two (ATP 1A and STK 26-like) were significantly downregulated in the MP groups. The results of RT-qPCR were consistent with those of RNA-Seq, indicating the reliability of the RNA-Seq data (**Figure 4**, **Supplementary Table 5**, and **Supplementary Excel Table 4**).

Discussion

Numerous behavioral studies indicate that the male *P. clarkii* have "unidirectional receptors" for the sex pheromones released by females during the MP (Monteclaro et al., 2010; Peddio et al., 2019; Oyama et al., 2020). Male individuals can recognize different phases of females by detecting the pheromones and then performing courtship behaviors after checking the sex pheromone levels (Kubec et al., 2019). However, previous research suggests that the frequencies of copulation and oviposition in *P. clarkii* are significantly reduced when the temperature goes below 15°C (Egly et al., 2019). In this study, we found that male *P. clarkii* were attracted to female-conditioned water in MP but not in NMP, which was consistent with previous studies. We speculated that the expression of chemosensory genes in antenna may be altered due to different temperatures and periods. Therefore, we identified the chemosensory genes and pathways in the antenna of *P. clarkii* between MP vs. NMP by RNA-Seq for the first time. However, the information on chemosensory genes was as sparse as expected, presumably since genetic annotation of species closely related to *P. clarkii* is scarce in public genetic databases; in general, the genetic information on chemoreception in crustaceans is lacking. This study is a necessary supplement to the genetic information library for *P. clarkii*.

Procambarus clarkii is one of the main model animals in the study of chemosensory receptors in crustaceans. The previous study had found several types of chemoreceptors in the antennule and legs of *P. clarkii*, including the IRs, iGluRs, TRP channels, and GRs (Kozma et al., 2020a). In our study, we found these four types of chemosensory receptors in the antenna of *P. clarkii* as well, indicating that these chemosensory receptors are not organ-specific, but expressed generally in a variety of sensory organs of *P. clarkii*. Furthermore, we found only one GR in the antenna of *P. clarkii*, which was consistent with the transcriptomic results of the antennule and legs in *P. clarkii* (Kozma et al., 2020a). This suggested that GRs remain low in numbers in *P. clarkii* and other decapod crustaceans. However, compared with the transcriptomic results of antennule and legs in *P. clarkii* (Kozma et al., 2020a), we found that the numbers of TRP channels were more in the antenna than in the two organs (antennule and leg), which we suggested that TRP channels might mediate more chemosensory processes in the antenna of *P. clarkii*. Overall, a lot of research on the chemosensory genes and receptors of crustaceans is still in the recognition stage, and there are still numerous receptor functions that have

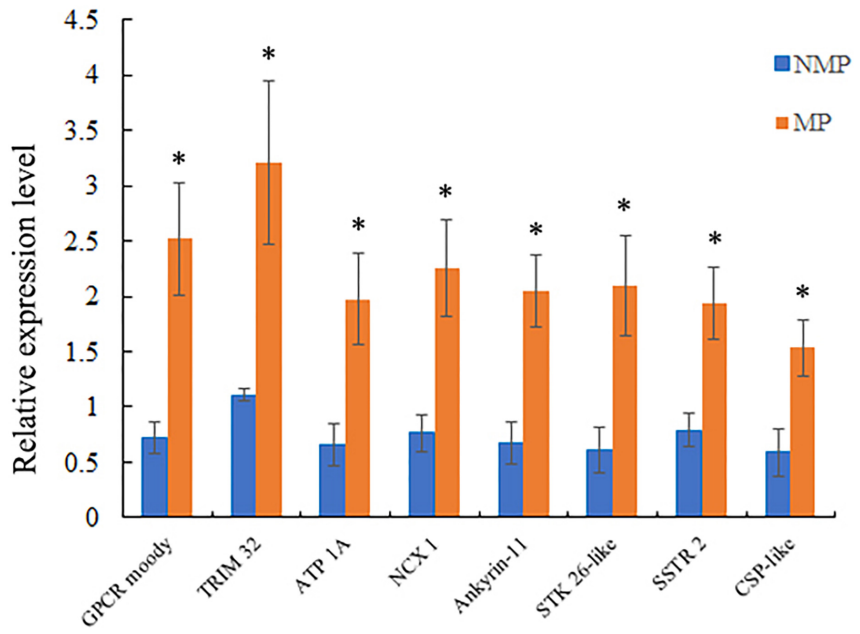

FIGURE 4
Relative levels of selected differentially expressed unigene (DEG) expression by RT-qPCR. Levels were measured using the $2^{-\Delta\Delta CT}$ method; β-actin was the internal normalization control. Data were shown as mean ± SD, $n = 3$; $^*P < 0.05$.

not been studied and reported yet and need more studies to investigate.

Notably, the DEGs related to the TRP channels were typically upregulated. TRP channels are homotetramers or heterotetramers with six transmembrane segments. There are eight subfamilies of TRP channels, including TRPC, TRPA, TRPP, TRPN, TRPV, TRPM, TRPM, and TRPT (Venkatachalam and Montell, 2007; Venkatachalam et al., 2014; Van den Eynde et al., 2021). In this study, six types of TRP channels (TRPA1, Pyrexia TRPA, TRPM2, TRPM3, TRPC4, and TRPV5) were identified. TRP channels participate in many sensory processes, such as vision, olfactory, audition, and temperature sensation, which affect the behaviors of creatures profoundly (Fowler and Montell, 2013). As the climate becomes warmer, more movements are generated by benthos and crayfish (Larson and Magoulick, 2011; Johnson et al., 2014; Wittwer et al., 2018). We speculated that *P. clarkii* could enhance self-abilities for various senses instinctively for environmental adaptation, which include the sex pheromone receptions as well.

Many DEGs have been annotated to uncharacterized proteins in our study, of which two DEGs were annotated to CSPs in other species *via* the Pfam database. The expressions of the two CSPs-like were significantly higher in the MP group than in the NMP group. In addition, the expressions of CSPs were also much higher relative to other unigenes. Previous studies supported that CSPs were sensory-related proteins with pheromone binding function in insects (Bohbot et al., 1998; Jacquin-Joly et al., 2001). Unlike insect-specific proteins (OBPs), CSPs were found in crustaceans such as *Daphnia carinata* and were expressed in ovaries, thoracic limbs, rectum, and second antenna in both sexual and asexual females (Li et al., 2016). This suggested that the CSPs might respond to environmental signals and control the reproductive switch from sexual to asexual reproduction in *D. carinata* (Li et al., 2016). Generally, owing to the lack of OBPs, crustaceans only have 0–2 CSPs (Derby et al., 2016). Our results were consistent. Even though CSPs were broadly reported in insects, crustaceans, and myriapods (Zhou et al., 2006; Chipman et al., 2014; Pelosi et al., 2014), their functions in crustaceans remained unknown. Based on our results, we speculated that CSPs might be one of the dominant signaling receptors of pheromone binding in male *P. clarkii*. More attention to the expressions and changes in CSPs is needed in further studies. Moreover, the functions of lots of DEGs in the antenna of *P. clarkii* have not been annotated in the six databases, and have been classified as uncharacterized proteins, indicating that the information on pheromone-related receptors in *P. clarkii* is very scarce, which needs a lot of research to investigate. We suggested that there are still many candidate sex pheromone receptors in *P. clarkii* that have not been discovered and reported.

The ribosome is the molecular machine for biological protein synthesis (Lv et al., 2017). A previous study has reported that the inactivation of ribosomes can disrupt chemoreception in *Caenorhabditis elegans* (McConnell et al., 2015). Researchers suggested that the ribosomes affect the process of translation, and further affect the biological synthesis of chemosensation

(McConnell et al., 2015). In this study, the ribosome was the most enriched pathway, of which most DEGs related to ribosome were upregulated. In *Apis Mellifera ligustica*, ribosomes were considered to play an important role in olfactory learning behavior, and the learning ability can be significantly reduced after being irritated by insecticide (Hou et al., 2021). Despite the interactive relationship between ribosomes and chemosensory processes in crustaceans remaining unknown, our results can still indicate that biological protein synthesis is more active after the male *P. clarkii* enters the mating period.

Ca^{2+} is the regulator of almost all eukaryotic signaling cascades, and Ca^{2+}-entry is the first step to converting chemical signals into electrical ones (Pyrski et al., 2007). The previous study has shown that the concentration ratio of intracellular Ca^{2+} and cyclic nucleotide levels can mediate the ion channels synergistically (Stengl, 2010). After the sex pheromone binds to the receptor, the latter can activate the type III adenylyl cyclase *via* a GTP-binding protein, resulting in raising the cAMP level (Castillo et al., 2007). High concentrations of cAMP can open the cyclic nucleotide-gated channels, leading to the Na^{+} and Ca^{2+} flow into the cell, further leading to depolarization (Firestein and Zufall, 1994). The sodium carrier family (NCX and sodium–calcium exchanger) is one of the membrane transporters, which has been considered the main mechanism for Ca^{2+} efflux in crustaceans (Flik et al., 1994). The NCX has a fundamental role in controlling the intracellular concentrations of Na^{+} and Ca^{2+} (Canitano et al., 2002), which is the efficient Ca^{2+} extrusion system in the plasma membrane. In this study, we further found that the DEG related to NCX and sodium–calcium exchanger was significantly upregulated in MP groups and was involved in olfactory transduction and calcium signaling pathways. In the calcium signaling pathway, the upregulation of NCX could further affect the calcium concentrations of mitochondria, which plays a central role in cell signalings (Jacobson and Duchen, 2004). The previous study had shown that the mitochondria acted as the calcium buffer and reacted with the signals *via* upregulating the tricarboxylic acid cycle (Jacobson and Duchen, 2004). The activations of mitochondria might be more active for signal transductions. Therefore, we suggested that the calcium signaling pathway might be involved in sex pheromone signal transductions and the changes in antenna potential to sex pheromone signals were more sensitive when *P. clarkii* entered the mating period.

Conclusion

In this study, we found the sensitivity of male *P. clarkii* to conditioned water was different in the mating and non-mating period, and they were more sensitive in the mating period. Furthermore, a total of 12 upregulated and nine downregulated DEGs were associated with chemosensory-related functions, of which two related to CSPs were remarkably upregulated after *P. clarkii* entered MP. Based on the levels of CSP-related DEG expression, we suggested that CSP might be the key receptor for chemical signals or sex pheromone reception in the antenna of *P. clarkii*. In addition, the calcium-related DEG and signaling pathway might be one of the dominant factors in the differences in chemosensory sensitivity of male *P. clarkii* between the mating and non-mating periods. The results presented herein will be fundamental for future functional studies on chemosensory genes in *P. clarkii*. The findings are expected to clarify the chemical communication mechanisms in *P. clarkii* and provide new targets for invasion management in future.

Data availability statement

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: National Center for Biotechnology Information (NCBI) BioProject, https://www.ncbi.nlm.nih.gov/bioproject/, PRJNA839237.

Author contributions

ZZ, JH, and ZW designed the study. ZZ, HW, DL, and WZ performed the experiments and data analysis. ZZ drafted the manuscript. ZZ, HW, LM, JH, and ZW discussed the study design and data. JH and ZW revised the manuscript. All authors contributed to the article and approved the submitted version.

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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/fevo.2022.976448/full#supplementary-material

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