Comparative Transcriptome Analysis Reveals Sex-Biased Gene Expression in Juvenile Chinese Mitten Crab *Eriocheir sinensis*

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**Abstract**

Sex-biased genes are considered to account for most of phenotypic differences between males and females. In order to explore the sex-biased gene expression in crab, we performed the whole-body transcriptome analysis in male and female juveniles of the Chinese mitten crab *Eriocheir sinensis* using next-generation sequencing technology. Of the 23,349 annotated unigenes, 148 were identified as sex-related genes. A total of 29 candidate genes involved in primary sex determination pathways were detected, indicating the sex determination cascade of the mitten crab might be more complex than previously supposed. Differential expression analysis showed 448 differentially expressed genes (DEGs) between the two transcriptomes. Most of DEGs were involved in processes such as metabolism and immunity, and not associated with obvious sexual function. The pathway predominantly enriched for DEGs was related to lysosome, which might reflect the differences in metabolism between males and females. Of the immune DEGs, 18 up-regulated genes in females were humoral immune factors, and eight up-regulated genes in males were pattern recognition receptors, suggesting sex differences of immune defense might exist in the mitten crab. In addition, two reproduction-related genes, *vitellogenin* and *insulin-like androgenic gland factor*, were identified to express in both sexes but with significantly higher level in males. Our research provides the first whole-body RNA sequencing of sex-specific transcriptomes for juvenile *E. sinensis* and will facilitate further studies on molecular mechanisms of crab sexual dimorphism.

**Introduction**

Sexual dimorphism, which differentiates males and females in morphological, physiological and behavioral characteristics, is a common phenomenon in the animal kingdom. Based on the nearly identical genomes, the phenotypic differences between the sexes are thought to largely result from sex differences in gene expression [1,2]. So far, sex-biased gene expression...
Sex-specific transcriptomics in juvenile *E. sinensis* has been well explored in mammals, birds, fish and insects, particularly in classic model organisms [3–6], however, little attention has been received to such studies in crustaceans.

A number of crustacean species exhibit significant sex differences in their biology and economic value [7]. For example, male crabs usually show higher growth rate and larger size than females when they reach maturation, while female crabs are of higher economic values than males [8,9]. Among the cultured crabs, the Chinese mitten crab *Eriocheir sinensis* (Henri Milne Edwards, 1854) is the most prevalent and commercially important species in China [10]. *E. sinensis* is a catadromous crustacean that spends most of its life in freshwater and migrates seawards to breed [11]. Due to the complex life cycle, mitten crabs might have unique regulatory mechanisms involved in crustacean reproduction or sexual development [12,13]. *E. sinensis* can serve as a model species of crustaceans to study sexual dimorphism and discover sex-biased gene expression.

Next-generation high-throughput sequencing technologies, such as Illumina/Solexa platform and Ion Proton system, have been used to generate large amounts of transcript sequences and gene expression data for non-model species without sequenced genomes [14,15]. Most studies of sex-biased transcriptome have focused on adult gonads in crustacean, and some sex-related genes have been identified, for example, *Tra-2* [16] in the giant tiger shrimp *Penaeus monodon*, *Dmrt, Fem-1* and *vasa* in the green mud crab *Scylla paramamosain* [17], and *Dmc1* and DEAD box family genes in *E. sinensis* [12,18,19]. Currently, several studies have reported significant sex-biased gene expression in juvenile stages of fish and insects [20–22]. However, the sex-biased genes by comparing expression before reproductive maturity in crustaceans remain largely unexplored.

Here, we analyzed the whole-body transcriptomes of juvenile *E. sinensis* using Ion Proton sequencing technology. This study was designed to enrich the genetic resources for *E. sinensis*, to identify new candidate genes involved in sex determination and differentiation, and to detail the different expression patterns between the sexes. Furthermore, differentially expressed genes (DEGs) involved in metabolism, immunity and reproduction were determined and analyzed. Our results are important resources for future research on molecular mechanisms underlying the sexual dimorphism in *E. sinensis* and other crustaceans.

**Materials and Methods**

**Ethics Statement**

The Chinese mitten crabs were captured from Liao River in Panjin, China and no specific permission was required for the sampling area and species because of scientific research purpose. The sampling location is not privately owned or protected. No endangered or protected species were involved in the field sampling. The experiments were performed in accordance with the guidelines on the care and use of animals for scientific purpose set by the Institutional Animal Care and Use Committee (IACUC) of the Chinese Academy of Sciences (No. 2011-2). This study was specifically approved by the Committee on the Ethics of Animal Experiments of the Institute of Oceanology at the Chinese Academy of Sciences. All efforts were made to minimize the suffering of the animals.

**Sample preparation and RNA isolation**

Healthy mitten crabs were obtained from a local crab farm in Panjin, China. Five males (1.6 ± 0.4 g) and females (1.9 ± 0.2 g) at the third juvenile instar were pooled, as this stage is the earlier stage to distinguish their sex from morphology [23]. Total RNA was extract from the whole bodies using Trizol reagent (Invitrogen, USA) according to the manufacturer’s protocol. The quality and integrality were determined by Agilent2100 (Agilent, USA). The mRNA was
purified from total RNA using Dynabeads mRNA Direct Micro kit (Ambion, USA). The final concentration was determined using a NanoDrop2000 spectrophotometer (Thermo Scientific, USA).

cDNA library construction and sequencing

Two libraries for male and female juveniles of *E. sinensis* were constructed in this study. For library preparation, equal amounts of purified mRNA samples from two biological replicates were pooled together for the cDNA synthesis. The cDNA libraries were prepared using an Ion Total RNA-seq kit v2 (Life Technologies, USA), with 100 ng fragmented mRNA. Adapter ligation, size selection, nick repair and amplification (12 cycles) were performed as described in the Ion Proton protocol associated with the kit. The resulting cDNA libraries were purified by AMPure beads (Beckman Coulter, USA), and their concentrations and sizes were determined by Agilent2100 (Agilent, USA). Emulsion PCR and enrichment steps were performed using an Ion PI Template OT2 200 kit v3 (Life Technologies) according to the manufacturer’s instructions. PI Chips were loaded according to the spinning protocol and sequencing was performed on Ion Proton Sequencer using the Proton 200 sequencing kit (Life Technologies). Base calls were collected with Torrent Suite using v4.0.2 software.

Sequence assembly

The raw data for each pool of samples were separately trimmed to remove adaptors and low quality regions (< Q20). Reads with a length of less than 50 bp were also discarded. The remaining reads without ambiguous bases (N) were *de novo* assembled in a unique file by Trinity (http://trinityrnaseq.sourceforge.net/) with k-mer length of 25 referring to the strategy of Grabherr et al. [24]. Trinity combining three independent software modules: Inchworm, Chrysalis and Butterfly, applied sequentially to process large volumes of RNA-seq reads into contigs, de Bruijn graphs and full-length transcripts.

Gene annotation

Gene functional annotation was performed by sequence comparison with public databases. All assembled transcripts were searched against the NCBI non-redundant (nr) protein database (http://www.ncbi.nlm.nih.gov/) using BlastX algorithm with an *E*-value cutoff of 1E-05. The unigenes were obtained after clustering the top hit results. Gene Ontology (GO) annotations were determined using Blast2GO to obtain a functional classification of the unigenes [25]. EggNOG (evolutionary genealogy of genes: Non-supervised Orthologous Groups) was performed to predict and classify potential functions of the unigenes based on known orthologous gene products [26]. EC (Enzyme Commission) terms and biochemical pathway information were generated by Kyoto Encyclopedia of Genes and Genomes (KEGG) database [27]. In addition, the unigenes associated with sex determination and differentiation were identified manually according to the annotation in consulting published literature and public datasets.

Identification of SNPs and SSRs

Single nucleotide polymorphisms (SNPs) were identified using SAMtools mpileup and VarScan (version 2.3.3) [28,29]. VarScan was run with the following parameters: “mpileup—p-value 0.01—min-avg-qual 20—min-reads2 2—min-var-freq 0.2-variants”. From these results, a set of high confidence SNPs (with coverage of 8 or more reads) were identified. All unigenes were searched for the presence of SSRs using MISA (http://pgrc.ipk-gatersleben.de/misa/) [30] with the following minimum length criteria (unit size/minimum repeat time): 2/6, 3/5, 4/5, 5/5
Compound microsatellites were defined as repeats interrupted by a non-repetitive sequence of a maximum of 100 nucleotides.

**Differential expression analysis**

The reads for a specific transcript were counted by mapping reads to assembled unigene sequences. The unigene expression was calculated using the reads per kb per million reads (RPKM) method \[31\]. Differentially expressed genes (DEGs) were identified by the DESeq program \[32\]. The fold change values \(>2\) and false discovery rates (FDR) adjusted significance values \(<0.05\) (-log10 (0.05) = 1.3) were considered as the threshold to judge the significance of unigene expression.

GO, eggNOG, KEGG Orthology (KO) and KEGG pathway enrichment analyses were used to categorize DEGs and evaluate DEGs in the potential biological pathways. Processes, functions or components in the GO and KEGG pathway enrichment analyses with \(p\)-values less than 0.05 (-log10 (0.05) = 1.3) were considered to be significantly different in the DEGs. Based on public databases and the published literatures, the crucial DEGs related to metabolism, immunity and reproduction were manually checked.

The DEG encoding insulin-like androgenic gland factor (IAG) was selected for further sequence and phylogenetic analysis. Multiple amino acid sequence alignment was performed using the Clustal X with the default settings \[33\]. Neighbor-joining tree with bootstrap values were constructed for phylogenetic analysis using MEGA 4.0 \[34\]. All the reference sequences for phylogenetic analysis were derived from GenBank.

**Quantitative Real-time PCR (qRT-PCR) validation**

To validate RNA-seq data and expression profiles obtained from DESeq analysis, mitten crabs at the same developmental stage, the independent samples of RNA-seq, were used for real-time PCR analyses. Three biological replicates were prepared for male and female juveniles. Approximately 2.5 μg of total RNA of each sample obtained as previously described were treated with DNase I (Ambion, USA) at 37°C for 1 h, reverse-transcribed using M-MLV reverse transcriptase (Promega, China) and amplified by qRT-PCR.

The SYBR Green I RT-PCR assay was carried out in an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, USA). The gene-specific primers designed for the 12 genes are listed in S1 Table. These 12 genes all exhibited large significant differences in expression between male and female libraries. The \(\beta\)-actin from *E. sinensis* was chosen as reference gene for internal standardization. The PCR was carried out in a total volume of 10 μl, containing 5 μl of 2×SYBR Premix Ex Taq (TaKaRa, China), 0.2 μl 50×ROX Reference Dye, 2 μl of the diluted cDNA mix, 0.2 μl of each primer (10 μM), and 2.4 μl of sterile distilled H2O. The PCR program was 95°C for 5 min, followed by 40 cycles of 95°C for 5 s and 60°C for 31 s. Each sample was run in triplicate along with the internal control gene. To confirm that only one PCR product was amplified and detected, dissociation curve analysis of amplification products was performed at the end of each PCR reaction. After the PCR program, data were analyzed with ABI7300 SDS software (Applied Biosystems). Fold change for the gene expression relative to controls was determined by the \(2^{\Delta\Delta C_T}\) method \[35\]. All data were given in terms of relative mRNA expression as mean ±S.E. The results were subjected to one-way analysis of variance (one way ANOVA) using SPSS 13.0, and the \(P\) values less than 0.05 and 0.01 were considered statistically significant.
Results

Transcriptome sequencing and assembly

Two cDNA libraries were generated with pooled mRNAs from the whole bodies of female and male juveniles of *E. sinensis*. After quality trimming and the removal of adapters, sequencing runs performed on Ion Proton platform produced a total of 42,979,050 reads for the female and 47,560,370 reads for the male. All data were deposited in NCBI Short Read Archive database with accession numbers SRX554564 and SR554562. Clustering and assembly of these reads resulted in 282,954 contigs with an average length of 274 bp. Further assembly analysis showed all contigs contributed to 151,128 transcripts with an average length of 614 bp (S2 Table).

Functional annotation

BlastX searches of all assembled transcripts against the NCBI nr database revealed 23,349 unigenes with significant matches to existing protein sequences above the preset cutoff value. The average length of these annotated unigenes was 988 bp with N50 length of 1,375 bp (S2 Table).

Based on GO analysis, 17,388 unigenes (74.5%) were assigned to one or more GO term. Finally, 77,697 GO assignments were obtained and fall into the three major functional categories (S1 Fig). Thereinto, 38,578 unigenes (49.7%) were involved in biological process, 26,583 (34.2%) were cellular components and 12,518 (16.1%) have molecular functions. Moreover, 0.4% (323 unigenes) were assigned to reproduction of biological process.

To classify orthologous gene products, 22,101 unigenes were assigned to 25 function categories (S2 Fig). Of these, the cluster of ‘function unknown’ (4,825, 18.3%) represented the largest group, followed by ‘signal transduction mechanisms’ (3,757, 14.3%), ‘general function prediction only’ (3194, 12.1%) and ‘transcription’ (2,112, 8.0%). KEGG analysis revealed that 9,605 unigenes were assigned to six biochemical pathways, including metabolism, genetic information processing, environmental information processing, cellular processes, organismal systems and human diseases. A total of 3,743 unigenes had significant matches in the KEGG database with corresponding EC numbers (S2 Table).

SNP and SSR discovery

Using SAMtools/VarScan software, 48,753 SNPs and 15,271 indels were identified from *E. sinensis* unigenes. The overall frequency of all types of SNPs, including indels, was one per 360 bp (Fig 1A). Transition occurred 2.7 times more frequently than transversion. A/G was the most abundant transition (28.3%), and A/T was the most abundant transversion (5.3%). Indels were less frequent than transitions, with a frequency of one per 1,511 bp and a total proportion of around 23.9%. Of the identified SNPs and indels, 55,230 were found in both female and male *E. sinensis* unigenes. 3,411 SNPs and indels were found exclusively in female unigenes, while 5,383 were just present in male unigenes (Fig 1B).

In addition, using MISA, 3,928 perfect and 262 compound SSRs were detected from *E. sinensis* unigenes (S3 Table). Trinucleotide repeats were the most frequent type, counting a total number of 2,286 (54.6%) with AGG/CCT as a major motif accounted for 31.4% of all trinucleotide repeats. Dinucleotides repeats were the second most frequent type accounting for 37.3% of all SSRs with the abundance of AG/GT motif. And the other two distinguished types, compound and tetranucleotide repeats, accounted for less than 10% of all SSRs.
Genes related to sex determination and differentiation

Of the total unigenes, 148 were identified as sex-related genes based on sequence annotation and the published literature (S4 Table). These genes could be categorized into 36 groups and
putatively involved in sex determination and differentiation, oogenesis, spermatogenesis and
gonad development. Among these unigenes, 40 were classified as testis or sperm-speciﬁc
genes, and 24 were ovary or oocyte-speciﬁc genes. Eleven unigenes annotated as SOX (SRY related
HMG box) gene family were found, including SOX2, SOX5, SOX8, SOX14, SOXB1, SRY, SRY-
box containing protein 8 and B2b2.

A total of 29 genes with similarity to those involved in primary sex determination pathway
were identiﬁed (Table 1). Eight genes, including two fruitless (Fru), two transformer-2 (Tra-2)
and four feminization-1 (Fem-1) genes, were associated with the sex determination in Drosoph-
ila melanogaster and Caenorhabditis elegans. Fourteen genes involved in mammalian female
Sex-specific development were detected: five genes belonging to the WNT signaling pathway (RSPO1, WNT4 and β-catenin), a transcription factor (FOXL2), three estradiol receptors (ER), and five activin-binding proteins (FST). Seven genes involved in mammalian sex development were examined: a doublesex and mab-3 related transcription factor 2 (Dmrt2), three prostaglandin D synthases (PGDS), and three genes belonging SOX transcription factors (SRY and SOX8).

The primary sex determination pathway related genes were expressed in both female and male E. sinensis transcriptomes (S3 Fig). EsPGDS was the most highly expressed transcript (RPKM value > 800), followed by EsER3 (RPKM value > 50) and EsHPGDS (RPKM value > 40). EsDmrt2, EsPGDS and EsSRY presented higher expression pattern in males (fold change values > 1.5), while EsFST5, EsRSPO-1p and EsFST5p showed preferential expression in females (fold change values > 1.5). The other 23 genes were similar expressed in females and males.

From the 148 unigenes annotated for sex-related genes, 18 SNPs were identified in female individuals and 23 in male individuals (S5 Table). Most unigenes had only one SNP, except for vitelline membrane outer layer protein 1, SOX14, oocyte zinc finger protein, male reproductive-related microfibril-associated protein spermatogenesis-associated protein 5 and 20. In addition, five compound and 18 perfect SSRs were detected from the sex-related unigenes (S6 Table). The size of SSRs ranged from 12 to 242, with trinucleotide repeats as the most frequent type. Three sex-related genes, including SRY-box containing gene 8, SRY interacting protein 1 and zinc finger protein 76, were found to contain both SNPs and SSRs.

**Differential expression between the sexes**

The DESeq method identified 448 differentially expressed unigenes between the two transcriptomes, including 188 up-regulated in females and 260 up-regulated in males (S7 Table). The distribution of the significant changes detected was illustrated in a volcano plot, where the statistical significance of each unigene was plotted against fold change (S4 Fig). Sequences with the highest average differences between the sexes also had the smallest false discovery rate (FDR) values.

All of the DEGs were performed on GO function and pathway enrichment analysis. Based on GO analysis, a total of 340 DEGs with GO terms were categorized into three major functional groups (Fig 2). Within the biological processes, the highest percentage of DEGs involved in GO terms was 'transport' (10.7%), followed by 'carbohydrate metabolic process' (8.2%), 'biological process' (6.8%), 'oxidoreductase activity' (6.8%), 'protein maturation' (6.6%) and 'carbohydrate metabolic process' (6.5%) (Fig 2A). Of the cellular component categories, the majority of GO terms were related to 'cell' (23.1%), 'intracellular' (22.1%) and 'cytoplasm' (15.4%) (Fig 2B). As for molecular functions, the three major GO terms were 'ion binding' (64.6%), 'RNA binding' (8.9%) and 'enzymatic activity' (6.8%) (Fig 2B). After the overall comparison was completed, the top ten significantly changed categories were obtained, including 'hydrolase activity, acting on glycosyl bonds', 'carbohydrate metabolic process', 'extracellular region', 'oxidoreductase activity', 'protein maturation', 'transport', 'lyase activity', 'lipid metabolic process', 'immune system process' and 'ion binding' (p < 0.05, Fig 2C).

KEGG analysis revealed that 139 DEGs with KO terms were involved in 29 predicated biological pathways (S5 Fig). The most abundant pathways included 'carbohydrate metabolism' (36 DGEs), 'glycan biosynthesis and metabolism' (24 DGEs), 'transport and catabolism' (24 DGEs) and 'lipid metabolism' (16 DGEs). The significantly distinct categories were 'carbohydrate metabolism', 'glycan biosynthesis and metabolism', 'metabolism of cofactors and vitamins', 'transport and catabolism', 'digestive system', 'lipid metabolism', 'xenobiotics'...
biodegradation and metabolism' and 'amino acid metabolism' (p < 0.05, S5 Fig). Except for metabolic pathway, the pathway predominantly enriched for DEGs were related to lysosome (ko04142) of 'transport and catabolism' (Fig 3). There were 19 DEGs with ten KO terms in lysosomal pathway, including 18 up-regulated in males and one up-regulated in females. In addition, one important pathway identified as enriched for the up-regulated genes in males were associated with sphingolipid metabolism (ko00600) of 'lipid metabolism' (S6 Fig).

Fig 2. GO distributions of differentially expressed genes (DEGs) from female and male Eriocheir sinensis transcriptomes. (A) Different functional distribution of the DEGs involved with biological processes. (B) Different functional distribution of the DEGs involved with cellular components and molecular functions. (C) Differentially expressed functional processes. The horizontal line indicates the significance threshold (p < 0.05).

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Candidate DEGs involved in metabolism, immunity and reproduction

By sequence annotation and functional classification, we identified 61 differentially expressed unigenes related to metabolism, immunity and reproduction. The metabolism-related DGEs could be categorized into four classes, including five female-biased genes in amino acid metabolism, ten male-biased genes in lipid metabolism, six male-biased genes in glycan metabolism, and three male-biased genes in cytochrome P450 (CYP450) superfamily (Table 2). A total of 30 immunity-related DGEs were found, including 18 up-regulated humoral immune factors in females, eight up-regulated pattern recognition receptors (PRRs) (CLEC and LGBP) and four other up-regulated immune factors in males (Table 3). The female-biased immune DGEs were largely involved in antimicrobial peptide synthesis (Lyz, Crustin and Carcinin), prophenoloxidase (proPO) system (SP, PPAF and Kazal) and antioxidant system (SOD and Trx). Of the reproduction-related DGEs, two genes involved in ovarian development showed female-biased expression, while five genes, including two Vg homologs, VMO1, IAG and NPC2, showed male-biased expression (Table 4).
A partial sequence of 741 bp (comp61312_c0_seq3), without an ATG codon but with a TAA stop codon at position 526 bp, was identified to encode an IAG ortholog (EsIAG, S7 Fig). The deduced amino acid sequence was 132 aa in length and contained B chain and A chain, with three disulfide bridges (between CB9 and CA12, CB20 and CA25, CA11 and CA16, respectively). Multiple sequence alignment of EsIAG and six decapod IAGs showed the conserved cysteine residues shared by all sequences (S7A Fig). EsIAG displayed 36.3% amino acid identity with *Callinectes sapidus* IAG1, 32.5% with *C. sapidus* IAG2 and 33.1% with *Scylla paramamosain* IAG. The phylogenetic tree showed that the IAGs formed two major clades: one with three isopods and the other containing five subclades from the decapods (S7B Fig). The crab IAGs were separated in two groups. EsIAG was clustered with IAGs from the Atlantic blue crab *C. sapidus* and the mud crab *S. paramamosain* to form one group. Another group included IAG from the blue swimmer crab *Portunus pelagicus*, which had a closer relationship with two IAGs from crayfish.

**Table 2. List of differentially expressed genes related to metabolism in female and male *Eriocheir sinensis* transcriptomes.**

| Gene    | Unigene        | Gene annotation [Matched species]                                      | Fold change (Female: Male) |
|---------|----------------|------------------------------------------------------------------------|----------------------------|
| **Up-regulated genes in females** |                          |                                                                        |                            |
| Amino acid metabolism-related genes |                          |                                                                        |                            |
| AspRS   | comp59739_c0_seq1 | aspartate-tRNA ligase, mitochondrial-like [Takifugu rubripes]           | 3.17                       |
| Ark     | comp72982_c2_seq1 | arginine kinase [Eriocheir sinensis]                                   | 2.32                       |
| Arg1    | comp74968_c0_seq2 | arginase-1 isomorph 2 [Trichechus manatus latirostris]                 | 2.08                       |
| NIT2    | comp56883_c0_seq1 | Nit protein 2-like [Saccoglossus kowalevskii]                          | 7.86                       |
|         | comp48414_c0_seq2 | Omega-amidase NIT2-B [Crassostrea gigas]                               | 6.85                       |
| **Up-regulated genes in males** |                          |                                                                        |                            |
| Lipid metabolism-related genes |                          |                                                                        |                            |
| ACAC    | comp77110_c0_seq6 | Acetyl-CoA carboxylase [Harpegnathos saltator]                         | 0.47                       |
| ASAH2   | comp73699_c0_seq4 | ceramidase [Danaus plexippus]                                          | 0.42                       |
| ARLS    | comp74420_c0_seq3 | arylsulfatase A-like [Strongylocentrotus purpuratus]                   | 0.29                       |
| FASN    | comp75785_c0_seq2 | fatty acid synthase [Tribolium castaneum]                              | 0.43                       |
|         | comp76291_c0_seq2 | fatty acid synthase-like isomorph 1 [Nasonia vitripennis]              | 0.46                       |
| GBA     | comp74162_c1_seq1 | glucosylceramide-like [Ciona intestinalis]                              | 0.46                       |
|         | comp63076_c0_seq1 | glucosylceramide-like [Monodelphis domestica]                          | 0.47                       |
| Se-Gpx  | comp66076_c0_seq2 | selenium-dependent glutathione peroxidase [Procambarus clarkii]       | 0.21                       |
| SMPD1   | comp72185_c1_seq1 | acid sphingomyelinase 1 [Glossina morsitans morsitans]                 | 0.36                       |
| PLRP2   | comp75117_c0_seq1 | pancreatic lipase-related protein 2-like [Acrythosiphon pisum]         | 0.34                       |
| Glycan metabolism-related genes |                          |                                                                        |                            |
| A4GALT  | comp9581_c0_seq1 | lactosylceramide 4-alpha-galactosyltransferase [Rattus norvegicus]     | 0.33                       |
| GALNS   | comp76195_c0_seq5 | n-acetylgalactosamine-6-sulfatase-like [Monodelphis domestica]         | 0.40                       |
|         | comp76195_c0_seq6 | galactosamine (N-acetyl)-6-sulfate sulfatase [Xenopus (Silurana) tropicalis] | 0.49                     |
| DHDH    | comp63990_c0_seq2 | Trans-1,2-dihydrobenzene-1,2-diol dehydrogenase [Acromyrmex echinatior] | 0.44                       |
| HEXA_B  | comp75397_c0_seq1 | beta-hexosaminidase subunit alpha-like [Ornithorhynchus anatinus]      | 0.43                       |
| LAMAN   | comp60626_c0_seq1 | lysosomal alpha-mannosidase precursor [Danio rerio]                     | 0.40                       |
| Cytochrome P450 superfamily |                          |                                                                        |                            |
| CYP3A4  | comp59688_c0_seq1 | cytochrome P450, family 3, subfamily A, polypeptide 4 [Callorhinchus milii] | 0.08                       |
| CYP2B19 | comp69705_c0_seq2 | cytochrome P450 2B19-like [Ciona intestinalis]                          | 0.48                       |
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A partial sequence of 741 bp (comp61312_c0_seq3), without an ATG codon but with a TAA stop codon at position 526 bp, was identified to encode an IAG ortholog (EsIAG, S7 Fig). The deduced amino acid sequence was 132 aa in length and contained B chain and A chain, with three disulfide bridges (between CB9 and C A12, CB20 and C A25, C A11 and C A16, respectively). Multiple sequence alignment of EsIAG and six decapod IAGs showed the conserved cysteine residues shared by all sequences (S7A Fig). EsIAG displayed 36.3% amino acid identity with *Callinectes sapidus* IAG1, 32.5% with *C. sapidus* IAG2 and 33.1% with *Scylla paramamosain* IAG. The phylogenetic tree showed that the IAGs formed two major clades: one with three isopods and the other containing five subclades from the decapods (S7B Fig). The crab IAGs were separated in two groups. EsIAG was clustered with IAGs from the Atlantic blue crab *C. sapidus* and the mud crab *S. paramamosain* to form one group. Another group included IAG from the blue swimmer crab *Portunus pelagicus*, which had a closer relationship with two IAGs from crayfish.
qRT-PCR validation of RNA-seq data

To verify the result of RNA-seq analysis, 12 DGEs based on RNA-seq were selected for qRT-PCR to further investigate the expression profiles. The expression patterns from qRT-PCR showed general agreement with those from the RNA-seq (Fig 4). Five genes including AspRS, NIT2, Lyz, Crustin and PPAF were up-regulated in females, while Vg, ARSA, Se-Gpx, CYP3A4, CLEC, LGBP and IAG were up-regulated in males. Among these genes, NIT2 showed the largest up-regulation in females and CYP3A4 manifested the largest up-regulation in males, which was consistent with RNA-seq results. The consistent expression between qRT-PCR and RNA-seq analyses confirmed the accuracy of Proton results.

Table 3. List of differentially expressed genes related to immunity in female and male Eriocheir sinensis transcriptomes.

| Gene                  | Unigene       | Gene annotation [Matched species]                                      | Fold change (Female: Male) |
|-----------------------|---------------|------------------------------------------------------------------------|---------------------------|
| **Up-regulated genes in females** |              |                                                                        |                           |
| Lyz                   | comp67931_c0_seq2 | lysozyme [Scylla paramamosain]                                         | 7.21                      |
| A2ML2                 | comp75726_c0_seq17 | alpha2 macroglobulin isoform 2 [Fenneropenaeus chinensis]              | 2.15                      |
| Carcinin              | comp61957_c0_seq1  | carcinin-like protein [Carcinus maenas]                                | 2.72                      |
| Crustin               | comp54631_c0_seq1  | crustin [Scylla tranquebarica]                                         | 3.94                      |
|                       | comp66850_c0_seq1  | crustin 2 [Portunus trituberculatus]                                   | 2.06                      |
| CPAMD8                | comp74090_c0_seq4  | C3 and PZP-like alpha-2-macroglobulin domain-containing protein 8-like [Metaseiulus occidentalis] | 2.07                      |
| HPT factor 9          | comp58745_c0_seq1  | HPT factor 9 [Pacifastacus leniusculus]                                | 2.25                      |
| Kazal                 | comp67267_c0_seq3  | Kazal-type protease inhibitor [Eriocheir sinensis]                     | 2.25                      |
| PPAF                  | comp72920_c0_seq1  | phenoloxidase activating factor serine proteinase [Scylla serrata]     | 7.33                      |
|                       | comp57767_c0_seq1  | phenoloxidase activating factor [Portunus trituberculatus]             | 2.32                      |
| SP                    | comp67291_c1_seq3  | clip domain serine proteinase 3 [Portunus trituberculatus]             | 2.63                      |
|                       | comp71922_c0_seq2  | clip domain serine proteinase [Portunus trituberculatus]               | 2.30                      |
|                       | comp63300_c1_seq1  | clip domain serine proteinase [Eriocheir sinensis]                     | 2.11                      |
|                       | comp67168_c0_seq1  | serine proteinase [Portunus trituberculatus]                           | 2.06                      |
|                       | comp67235_c0_seq1  | trypsin-like serine protease [Eriocheir sinensis]                      | 2.16                      |
| SOD                   | comp66967_c0_seq1  | superoxidase dismutase [Eisenia fetida]                                | 2.28                      |
| PDGF/VEGF-related factor 1 | comp75589_c0_seq4 | PDGF/VEGF-related factor 1 [Eriocheir sinensis]                       | 2.26                      |
| Trx1                  | comp61426_c0_seq2  | Trx1 [Eriocheir sinensis]                                              | 2.08                      |
| **Up-regulated genes in males** |              |                                                                        |                           |
| CLEC                  | comp62386_c0_seq1  | C-type lectin [Eriocheir sinensis]                                     | 0.29                      |
|                       | comp74386_c0_seq1  | C-type lectin [Eriocheir sinensis]                                     | 0.37                      |
|                       | comp69162_c2_seq4  | C-type lectin 1 [Marsupenaeus japonicus]                               | 0.34                      |
|                       | comp18326_c0_seq1  | C-type lectin-1 [Litopenaeus vannamei]                                 | 0.45                      |
|                       | comp22144_c0_seq1  | C-type lectin-2 [Litopenaeus vannamei]                                 | 0.37                      |
| CLR                   | comp45203_c0_seq1  | C-type lectin receptor protein [Eriocheir sinensis]                    | 0.46                      |
| HC6                   | comp67750_c0_seq2  | hemocyanin subunit 6 [Eriocheir sinensis]                              | 0.50                      |
| LGBP                  | comp56987_c0_seq1  | lipopolysaccharide and beta-1,3-glucan binding protein [Procamarus clarkii] | 0.19                      |
|                       | comp72288_c0_seq1  | lipopolysaccharide and beta-1,3-glucan binding protein [Eriocheir sinensis] | 0.29                      |
| MIF                   | comp54328_c1_seq1  | macrophage migration inhibitory factor [Anisakis simplex]              | 0.40                      |
| Peritrophin           | comp54270_c0_seq1  | peritrophin [Macrobrachium nipponense]                                 | 0.39                      |
| PLGRKT                | comp59036_c0_seq2  | plasminogen receptor (KT) [Dasypus novemcinctus]                      | 0.40                      |

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Discussion

Whole-body reference transcriptome of male and female *Eriocheir sinensis*

Compared with the available data from database and the previously reported transcriptomes from male gonads, the present study, providing more than 10 Gb clean data, is the first whole-body RNA sequencing of sex-specific transcriptomes for juvenile Chinese mitten crab *E. sinensis*. This resource expands the limited amount of sex-related sequence data of *E. sinensis*, which will facilitate further molecular investigation on sexual dimorphism of crab.

Similar to recent studies in *E. sinensis* [36–38], only about 15% of all assembled transcripts are successfully matched in the nr database, probably due to the limited number of crustacean sequences in public database. However, this percentage is higher than that in gonad transcriptomes of *S. paramamosain* [17], supposedly because the whole bodies of juvenile are used and more extensive sequencing depth are applied in our transcriptomes. From our sequencing effort, a large number of SNPs and SSRs are detected for future genetics studies. This database, especially the sex-related markers, will play important roles in the exploration and utilization of sex-related genes, and may provide powerful tools for early gender identification and breeding in this commercially important species.

### Table 4. List of differentially expressed genes related to reproduction in female and male *Eriocheir sinensis* transcriptomes.

| Gene          | Unigene | Gene annotation [Matched species]                          | Fold change (Female: Male) |
|---------------|---------|------------------------------------------------------------|-----------------------------|
| **Up-regulated genes in females** |                      |                                                           |                            |
| Vg receptor   | comp76488_c0_seq9 | vitellogenin receptor [Macrobrachium rosenbergii]          | 2.18                        |
| Pxt           | comp58063_c0_seq3 | chorion peroxidase-like [Acrithosiphon pisum]              | 2.16                        |
| **Up-regulated genes in males** |                      |                                                           |                            |
| Vg            | comp10184_c0_seq1 | vitellogenin [Crassostrea gigas]                           | 0.19                        |
|               | comp77252_c0_seq2 | vitellogenin [Charybdis feriata]                           | 0.47                        |
| VMO1          | comp67371_c0_seq1 | vitelline membrane outer layer protein 1 homolog isoform 1 [Oryzias latipes] | 0.38                        |
| IAG           | comp61312_c0_seq3 | insulin-like androgenic gland factor [Callinectes sapidus] | 0.25                        |
| NPC2          | comp55602_c0_seq1 | epididymal secretory protein E1-like [Hydra magnipapillata] | 0.36                        |

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Fig 4. qRT-PCR validation of selected differentially expressed genes identified by RNA-seq. Vertical bars represent the mean ± S.E. (n = 3). Significant differences are indicated with an asterisk at P < 0.05, and two asterisks at P < 0.01.

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Candidate genes involved in sex determination and differentiation

Though several sex-related genes were obtained previously [39–41], little information about sex determination cascades is available in the mitten crab. GO analysis and orthology prediction supply gene function classification labels and an overall framework for the sex-specific transcriptomes.

Two Tra-2 and two Fru genes are identified in our transcriptome database. The Tra-2 and Fru genes, by promoting female sexual development or directing male sexual behavior, have been proved to participate in the Drosophila sex determination cascade [42,43]. Together with the previously cloned sex-lethal (Sxl) [41] and our identified double sex (dsx) [44], most of ortholog genes in ‘Sxl-Tra/Tra-2-Dsx/Fru’ pathway are detected in the mitten crab. As suggested in the penaeid shrimp [16,45], the present data provides a hint that the mitten crab might adopt a similar sex determination pathway with that in Drosophila. However, the absence of Tra and lack of a sex specificity of Sxl in the mitten crab, also reported in the silk-worm Bombyx mori [46–48], imply that genetic regulation for sex determination potentially does not initiate from Sxl.

We discover four homologs of Fem-1 that have not been identified previously in E. sinensis. In C. elegans, Fem-1, encoding an ankyrin repeat protein Fem-1, is a component of the signal transduction pathway controlling sex determination [49]. In our high density linkage map of E. sinensis, another ankyrin repeat–containing gene is found to be located on the putative sex chromosome, suggesting its possible role in sex determination [44]. However, in the present study, the four homologs of Fem-1 are expressed similarly in both males and females. Whether Fem-1 participates in the process of sex differentiation in the crab has yet to be established.

Besides the above genes, many genes involved in mammalian sex determination cascade are reported in E. sinensis, further indicating the complex sex determination system of the mitten crab. In mammals, the presence of the male-determining SRY gene directs the undifferentiated gonad to develop into testes by promoting the expression of SOX9 [50]. Early ovarian development has long been considered to be a default pathway switched on passively by the absence of SRY gene. However, recent reports have revealed that FOXL2-leading pathway and RSPO-1-activating signaling pathway act independently and complementary to each other to promote ovarian development [51–53]. In invertebrates, orthologs of FOXL2 have been characterized, but without a good understanding of their role in reproduction [54–57]. Though the orthologs genes in ‘RSPO-1/WNT4/β-catenin’ signaling pathway are detected, further investigations are needed to determine whether this pathway exists in the mitten crab.

Patterns of gene expression between the sexes

Differential expression analysis reveals 188 and 260 significantly expressed unigenes in female and male transcriptomes, respectively. These results show a slight imbalance in favor of the male sequences. This tendency has also been reported in adult transcriptomes of Caligus rogercresseyi [58] and gonad transcriptomes of the green mud crab [17] and other species such as Acipenser fulvescens [59] and Haliotis rufescens [60]. Coinciding with that in male and female rainbow trout embryos [20], most DEGs in this study are not related to sexual function. Further GO and KEGG analyses reveal these DEGs are largely involved in biological processes, such as lipid metabolism, glycan metabolism, transport and catabolism, and immune system process. This suggests that there are inherent and broad differences in the transcriptomes of male and female mitten crab, and that these differences are present before sexual maturation.

Lysosomes are membrane-enclosed organelles that contain an array of enzymes capable of breaking down all types of biological polymers-proteins, nucleic acids, carbohydrates and lipids [61]. Of DEGs involved in lysosomal pathway, one gene encoding protease legumain is up-
regulated in females, while other genes encoding glycosidases, sulfatases, sphingomyelinase and membrane proteins are up-regulated in males. Together with the identified DEGs related to metabolism, this differential expression pattern might due to the higher level of amino acid metabolism in females as well as higher levels of glycan and lipid metabolisms in males. Three metabolism-related CYP450 genes, CYP2B19, CYP3A4 and CYP9Z7, were identified as male-biased DGEs. Enzymes in CYP2 and CYP3 families, especially CYP3A4, have important roles in steroid biosynthesis and metabolism in human [62]. The higher transcripts of CYP450 enzymes in males might indicate male mitten crabs require a large amount of steroid during early juvenile stages.

Sex differences in the immune defense, where females show greater immunity or resistance to infection, have been demonstrated for several arthropods [63–67]. In many cases, females have higher levels of hemocytes or PO activity than males in the absence of infection. Most of these studies have focused on insects such as butterflies, crickets, dragonflies and scorpionflies, with relatively few studies on crustaceans. Here, we first report some genes that potentially contribute to the sex differences in the immune system of the mitten crab.

Humoral immune responses that mainly occur in hemolymph include proPO system, clotting cascade and a wide array of antimicrobial peptides [68]. The identified female-biased humoral immune factors indicate females have greater lysozyme and PO activities in hemocytes, which is in agreement with those studies in insects [64–66]. PRRs, as a set of germline-encoded receptors, can interact with pathogen associated molecular pattern (PAMP) and activate innate immune response [69]. The higher transcripts of PRRs in males might suggest male mitten crabs could trigger quick and effective defense responses in the presence of pathogens infection. Sex differences in immunity appear to be related to differential reproductive strategies and the resulting resource trade-offs in life history. The identified immune DEGs provide a framework for future research to unravel the mechanism of six-biased immune regulation in crab.

Among the reproduction-related DGEs, two special genes vitellogenin (Vg) and insulin-like androgenic gland factor (IAG) were identified. Vg, usually considered as a female specific protein, could serve as energy resource for embryonic development [70]. Apart from its nutritional function, Vg has been shown to play important roles in innate immunity by acting as a multivalent pattern recognition receptor, a bactericidal molecule or an acute phase protein [71]. The Vg gene is normally silent in males, but can be activated by estrogen exposure [72,73]. Interestingly, we detected the expression of Vg in the normal physiological conditions of male E. sinensis, which is consistent with the finding in European honey bee Apis mellifera [74,75] but contrary to most studies in crustaceans [76–78]. This male-biased expression suggests that Vg might have functions in addition to its roles in oocyte maturation and energy supply for embryogenesis. IAG, a key regulator of male sex differentiation in crustaceans [79], is first discovered from E. sinensis transcriptomes. EsIAG shows remarkable structural similarity and sequence homology with other IAGs, suggesting that it is a member of the insulin/insulin-like growth factor family. By qRT-PCR validation, EsIAG is expressed in both sexes with a significantly higher level in males. It suggests that EsIAG might be not expressed exclusively in the male AG, which is also reported in S. paramamosain [79], C. sapidus [80] and Fenneropenaeus chinensis [81].

In conclusion, this is the first whole-body, sex-specific transcriptomes of juvenile E. sinensis using RNA-seq technology. More than 90 million clean reads were obtained, and some candidate genes in sex determination and differentiation were found. A large number of differentially expressed genes between the sexes were identified, and most of them had no obvious sexual function. Many potential SNPs and SSRs were detected that could be used for further gender identification and genetic breeding studies. This study will not only provide valuable
genetic resources for the understanding of sexual dimorphism in \textit{E. sinensis}, but also facilitate further investigations of functional genomics for this species and other closely related species.

Supporting Information

S1 Fig. Gene Ontology (GO) annotation of all unigenes from \textit{Eriocheir sinensis} transcriptomes. (TIF)

S2 Fig. eggNOG functional category of all unigenes from female and male \textit{Eriocheir sinensis} transcriptomes. (TIF)

S3 Fig. Expression levels of the identified sex determination genes in female and male \textit{Eriocheir sinensis} transcriptomes. (TIF)

S4 Fig. Volcano plot of differentially expressed genes (DEGs) from female and male \textit{Eriocheir sinensis} transcriptomes. For each unigene, the ratio of expression levels (Female vs. Male) is plotted against the-log error rate. The horizontal line indicates the significance threshold (FDR adjusted < 0.05), and the vertical lines indicate the two fold change threshold. Non-differentially expressed genes are shown with orange dots, and DEGs are shown with blue dots. (TIF)

S5 Fig. KEGG categories of differentially expressed genes (DEGs) from female and male \textit{Eriocheir sinensis} transcriptomes. The horizontal line indicates the significance threshold ($P < 0.05$). (TIF)

S6 Fig. Expression pattern of genes involved in sphingolipid metabolism pathway. The up-regulated in male are labeled by green, and the purple color represents genes with no expression differences between female and male \textit{Eriocheir sinensis} transcriptomes. (TIF)

S7 Fig. Amino acid sequence alignment (A) and bootstrapped neighbor-joining (NJ) tree of \textit{Eriocheir sinensis} IAG (EsIAG) with other crustacean IAGs. B and A chains are marked in yellow and green boxes, respectively. The six conserved cysteine residues are highlighted with dark red background and the predicted disulfide bridges are drawn. The species and the GenBank accession numbers are as follow: \textit{Callinectes sapidus} IAG1 (AEI72263), \textit{C. sapidus} IAG2 (AHM93481), \textit{Cherax destructor} (ACD91988), \textit{Cherax quadricarinatus} (ABH07705), \textit{Fenneropenaeus chinensis} IAG1(AFU60548), \textit{F. chinensis} IAG2 (AFU60549), \textit{Jasus edwardsii} (AIM55892), \textit{Litopenaeus vannamei} (AIR09497), \textit{Macrobrachium} lar (BAJ78349), \textit{Macrobrachium nipponense} IAG1 (AGB56976), \textit{Macrobrachium nipponense} IAG2 (AHA33389), \textit{Macrobrachium rosenbergii} (ACJ38227), \textit{Macrobrachium vollenhovenni} (AHZ34725), \textit{Marsupenaeus japonicus} (BAK20460), \textit{Palaemon pacificus} (BAJ84109), \textit{Palaemon paucident} (BAJ84108), \textit{Penaeus monodon} (ADA67878), \textit{Portunus pelagicus} (ADK46685), \textit{Sagmariasus verreauxi} (AHEY99679) and \textit{Scylla paramamosain} (AIF30295). Three isopods \textit{Armadillidium vulgare} (BA86893), \textit{Porcellio dilatatus} (BAC57013), \textit{Porcellio scabar} (BAC57012) served as outgroups. (TIF)
S1 Table. Primer sequences and product size of target and reference genes used for real-time PCR.

S2 Table. Summary statistics of sequencing, assembly and annotation of female and male *Eriocheir sinensis* transcriptomes.

S3 Table. Summary of simple sequence repeat (SSR) types in female and male *Eriocheir sinensis* unigenes.

S4 Table. Primary sex-related genes detected in female and male *Eriocheir sinensis* transcriptomes.

S5 Table. List of SNPs in sex-related genes in female and male *Eriocheir sinensis* transcriptomes.

S6 Table. List of SSRs in sex-related genes in female and male *Eriocheir sinensis* transcriptomes.

S7 Table. Differentially expressed genes from female and male *Eriocheir sinensis* transcriptomes.

**Author Contributions**

Conceived and designed the experiments: Y. Liu ZC. Performed the experiments: Y. Liu DL CS. Analyzed the data: Y. Liu MH. Contributed reagents/materials/analysis tools: Y. Li LL. Wrote the paper: Y. Liu.

**References**

1. Connallon T, Knowles LL (2005) Intergenomic conflict revealed by patterns of sex-biased gene expression. Trends in Genetics 21: 495–499. PMID: 16039005
2. Assis R, Zhou Q, Bachtrog D (2012) Sex-biased transcriptome evolution in *Drosophila*. Genome Biology and Evolution 4: 1189–1200. doi:10.1093/gbe/evs093 PMID: 23097318
3. Yang X, Schadt EE, Wang S, Wang H, Arnold AP, Ingram-Drake L, et al. (2006) Tissue-specific expression and regulation of sexually dimorphic genes in mice. Genome Research 16: 995–1004. PMID: 16925664
4. Small CM, Carney GE, Mo Q, Vannucci M, Jones AG (2009) A microarray analysis of sex-and gonad-biased gene expression in the zebrafish: evidence for masculinization of the transcriptome. BMC Genomics 10: 579. doi:10.1186/1471-2164-10-579 PMID: 19958554
5. Mank JE, Hultin-Rosenberg L, Webster MT, Ellegren H (2008) The unique genomic properties of sex-biased genes: insights from avian microarray data. BMC Genomics 9: 148. doi:10.1186/1471-2164-9-148 PMID: 18377635
6. Parisi M, Nuttall R, Edwards P, Minor J, Naiman D, Lü J, et al. (2004) A survey of ovary-, testis-, and soma-biased gene expression in *Drosophila melanogaster* adults. Genome Biology 5: R40. PMID: 15186491
7. Hartnoll RG (1982) Growth. In: Bliss DE, editor. The Biology of Crustacea. New York: Academic Press. 111–197 p.
8. Lee HH, Hsu CC (2003) Population biology of the swimming crab *Portunus sanguinolentus* in the waters off Northern Taiwan. Journal of Crustacean Biology 23: 691–699.
9. Mokhtari M, Savari A, Rezai H, Kochanian P, Bitaab A (2008) Population ecology of fiddler crab, Uca lactea annulipes (Decapoda: Ocyopodidae) in Sirik mangrove estuary, Iran. Estuarine, Coastal and Shelf Science 76: 273–281.

10. Chen DW, Zhang M, Shrestha S (2007) Compositional characteristics and nutritional quality of Chinese mitten crab (Eriocheir sinensis). Food Chemistry 103: 1343–1349.

11. Veilleux É, De Lafontaine Y (2007) Biological synopsis of the Chinese mitten crab (Eriocheir sinensis). Canadian Manuscript Report of Fisheries and Aquatic Sciences 2812: vi + 45p.

12. He L, Wang Q, Jin X, Wang Y, Chen L, Liu L, et al. (2012) Transcriptome profiling of testis during sexual maturation stages in Eriocheir sinensis using Illumina sequencing. PloS one 7: e33735. doi: 10.1371/journal.pone.0033735 PMID: 22442720

13. Rudnick DA, Hieb K, Grimmer KF, Resh VH (2003) Patterns and processes of biological invasion: the Chinese mitten crab in San Francisco Bay. Basic and Applied Ecology 4: 249–262.

14. Metzker ML (2010) Sequencing technologies—the next generation. Nature Reviews Genetics 11: 31–46. doi: 10.1038/nrg2626 PMID: 19997069

15. Garber M, Grabherr MG, Guttman M, Trapnell C (2011) Computational methods for transcriptome annotation and quantification using RNA-seq. Nature Methods 8: 469–477. doi: 10.1038/nmeth.1613 PMID: 21623353

16. Leelatanawit R, Sittikankanwek K, Yocawibun P, Klinbunsa S, Roypakul S, Aoki T, et al. (2009) Identification, characterization and expression of sex-related genes in testes of the giant tiger shrimp Penaeus monodon. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 152: 66–76.

17. Gao J, Wang X, Zou Z, Jia X, Wang Y, Zhang Z (2014) Transcriptome analysis of the differences in gene expression between testis and ovary in green mud crab (Scylla paramamosain). BMC Genomics 15: 585. doi: 10.1186/1471-2164-15-585 PMID: 25015001

18. He L, Jiang H, Cao D, Liu L, Hu S, Wang Q (2013) Comparative transcriptome analysis of the accessory sex gland and testis from the Chinese mitten crab (Eriocheir sinensis). PloSone 8: e53915. doi: 10.1371/journal.pone.0053915 PMID: 23342039

19. Zhang W, Wan H, Jiang H, Zhao Y, Zhang X, Hu S, et al. (2011) A transcriptome analysis of mitten crab testes (Eriocheir sinensis). Genetics and Molecular Biology 34: 136–141. doi: 10.1590/S1415-47572010005000099 PMID: 21637557

20. Hale MC, Xu P, Scardina J, Wheeler PA, Thorgaard GH, Nichols KM (2011) Differential gene expression in male and female rainbow trout embryos prior to the onset of gross morphological differentiation of the gonads. BMC Genomics 12: 404. doi: 10.1186/1471-2164-12-404 PMID: 21824436

21. Zhao M, Zha XF, Liu J, Zhang WJ, He NJ, Cheng DJ, et al. (2011) Global expression profile of silkworm genes from larval to pupal stages: Toward a comprehensive understanding of sexual differences. Insect Science 18: 607–618.

22. Perry JC, Harrison PW, Mank JE (2014) The ontogeny and evolution of sex-biased gene expression in Drosophila melanogaster. Molecular Biology and Evolution 31: 1206–1219. doi: 10.1093/molbev/msu072 PMID: 24526011

23. Lee T-H, Yamauchi M, Yamazaki F (1994) Sex differentiation in the crab Eriocheir japonicus (Decapoda, Grapsoidea). Invertebrate Reproduction and Development 25: 123–137.

24. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, et al. (2011) Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nature Biotechnology 29: 644–652. doi: 10.1038/nbt.1883 PMID: 21572440

25. Koboldt DC, Chen K, Wylie T, Larson DE, McLellan MD, Mardis ER, et al. (2009) VarScan: variant detection in massively parallel sequencing of individual and pooled samples. Bioinformatics 25: 2283–2285. doi: 10.1093/bioinformatics/btp373 PMID: 19542151
30. Thiel T, Michalek W, Varshney R, Graner A (2003) Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (Hordeum vulgare L.). Theoretical and Applied Genetics 106: 411–422. PMID: 12589540

31. Mortazavi A, Williams BA, McCue K, Schaefter L, Wold B (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nature Methods 5: 621–628. doi: 10.1038/nmeth.1226 PMID: 18516045

32. Anders S, Huber W (2010) Differential expression analysis for sequence count data. Genome Biology 11: R106. doi: 10.1186/gb-2010-11-10-r106 PMID: 20979621

33. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25: 4876–4882. PMID: 9396791

34. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599. PMID: 17488738

35. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 25: 402–408. PMID: 11846609

36. Hui M, Liu Y, Song C, Li Y, Shi G, Cui Z (2014) Transcriptome changes in Eriocheir sinensis megalopae after desalination provide insights into osmoregulation and stress adaption in larvae. PloS one 9: e114187. doi: 10.1371/journal.pone.0114187 PMID: 25470496

37. Song C, Cui Z, Hui M, Liu Y, Li X (2015) Comparative transcriptomic analysis provides insights into the molecular basis of brachyurization and adaptation to benthic lifestyle in Eriocheir sinensis. Gene 558: 88–98. doi: 10.1016/j.gene.2014.12.048 PMID: 25542812

38. Zhang EF, Qiu GF (2010) A novel Dmrt gene is specifically expressed in the testis of Chinese mitten crab, Eriocheir sinensis. Development Genes and Evolution 220: 151–159. doi: 10.1007/s00427-010-0336-2 PMID: 20809137

39. Wang Q, Fang D-A, Sun J-L, Wang Y, Wang J, Liu L-H (2012) Characterization of the vasa gene in the Chinese mitten crab Eriocheir sinensis: A germ line molecular marker. Journal of Insect Physiology 58: 960–965. doi: 10.1016/j.jinsphys.2012.04.012 PMID: 22562064

40. Shen H, Hu Y, Zhou X (2014) Sex-lethal gene of the Chinese mitten crab Eriocheir sinensis: cDNA cloning, induction by eyestalk ablation, and expression of two splice variants in males and females. Development Genes and Evolution 224: 97–105. doi: 10.1007/s00427-014-0467-y PMID: 24549568

41. Inoue K, Hoshijima K, Higuchi I, Sakamoto H, Shimura Y (1992) Binding of the transformer gene to the regulatory elements of doublesex primary transcript for sex-specific RNA processing. Proceedings of the National Academy of Sciences 89: 8092–8096.

42. Ryner LC, Goodwin SF, Castrillon DH, Anand A, Villella A, Baker BS, et al. (1996) Control of male sex-ual behavior and sexual orientation in Drosophila by the fruitless gene. Cell 87: 1079–1089. PMID: 8978612

43. Cui Z, Hui M, Liu Y, Song C, Li X, Li Y et al. (2015) High density linkage mapping aided by transcriptomics documents ZW sex determination system in the Chinese mitten crab, Eriocheir sinensis. Heredity doi: 10.1038/hdy.2015.26

44. Li S, Li F, Wen R, Xiang J (2012) Identification and characterization of the sex-determiner transformer-2 homologue in Chinese shrimp, Fenneropenaeus chinensis. Sexual Development 6: 267–278. doi: 10.1007/s00427-014-0467-y PMID: 22846336

45. Mita K, Kasahara M, Sasaki S, Nagayasu Y, Yamada T, Kanamori H, et al. (2004) The genome sequence of silkworm, Bombyx mori. DNA Research 11: 27–35. PMID: 15141943

46. Niimi T, Sahara K, Oshima H, Yasukochi Y, Ikeo K, Traut W (2006) Molecular cloning and chromosomal localization of the Bombyx Sex-lethal gene. Genome 49: 263–268. PMID: 16604109

47. Suzuki MT (2010) Sex determination: insights from the silkworm. Journal of Genetics 89: 357–363. PMID: 20877002

48. Doniach T, Hodgkin J (1984) A sex-determining gene, fem-1, required for both male and hermaphrodite development in Caenorhabditis elegans. Developmental Biology 106: 223–235. PMID: 6541600

49. Kashimada K, Koopman P (2010) Sry: the master switch in mammalian sex determination. Development 137: 3921–3930. doi: 10.1242/dev.049363 PMID: 21062860

50. Garcia-Ortiz JE, Pelosi E, Omar S, Nedorezov T, Piao Y, Karmazin J, et al. (2009) FoxI2 functions in sex determination and histogenesis throughout mouse ovary development. BMC Developmental Biology 9: 36. doi: 10.1186/1471-213X-9-36 PMID: 19538736
52. Kocer A, Pinheiro I, Pannetier M, Renault L, Parma P, Radi O, et al. (2008) R-spondin1 and FOXL2 act into two distinct cellular types during goat ovarian differentiation. BMC Developmental Biology 8: 36. doi: 10.1186/1471-213X-8-36 PMID: 18384673

53. Zhou L, Charkraborty T, Yu X, Wu L, Liu G, Mohapatra S, et al. (2012) R-spondins are involved in the ovarian differentiation in a teleost, medaka (Oryzias latipes). BMC Developmental Biology 12: 36. doi: 10.1186/1471-213X-12-36 PMID: 23217106

54. Adell T, Müller WE (2004) Isolation and characterization of five Fox (Forkhead) genes from the sponge Suberites domuncula. Gene 334: 35–46. PMID:15256253

55. Magie CR, Pang K, Martindale MQ (2005) Genomic inventory and expression of Sox and Fox genes in the cnidarian Nematostella vectensis. Development Genes and Evolution 215: 618–630. PMID:16193320

56. Shimeld SM, Boyle MJ, Brunet T, Luke GN, Seaver EC (2010) Clustered Fox genes in lophotrochozoans and the evolution of the bilaterian Fox gene cluster. Developmental Biology 340: 234–248. doi: 10.1016/j.ydbio.2010.01.015 PMID: 20096280

57. Valenzuela-Muñoz V, Huvet A, Levy P, Klopp C, Lhuillier E, Gaertner-Mazouni N, et al. (2014) Gonad transcriptome analysis of pearl oyster Pinctada margaritifera: identification of potential sex determination and sex determining genes. BMC Genomics 15: 491. doi: 10.1186/1471-2164-15-491 PMID: 24942841

58. Farlora R, Araya-Garay J, Gallardo-Escárate C (2014) Discovery of sex-related genes through high-throughput transcriptome sequencing from the salmon louse Caligus rogercresseyi. Marine Genomics 15: 85–93. doi: 10.1016/j.margen.2014.02.005 PMID: 24642131

59. Hale MC, Jackson JR, DeWoody JA (2010) Discovery and evaluation of candidate sex-determining genes and xenobiotics in the gonads of lake sturgeon (Acipenser fulvescens). Genetica 138: 745–756. doi: 10.1007/s10709-010-9455-y PMID: 20386959

60. Valenzuela-Muñoz V, Bueno-Ibarra MA, Escárate CG (2014) Characterization of the transcriptomes of Haliotis rufescens reproductive tissues. Aquaculture Research 45: 1026–1040.

61. Cooper GM (2000) The Cell: A Molecular Approach 2nd edition. Boston University. Sunderland (MA): Sinauer Associates.

62. El-Sankary W, Bombail V, Gibson GG, Plant N (2002) Glucocorticoid-mediated induction of CYP3A4 is decreased by disruption of a protein: DNA interaction distinct from the pregnane X receptor response element. Drug Metabolism and Disposition 30: 1029–1034. PMID:12167569

63. Radhika M, Nazar AA, Munuswamy N, Nellaiappan K (1998) Sex-linked differences in phenol oxidase in the fairy shrimp Streptocephalus dichotomus Baird and their possible role (Crustacea: Anostraca). Hydrobiologia 377: 161–164.

64. Kurtz J, Wiesner A, Götz P, Sauer KP (2000) Gender differences and individual variation in the immune system of the scorpionfly Panorpa vulgaris (Insecta: Mecoptera). Developmental and Comparative Immunology 24: 1–12. PMID:10689094

65. Kurtz J, Sauer KP (2001) Gender differences in phenoloxidase activity of Panorpa vulgaris hemocytes. Journal of Invertebrate Pathology 78: 53–55. PMID: 11500094

66. Rolf J (2001) Effects of age and gender on immune function of dragonflies (Odonata, Lestidae) from a wild population. Canadian Journal of Zoology 79: 2176–2180.

67. Lindsay E, Altizer S (2009) Sex differences in immune defenses and response to parasitism in monarch butterflies. Evolutionary Ecology 23: 607–620.

68. Iwanaga S, Lee BL (2005) Recent advances in the innate immunity of invertebrate animals. BMB Reports 38: 128–150.

69. Akira S, Uematsu S, Takeuchi O (2006) Pathogen recognition and innate immunity. Cell 124: 783–801. PMID:15497588

70. Raikhel A, Brown M, Belles X (2005) Hormonal control of reproductive processes. Comprehensive Molecular Insect Science 3: 433–491.

71. Zhang S, Wang S, Li H, Li L (2011) Vitellogenin, a multivalent sensor and an antimicrobial effector. The International Journal of Biochemistry & Cell Biology 43: 303–305.

72. Flouriot G, Pakdel F, Ducouret B, Valotaire Y (1995) Influence of xenobiotics on rainbow trout liver estrogen receptor and vitellogenin gene expression. Journal of Molecular Endocrinology 15: 143–151. PMID: 8800639

73. Palmer BD, Palmer SK (1995) Vitellogenin induction by xenobiotic estrogens in the red-eared turtle and African clawed frog. Environmental Health Perspectives 103: 19.
74. Piulachs M, Guidugli K, Barchuk A, Cruz J, Simoes Z, Belles X (2003) The vitellogenin of the honey bee, *Apis mellifera*: structural analysis of the cDNA and expression studies. Insect Biochemistry and Molecular Biology 33: 459–465. PMID: 12650694

75. Guidugli KR, Piulachs MD, Bellés X, Lourenço AP, Simões ZL (2005) Vitellogenin expression in queen ovaries and in larvae of both sexes of *Apis mellifera*. Archives of Insect Biochemistry and Physiology 59: 211–218. PMID: 16034983

76. Tsutsui N, Kawazoe I, Ohira T, Jasmani S, Yang W-J, Wilder MN, et al. (2000) Molecular characterization of a cDNA encoding vitellogenin and its expression in the hepatopancreas and ovary during vitellogenesis in the kuruma prawn, *Penaeus japonicus*. Zoological Science 17: 651–660. doi: 10.2108/zsj.15.651 PMID: 18517301

77. Shechter A, Aflalo ED, Davis C, Sagi A (2005) Expression of the reproductive female-specific vitellogenin gene in endocrinologically induced male and intersex *Cherax quadricarinatus* crayfish. Biology of Reproduction 73: 72–79. PMID: 15744019

78. Zmora N, Trant J, Chan S-M, Chung JS (2007) Vitellogenin and its messenger RNA during ovarian development in the female blue crab, *Callinectes sapidus*: gene expression, synthesis, transport, and cleavage. Biology of Reproduction 77: 138–146. PMID: 17409377

79. Huang X, Ye H, Huang H, Yang Y, Gong J (2014) An insulin-like androgenic gland hormone gene in the mud crab, *Scylla paramamosain*, extensively expressed and involved in the processes of growth and female reproduction. General and Comparative Endocrinology 204: 229–238. doi: 10.1016/j.ygcen.2014.06.002 PMID: 24929228

80. Chung JS, Manor R, Sagi A (2011) Cloning of an insulin-like androgenic gland factor (IAG) from the blue crab, *Callinectes sapidus*: Implications for eyestalk regulation of IAG expression. General and Comparative Endocrinology 173: 4–10. doi: 10.1016/j.ygcen.2011.04.017 PMID: 21596044

81. Li S, Li F, Sun Z, Xiang J (2012) Two spliced variants of insulin-like androgenic gland hormone gene in the Chinese shrimp, *Penneropenaeus chinensis*. General and Comparative Endocrinology 177: 246–255. doi: 10.1016/j.ygcen.2012.04.010 PMID: 22561290