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Genetic regulation of male sexual development in the oriental river prawn *Macrobrachium nipponense* during reproductive *vs.* non-reproductive season

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Abstract
Rapid gonad development is an important concern in the *Macrobrachium nipponense* aquaculture industry. The process of sexual differentiation and development is influenced by environmental factors, including water temperature and illumination time. In this study, we aimed to identify the key metabolites and genes from the androgenic gland, in response to different water temperatures and illumination times based on the integrated metabolomic and transcriptomic analysis of the androgenic gland during the reproductive *vs.* non-reproductive seasons. Histological observations revealed that the division of the androgenic gland in the reproductive season is more active than that in the non-reproductive season. The metabolic profiling analysis revealed that glycerophospholipid metabolism and sphingolipid metabolism represented the most enriched metabolic pathways of differentially expressed metabolites (DEMs). Transcriptome profiling analysis revealed that phagosome, spliceosome, RNA degradation, ribosome biogenesis in eukaryotes, and oxidative phosphorylation represented the main enriched metabolic pathways of differentially expressed genes (DEGs). A total of 14 DEGs from these metabolic pathways were considered as strong candidate genes involved in the male sexual development in *M. nipponense*, based on the expression difference between the reproductive *vs.* non-reproductive seasons. qPCR verifications of these 14 DEGs indicate the accuracy of the RNA-Seq. In addition, eight genes out of 14 DEGs showed high expression levels in the testis or/and androgenic gland. Predicting these eight genes may play an essential role in male sexual development of *M. nipponense*. The results of this study contribute to our knowledge of sexual development in male *M. nipponense*, as well as other crustacean species.

Keywords *Macrobrachium nipponense* · Metabolome, Transcriptome · Androgenic glands · Male sexual development

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Introduction

The oriental river prawn *Macrobrachium nipponense* has a great market value in China and other Asian countries (Fu et al. 2012). The annual production of *M. nipponense* reached 205,010 tons in 2020, making it an economically important species (Zhang et al. 2020). Male *M. nipponense* grow faster and reach larger sizes (approximately 2–2.5 times larger) at harvest time, compared to the female counterparts. Thus, production of the all-male population provides economic benefits for the *M. nipponense* culture industry. In addition, the rapid gonad development of newly hatched prawn larvae during the reproductive season is the main problem in the *M. nipponense* culture industry. Both the testis and ovaries of the hatchlings developed to sexual maturity within 40 days after hatching. This causes inbreeding between young prawns. Inbreeding leads to the mating and propagation of multiple generations in the same ponds, resulting in the prawns with smaller market size, restricting the sustainable development of the *M. nipponense* industry (Jin et al. 2021a, b, c). Therefore, the identification of important sex-determining and reproduction-related metabolites and genes are urgently needed, as it is integral in the establishment of an artificial technique to produce all progenies on a commercial scale and to regulate the process of gonad development in *M. nipponense*.

The androgenic gland is a unique endocrine organ in male crustaceans. The androgenic gland and the hormones it secretes have been reported to play an essential role in controlling sex differentiation and maintaining male secondary sexual characteristics in crustaceans, especially in testis development (Sagi et al. 1986, 1990). Insulin-like androgenic gland hormone (*IAG*) is the main expressed gene in the androgenic gland, which was reported to be the most important sex-related gene in crustacean species (Rosen et al. 2010; Ventura et al. 2009, 2011). Knocking down the expression of IAG resulted in the sex-reversal in *Macrobrachium resenbergii* (Ventura et al. 2012). *IAG* also plays essential roles in the mechanism of male sexual differentiation and development in *M. nipponense* (Ma et al. 2016), *Fenneropenaeus chinensis* (Li et al. 2012), *Scylla paramamosain* (Huang et al. 2014), *Lysmata vittate* (Liu et al. 2021), and *Fenneropenaeus merguiensis* (Zhou et al. 2021). Moreover, the mechanism of male sexual differentiation and development in *M. nipponense*, based on the androgenic gland, has been successfully determined (Jin et al. 2013; Jin et al. 2018; Jin et al. 2019a, b; Ma et al. 2016). However, some important male reproduction-related metabolites and genes of *M. nipponense* are still unclear, which hinders the establishment of an artificial technique to regulate the process of testis development.

Previous studies reported that temperature, illumination, and the presence of chemical pollutants dramatically affect the process of sexual differentiation and development in aquatic animals, leading to sex reversal (Wedekind 2017). Environmental factors can influence the expression profiles of *DMRT1* and vitellogenin, which affect the sexual differentiation and development of lower vertebrates (Leslie and Valentine 2015; Om et al. 2015). The reproductive season of *M. nipponense* is from April to October in a year with the water temperature $\geq$ 28 °C and illumination time of $\geq$ 16 h, while the optimal period is from June to July. The non-reproductive season of *M. nipponense* is from December to February of the next year with the water temperature $\leq$ 15 °C and illumination time of $\leq$ 10 h (Fu et al. 2012). Thus, the identification of metabolites and genes from the androgenic gland, in response to water temperature and illumination time, play essential roles to fully understand the mechanism of sexual differentiation and development in *M. nipponense*.

In this study, we aimed to identify the morphological differences of the androgenic gland during the reproductive season in July (water temperature $\geq$ 28 °C and illumination

time ≥ 16 h) *vs.* that during the non-reproductive season in January (water temperature ≤ 15 °C and illumination time ≤ 10 h) by hematoxylin and eosin (HE) staining. The DEMs and DEGs were selected from the androgenic gland in *M. nipponense*, in response to different water temperatures and illumination times through the conduct of metabolomic and transcriptomic profiling analysis of the androgenic gland samples taken during the reproductive *vs.* non-reproductive seasons, which may be involved in the mechanism of sex differentiation and development. This study provided valuable evidences to better understand the molecular mechanisms, underlying sexual differentiation and development in male *M. nipponense*.

Materials and methods

Sample collection

A total of 200 healthy male prawns with body weights ranging from 3.23 to 5.16 g and 3.45 to 4.98 g were collected from a wild population in Tai Lake, Wuxi, China (120°13′44″E, 31°28′ 22″N) in January (non-reproductive season) and July (reproductive season), respectively. The specimens from the two batches were fed the same commercial diet. An additional 20 healthy female prawns with body weights of 2.76–3.12 g were collected from a wild population in Tai Lake in July. These specimens were maintained for 72 h in a pond with a dissolved oxygen level ≥ 6 mg/L, prior to tissue collection. The water temperature was ≥ 28 °C and illumination time was ≥ 16 h in July, while the water temperature was ≤ 15 °C and illumination time was ≤ 10 h in January. The prepared samples in this study are listed in Table 1. Androgenic glands were each collected from five individual male prawns in January and July, respectively, and maintained in 4% paraformaldehyde until histological analyses. A total of 20 androgenic glands were collected and pooled to form a biological replicate in January and July, respectively, and a total of eleven biological replicates were collected, of which eight replicates were prepared for metabolomic profiling analysis, and three replicates were prepared for the transcriptome profiling analysis. Samples of eight tissues were collected from male prawns ($N = 5$) in July for qPCR analysis, including the eyestalk, brain, heart, hepatopancreas, gill, muscle, testis, and androgenic gland, while ovaries were collected from female prawns ($N = 5$) also in July. The tissue samples were immediately frozen in liquid nitrogen and stored at – 80 °C until RNA extraction, in order to prevent RNA degradation.

Histological observations

The morphological differences of the androgenic gland in *M. nipponense* between the reproductive *vs.* non-reproductive seasons were determined by HE staining. The detailed procedure for HE staining has been provided in previous studies (Ma et al. 2006; Shang-Guan et al. 1991). Briefly, the tissues were first dehydrated by using 50%, 70%, 80%, 95%, and 100% ethanol. The tissues were then made transparent and embedded using alcohol/xylene (1:1), xylene, xylene/wax (1:1), and wax. The embedded tissues were sectioned to a thickness of 5 μm using a slicer (Leica, Wetzlar, Germany) and placed on a slide. The slides were then stained with HE for 3–8 min. The histological slides were observed under an Olympus SZX16 microscope (Olympus Corporation, Tokyo, Japan).

Table 1 Tissue samples for this study

Purpose	Tissues sample	Number of replicates
Histological observation	Androgenic glands were collected from male prawns during the reproductive season and non-reproductive season, respectively	Five androgenic glands were collected from each season
Metabolomic profiling analysis	A total of 20 androgenic glands were collected and pooled to form a biological replicate during the reproductive season and non-reproductive season, respectively	Eight biological replicates were collected
Transcriptome profiling analysis	A total of 20 androgenic glands were collected and pooled to form a biological replicate during the reproductive season and non-reproductive season, respectively	Three biological replicates were collected
qPCR analysis	Eyestalk, brain, heart, hepatopancreas, gill, muscle, testis, and androgenic gland were collected in male prawns during the reproductive season, while ovaries were collected from the female prawns	Each tissue was collected from five individual prawns

Metabolome profiling analysis

Metabolomic profiling was used to select DEMs from the androgenic gland between the reproductive *vs.* non-reproductive seasons, which may be involved in the mechanism of male sexual differentiation and development in *M. nipponense*. The DEMs were determined using liquid chromatography-mass spectrometry analysis (Ortiz-Villanueva et al. 2017). The procedures for metabolomic analysis have been described by Jin et al. (2020). The criterion of the robustness and predictive ability of the model was set as a seven-fold cross-validation, and further validation was performed using permutation tests.

Transcriptome profiling analysis

The DEGs of the androgenic gland between reproductive *vs.* non-reproductive seasons were identified through transcriptome profiling analysis. The transcriptome profiling analysis was performed on an Illumina High-seq 2500 sequencing platform. Previously published studies have described detailed procedures for RNA-Seq and analysis (Jin et al. 2013, 2021b). Trinity program (version: trinityrnaseq_r20131110) was used to assemble the clean data into non-redundant transcripts (Grabherr et al. 2011). Gene annotation was then performed in the Nr (non-redundant) database, Gene Ontology (GO) (Ashburner et al. 2000), Cluster of Orthologous Groups (COG) (Tatusov et al. 2003), and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (Kanehisa et al. 2008), using an E-value of 10^{-5} (Jin et al. 2013). EB-seq algorithm was used to filter the differentially expressed genes, under the criteria of FDR (False discovery rate) < 0.05 (Benjamini et al. 2001).

qPCR analysis

qPCR was used to verify the accuracy of RNA-Seq and to determine the mRNA expressions of important DEGs in different mature tissues. The detailed procedures have been well described in previously published studies (Jin et al. 2019a, 2021a; Zhang et al. 2013). Briefly, total RNA was extracted from each tissue, using the UNlQ-10 Column Trizol Total RNA Isolation Kit (Sangon, Shanghai, China) following the manufacturer's protocol. A total of 1 μg total RNA from each tissue was used to synthesize the cDNA template by using the PrimeScript™ RT reagent kit (Takara Bio Inc., Japan). The expression level of each tissue was determined using the UltraSYBR Mixture (CWBIO, Beijing, China). The qPCR analysis was performed on the Bio-Rad iCycler iQ5 Real-Time PCR System (Bio-Rad), and the SYBR Green RT-qPCR assay was used. The primers for qPCR analysis are listed in Table 2. The eukaryotic translation initiation factor 5A (*EIF*) was used as a reference gene in this study (Hu et al. 2018). The relative expression levels were measured using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Statistical analysis

Quantitative data were expressed as the mean ± SD. Statistical differences were estimated by one-way ANOVA, followed by LSD and Duncan's multiple range test. All statistical

Table 2 Primers used for the qPCR analysis in this study

Primer name	Sequence (5′→3′)		Length (bp)
Proteasome alpha 3	F	TGAAATGGAATTGTCCTGGGTCT	93
	R	TTGGCATATTGTTCAGCTTCCTG	
Proteasome subunit beta type-5	F	GGCTATGGTCTTTCTATGGGTGT	208
	R	AAATTGCACGTCTAGCCAAATCC	
Nucleolar protein 56	F	TTCTGGAAGATCAGGCATGGAAA	103
	R	GACACCAACTTCTTCAAACTCGG	
5'-3' exoribonuclease 1	F	AAACGACAATGACCCACATTTCC	201
	R	TTTTCAGCATCTACAGCCTCCTT	
RNA exonuclease 1 homolog	F	TACCAAGTTCACACCAGAGACAC	104
	R	GAAGGGATGGTAGGAGCATTCTT	
DEAD box polypeptide 39	F	GACAAAATGCTGGTTCAGTTGGA	122
	R	CATACTGGGCGAATTTCTTTGCT	
Mago nashi 1	F	TTAGACCAGATGGCAAACTTCGT	107
	R	CTTCAGCTCTTCCATCACACAAC	
U2 snRNP-associated protein	F	TACAAGGAGATTCACCAAGTCGG	143
	R	ATCATCCTCCTCCTCTTCGATCT	
Transcription elongation regulator 1	F	CTGCCATTGAGGCTGAAGTAAAG	173
	R	CGATGTCAACAAAAGATACCGGG	
6-phosphofructokinase	F	GCGATCTCACCCTTTACTTGATG	176
	R	TTCGAAGCATTTGTCCAAGGAAG	
HSP60	F	GGTCTTCTGACCTGTACCATCTC	116
	R	ATCGTCACTGGTACTGTCGTAAC	
M-phase phosphoprotein 6 protein	F	CCATTGAAACTGCTGCTGAAGAA	193
	R	ACATTCTGCTTTTCACCTTCACG	
Protein kinase C	F	ACAGTGCGGTAAATTCAAAGAGC	169
	R	CTTGCACATACCAAAGTCAGCAA	
Cytochrome oxidase sununit 3	F	ATGAGACAAATGAGGCTGGAGTT	151
	R	CCTCTGGAGTGGAGTAGGAGTAT	

analysis was carried out using SPSS Statistics 23.0. A probability level of 0.05 was used to indicate significance ($p < 0.05$).

Results

Histological observations of the androgenic gland

Histological observations revealed that the androgenic gland taken during the non-reproductive *vs.* reproductive seasons showed significant morphological differences (Fig. 1). The division of the androgenic gland during the reproductive season was more active than that during the non-reproductive season. The androgenic gland cells in the non-reproductive season were smaller than that during the reproductive season. The cell boundary was clear, and the cytoplasm was basophilic. The nucleolus of the nucleus was not visible in the non-reproductive season. However, the androgenic gland cells were loose in the reproductive

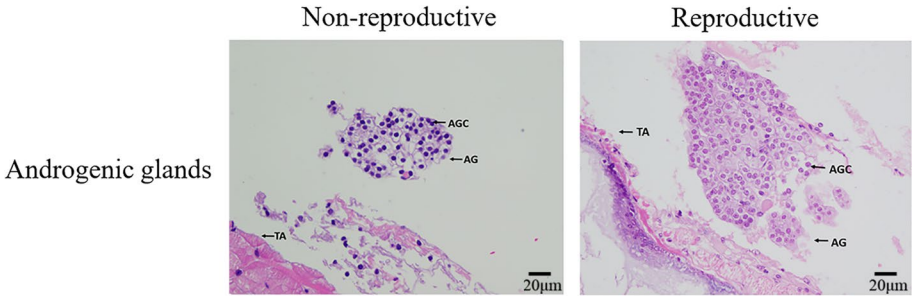


Fig. 1 Morphological difference of androgenic gland between reproductive season *vs.* non-reproductive season. AGC androgen gland cell, TA ampulla, AG androgenic gland

season. The intercellular connections were closer in the reproductive season, and multiple nucleoli were observed in the nucleus.

Metabolic profiling analysis

The overall quality of the metabolic profiling analysis of the androgenic gland between the non-reproductive *vs.* reproductive seasons was measured by principal component analysis (Fig. 2) and latent structures discriminant analysis (Fig. 3). The R2X (cum), R2Y (cum), and Q2 (cum) were 0.942, 1, and 0.947, respectively. The R2 and Q2 intercept values were 0.943 and − 0.906 after 200 permutations of the treatments. A total of 260 DEMs were identified from the androgenic gland between the non-reproductive *vs.* reproductive seasons, of which 148 DEMs were upregulated and 112 DEMs were downregulated in the

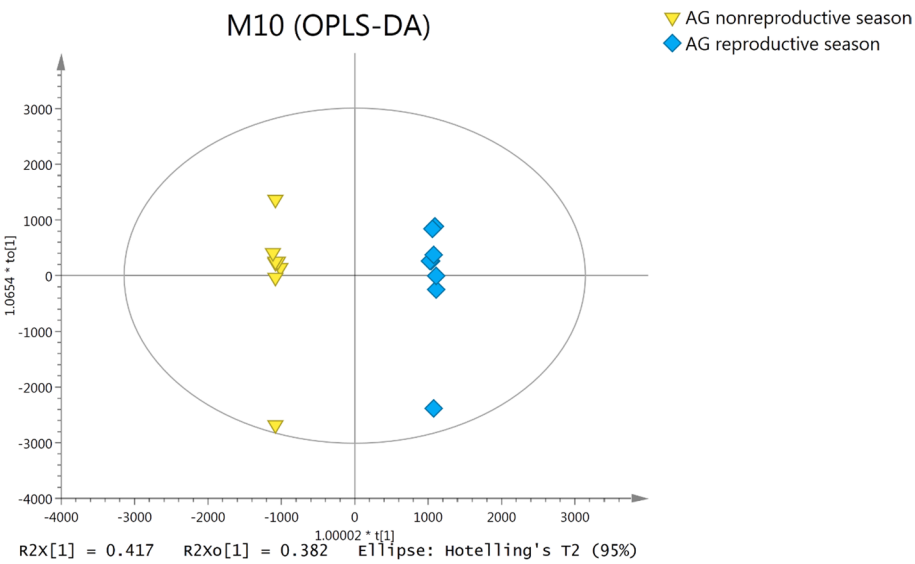


Fig. 2 Principal component analysis (PCA) of metabolic profiles of the androgenic gland samples of *M. nipponense* at non-reproductive season and reproductive season

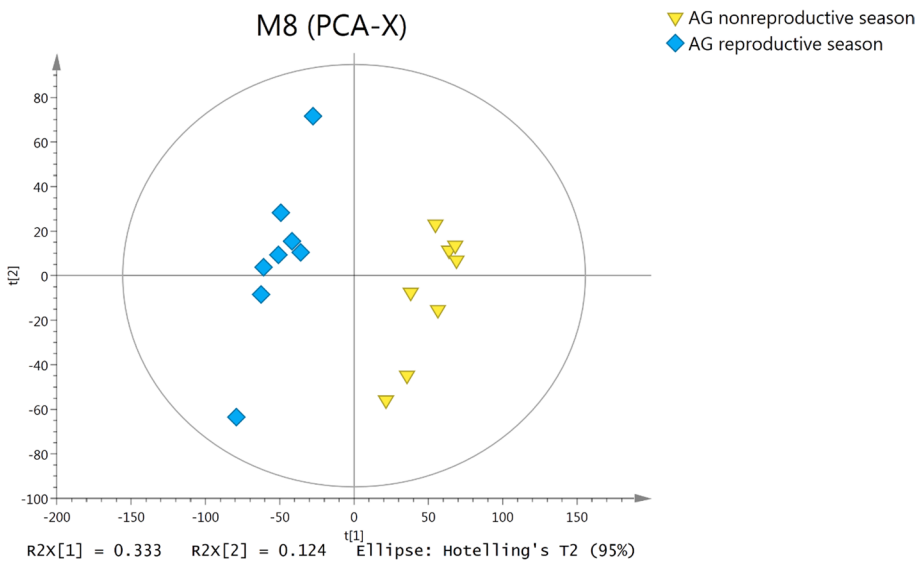


Fig. 3 Orthogonal projections to latent structures discriminate analysis (OPLS-DA) analysis of the androgenic gland samples at non-reproductive season and reproductive season. OPLS-DA score plots based on the LC–MS spectra

non-reproductive season, using the criteria of > 1.5 for upregulation and < 0.66 for downregulation. These DEMs assigned to 21 metabolic pathways, according to KEGG analysis. Glycerophospholipid metabolism and sphingolipid metabolism represented the most important enriched metabolic pathways, combined with the analysis of the number of enriched DEMs and *P*-values. The top 10 upregulated and downregulated DEMs are listed in Table 3. Gossyrubilone and saxagliptin were the upregulated and downregulated DEMs with significant expression changes in the metabolomic profiling analysis of both androgenic gland and testis, respectively.

Transcriptome profiling analysis

This study assembled 36,717 non-redundant transcripts with an average length of 1143.87 bp, and ranged from 301 bp to 29,020 bp. Approximately, 24.96% of non-redundant transcripts were 301–400 bp in length, followed by 401–500 bp (14.85%) and > 2000 bp (14.46%).

All of the assembled unigenes were then annotated in the Nr database in NCBI, GO, COG, and KEGG databases, in order to predict their putative functions, using an E-value of $< 10^{-5}$. A total of 12,819 (34.91%) unigenes were annotated in the Nr database. The other unannotated unigenes represented the novel genes with functions that have not yet been clearly defined. A total of 9372 (25.52%) and 8856 (24.12%) were annotated in the GO and COG databases, respectively. GO analysis included three functional categories, which were biological process (22 functional groups), cellular component (17 functional groups), and molecular function (15 functional groups), of which cellular process, cell, cell part, and binding represent the main functional groups (Fig. 4). COG analysis identified 25 functional groups, which include general function prediction only, signal transduction

Table 3 The most significant DEMs in this study

Metabolites	Formula	*P*-value	Fold change (Log2)
PC(16:0/3:1(2E))	C27H52NO8P	9.4E – 14	8.81387
Val Leu Glu Ser Phe	C28H43N5O9	1.4E – 12	6.96072
Gossyrubilone	C20H25NO4	6.8E – 08	5.64223
LysoPE(0:0/16:1(9Z))	C21H42NO7P	4.8E – 09	5.34849
PI(18:0/18:2(9Z,12Z))	C45H83O13P	0.0087	3.68784
Rhizochalinin D	C35H70N2O8	0.00112	3.2292
PE(18:3(9Z,12Z,15Z)/21:0)	C44H82NO8P	1.8E – 07	3.13264
PS(O-20:0/18:3(9Z,12Z,15Z))	C44H82NO9P	6.5E – 09	3.11879
N1-trans-Feruloylagmatine	C15H22N4O3	3.5E – 08	2.8783
PE(18:0/22:4(7Z,10Z,13Z,16Z))	C45H82NO8P	1.3E – 09	2.78103
PE(20:3(5Z,8Z,11Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	C47H76NO8P	1.1E – 07	– 3.1669
Saxagliptin	C18H25N3O2	9.1E – 10	– 3.2619
DG(15:0/20:5(5Z,8Z,11Z,14Z,17Z)/0:0)	C38H64O5	2.7E – 07	– 3.5407
CerP(d18:1/12:0)	C30H60NO6P	3.8E – 09	– 3.8706
PGF2a ethanolamide	C22H39NO5	5.6E – 09	– 3.9088
PA(12:0/18:2(9Z,12Z))	C33H61O8P	8.3E – 10	– 4.0671
L-octanoylcarnitine	C15H29NO4	5.8E – 10	– 4.3975
PC(12:0/12:0)	C32H64NO8P	3E – 07	– 4.8322
PA(12:0/20:3(8Z,11Z,14Z))	C35H63O8P	4.3E – 07	– 5.665
Nb-Palmitoyltryptamine	C26H42N2O	1.2E – 10	– 18.371

DEMs differentially expressed metabolites

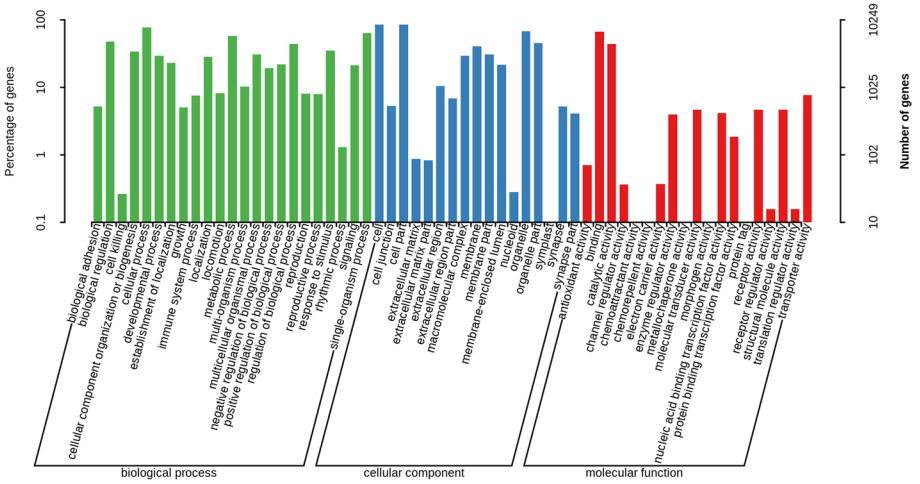


Fig. 4 Gene ontology classification of non-redundant transcripts. The left *y*-axis indicates the percentage of a specific category of genes existed in the main category, whereas the right *y*-axis indicates the number of a specific category of genes existed in main category

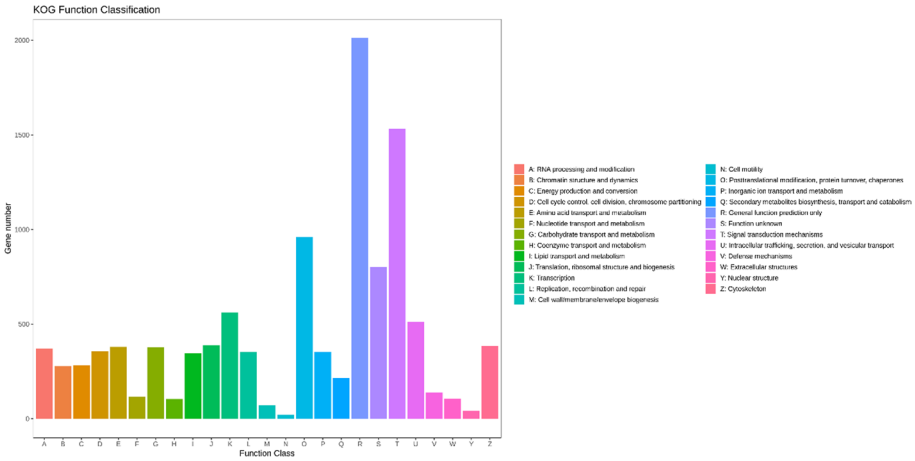


Fig. 5 Cluster of orthologous groups (COG) classification of putative proteins

mechanisms, secondary metabolite synthesis, transport and catabolism, and function unknown represent the main functional groups (Fig. 5). A total of 6787 (18.48%) unigenes matched the known proteins in the KEGG database and were mapped onto 217 metabolic pathways.

A total of 9734 unigenes were differentially expressed in the androgenic gland between the reproductive *vs.* non-reproductive seasons using the criteria of > 1.5 for upregulation and < 0.66 for downregulation, of which 5256 unigenes were upregulated, and 4478 unigenes were downregulated during the reproductive season. These DEGs ranging from 1 to 54 were mapped onto 174 metabolic pathways. Phagosome, spliceosome, and ribosome biogenesis in eukaryotes, RNA degradation, and oxidative phosphorylation represented the main enriched metabolic pathways of DEGs, combined with the analysis of enriched DEGs and P-values.

Identification of reproduction-related genes

A total of 14 DEGs were selected from the main enriched metabolic pathways, based on the expression patterns noted in the samples from the non-reproductive *vs.* reproductive seasons, which were considered as strong candidate genes involved in male sexual development (Table 4). Proteasome alpha 3 and proteasome subunit beta type-5 were selected from the metabolic pathway of proteasome. Nucleolar protein 56 (*NOL56*), 5′-3′ exoribonuclease 1, and RNA exonuclease 1 homolog were selected from the metabolic pathway of ribosome biogenesis in eukaryotes. DEAD box polypeptide 39, Mago nashi 1, U2 snRNP-associated protein, and transcription elongation regulator 1 (*TCERG1*) were selected from the metabolic pathway of spliceosome. 6-phosphofructokinase, heat shock protein 60 (*HSP60*), and M-phase phosphoprotein 6 protein selected from the metabolic pathway of RNA degradation. Cytochrome oxidase subunit 3 and protein kinase C were selected from the metabolic pathway of oxidative phosphorylation. The expression of these 14 DEGs between the reproductive *vs.* non-reproductive seasons was further verified by qPCR analysis, which showed the same expression pattern as RNA-Seq (Fig. 6).

Table 4 Identification of important DEGs

Gene	Metabolic pathways	Folder change (reproduction vs non-reproduction)	
		RNA-Seq	qPCR
Proteasome alpha 3	Proteasome	0.31	0.16
Proteasome subunit beta type-5	Proteasome	0.37	0.14
Nucleolar protein 56	Ribosome biogenesis in eukaryotes	0.19	0.38
5'-3' exoribonuclease 1	Ribosome biogenesis in eukaryotes, RNA degradation	2.32	3.90
RNA exonuclease 1 homolog	Ribosome biogenesis in eukaryotes	2.05	17.88
DEAD box polypeptide 39	Spliceosome	0.31	0.21
Mago nashi 1	Spliceosome	0.36	0.07
U2 snRNP-associated protein	Spliceosome	2.53	4.45
Transcription elongation regulator 1	Spliceosome	3.06	2.52
6-phosphofructokinase	RNA degradation	1.84	2.13
HSP60	RNA degradation	0.22	0.27
M-phase phosphoprotein 6 protein	RNA degradation	0.32	0.31
Cytochrome oxidase subunit 3	Oxidative phosphorylation	0.19	0.23
Protein kinase C	Oxidative phosphorylation	2.20	5.12

DEGs differentially expressed genes

qPCR analysis of DEGs in different tissues

The expression levels of these 14 DEGs were further verified in different mature tissues, in order to analyze their potential biological functions (Fig. 7). A total of 6 DEGs were highly expressed in the male sexual developmental system (testis and androgenic gland) in *M. nipponense* and showed significant differences with other tested tissues ($p < 0.05$), including *NOL56*, 5′-3′ exoribonuclease 1, RNA exonuclease 1 homolog, Mago nashi 1, U2 snRNP-associated protein, and *TCERG1*. Among these 6 DEGs, the expression levels of *NOL56*, 5′-3′ exoribonuclease 1, U2 snRNP-associated protein, and *TCERG1* in testis showed significant difference with other tested tissues ($p < 0.05$), while RNA exonuclease 1 homolog and Mago nashi 1 showed significant differences in both the testis and androgenic gland compared with other tested tissues ($p < 0.05$). In addition, *HSP60* and cytochrome oxidase subunit 3 also exhibited extremely high expression levels in male sexual developmental system.

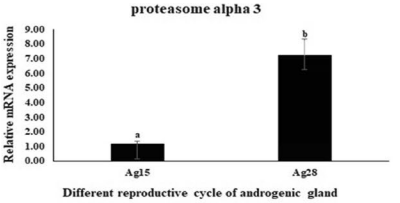
Discussion

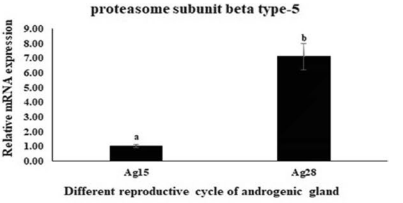
There were significant differences in the reproductive capacity between the *M. nipponense* prawns collected during the reproductive *vs.* non-reproductive seasons. Androgenic gland has been reported to play essential roles in promoting male sexual differentiation and development in crustaceans (Rosen et al. 2010; Ventura et al. 2009, 2011). It is

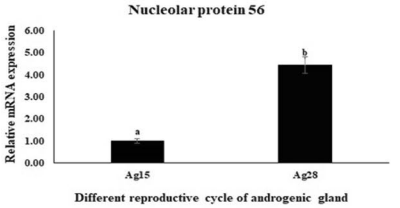
Fig. 6 Verification of the expressions of 14 differentially expressed genes (DEGs) in the androgenic gland between non-reproductive season *vs.* reproductive season by qPCR. The amounts of DEGs expression were normalized to the EIF transcript level. Data are shown as mean ± SD (standard deviation) of tissues in three separate individuals. Lowercase indicates the expression difference of DEGs in the androgenic gland between non-reproductive season *vs.* reproductive season

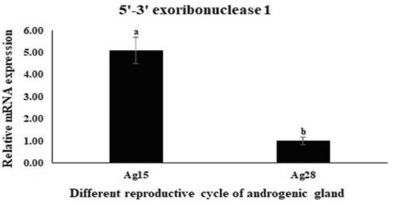
widely acknowledged that environmental factors have considerable effects on the process of sexual differentiation and development in aquatic animals (Wedekind 2017). However, the regulatory effects of environmental factors on the reproductive capacity of the androgenic gland are still unclear. In this study, the morphological differences of the androgenic gland of prawns collected during the reproductive *vs.* non-reproductive seasons were determined by HE staining. DEMs and DEGs were selected from the androgenic gland of prawns from the two seasons which were examined via an integrated metabolome and transcriptome analysis.

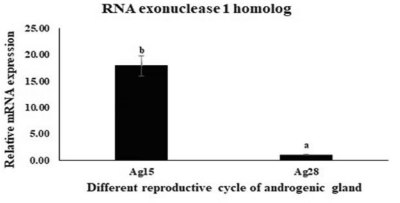
In this study, the R2X(cum), R2Y(cum), and Q2(cum) were 0.942, 1, and 0.947, respectively. The values indicated the separation of the variation in the statistical data between the androgenic gland during the non-reproductive *vs.* reproductive seasons. The R2 and Q2 intercept values were 0.943 and − 0.906, respectively. This suggested a strongly robust and reliable model with a low risk of overfitting to identify the different metabolic patterns in the androgenic gland during the non-reproductive *vs.* reproductive seasons. A total of 260 DEMs were identified in this study. Glycerophospholipid metabolism and sphingolipid metabolism were the main enriched metabolic pathways of DEMs in the androgenic gland between the reproductive *vs.* non-reproductive seasons. A previous study also reported that glycerophospholipid metabolism and sphingolipid metabolism were the main enriched metabolic pathways of DEMs in the testis between the reproductive *vs.* non-reproductive seasons (Jin et al. 2020). Histological observations revealed significant morphological differences in the androgenic gland between the reproductive *vs.* non-reproductive seasons in this study. Thus, glycerophospholipid metabolism and sphingolipid metabolism were predicted to play essential roles in the development of the androgenic gland in the reproductive season, promoting the lipid accumulation during this time. Glycerophospholipids are the most common and abundant phospholipid in the body. In glycerophospholipids, two hydroxyl groups of glycerol bind with the fatty acids to form the esters, and the third hydroxyl group is esterified by phosphorylation to form the phosphatidylic acid (Dennis et al. 1991; Exton 1994; Meagher and Fitzgerald 1993). Glycerophospholipid plays essential roles in the formation of biofilms. In addition, glycerophospholipids are also important components of bile and membrane surfactant, as these participate in protein recognition and signal transduction in the cell membrane. Sphingolipids are amphoteric lipids, containing a sphingosine skeleton (Meagher and Fitzgerald 1993). Sphingolipids, including sphingomyelin and gangliosides, generally exist in the plant and animal membranes, especially in the central nervous system. The hydrolysis of sphingomyelin in cells is catalyzed by the nerve phospholipase in lysosomes. The hydrolysates are ceramide and choline phosphate, which can continue to metabolize (Spiegel and Merrill 1996). A congenital defect of phospholipase leads to the accumulation of sphingomyelin in tissues, leading to liver and spleen swelling, seriously affecting the central nervous system, and even resulting in death (Morales et al. 2007). Gossyrubilone and saxagliptin were the main DEMs in the metabolomic profiling analysis of both the androgenic gland and testis, which may play essential roles in the mechanism of male sexual development. Gossyrubilone is a dark red pigment, which is the isopentylimine of hemigossypolone and isolated from

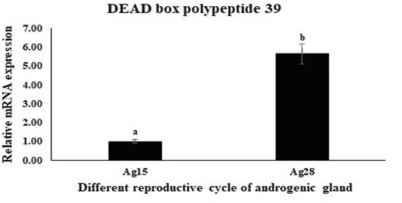

proteasome alpha 3
Relative mRNA expression
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
a
b
Ag15
Ag28
Different reproductive cycle of androgenic gland

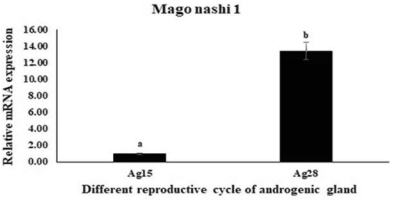

proteasome subunit beta type-5
Relative mRNA expression
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
a
b
Ag15
Ag28
Different reproductive cycle of androgenic gland

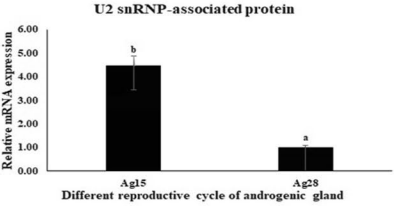

Nucleolar protein 56
Relative mRNA expression
6.00
5.00
4.00
3.00
2.00
1.00
0.00
a
b
Ag15
Ag28
Different reproductive cycle of androgenic gland

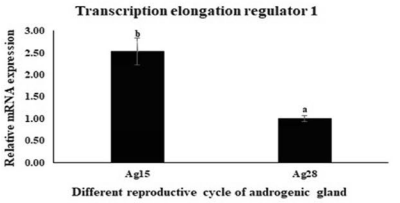

5'-3' exoribonuclease 1
Relative mRNA expression
6.00
5.00
4.00
3.00
2.00
1.00
0.00
a
b
Ag15
Ag28
Different reproductive cycle of androgenic gland

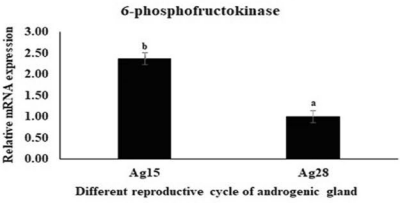

RNA exonuclease 1 homolog
Relative mRNA expression
25.00
20.00
15.00
10.00
5.00
0.00
b
a
Ag15
Ag28
Different reproductive cycle of androgenic gland

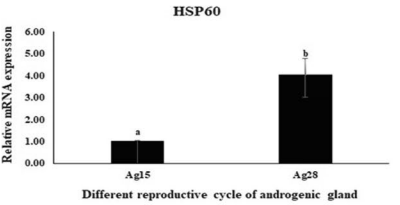

DEAD box polypeptide 39
Relative mRNA expression
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
a
b
Ag15
Ag28
Different reproductive cycle of androgenic gland

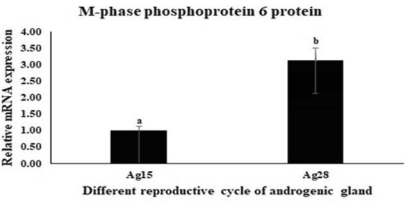

Mago nashi 1
Relative mRNA expression
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
a
b
Ag15
Ag28
Different reproductive cycle of androgenic gland

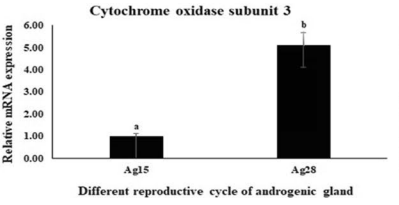

U2 snRNP-associated protein
Relative mRNA expression
6.00
5.00
4.00
3.00
2.00
1.00
0.00
b
a
Ag15
Ag28
Different reproductive cycle of androgenic gland


Transcription elongation regulator 1
Relative mRNA expression
3.00
2.50
2.00
1.50
1.00
0.50
0.00
b
a
Ag15
Ag28
Different reproductive cycle of androgenic gland


6-phosphofructokinase
Relative mRNA expression
3.00
2.50
2.00
1.50
1.00
0.50
0.00
b
a
Ag15
Ag28
Different reproductive cycle of androgenic gland


HSP60
Relative mRNA expression
6.00
5.00
4.00
3.00
2.00
1.00
0.00
a
b
Ag15
Ag28
Different reproductive cycle of androgenic gland


M-phase phosphoprotein 6 protein
Relative mRNA expression
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
a
b
Ag15
Ag28
Different reproductive cycle of androgenic gland


Cytochrome oxidase subunit 3
Relative mRNA expression
6.00
5.00
4.00
3.00
2.00
1.00
0.00
a
b
Ag15
Ag28
Different reproductive cycle of androgenic gland

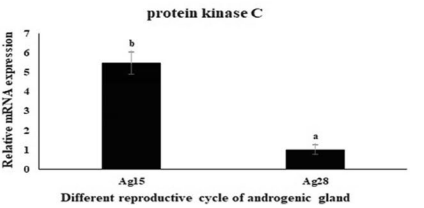
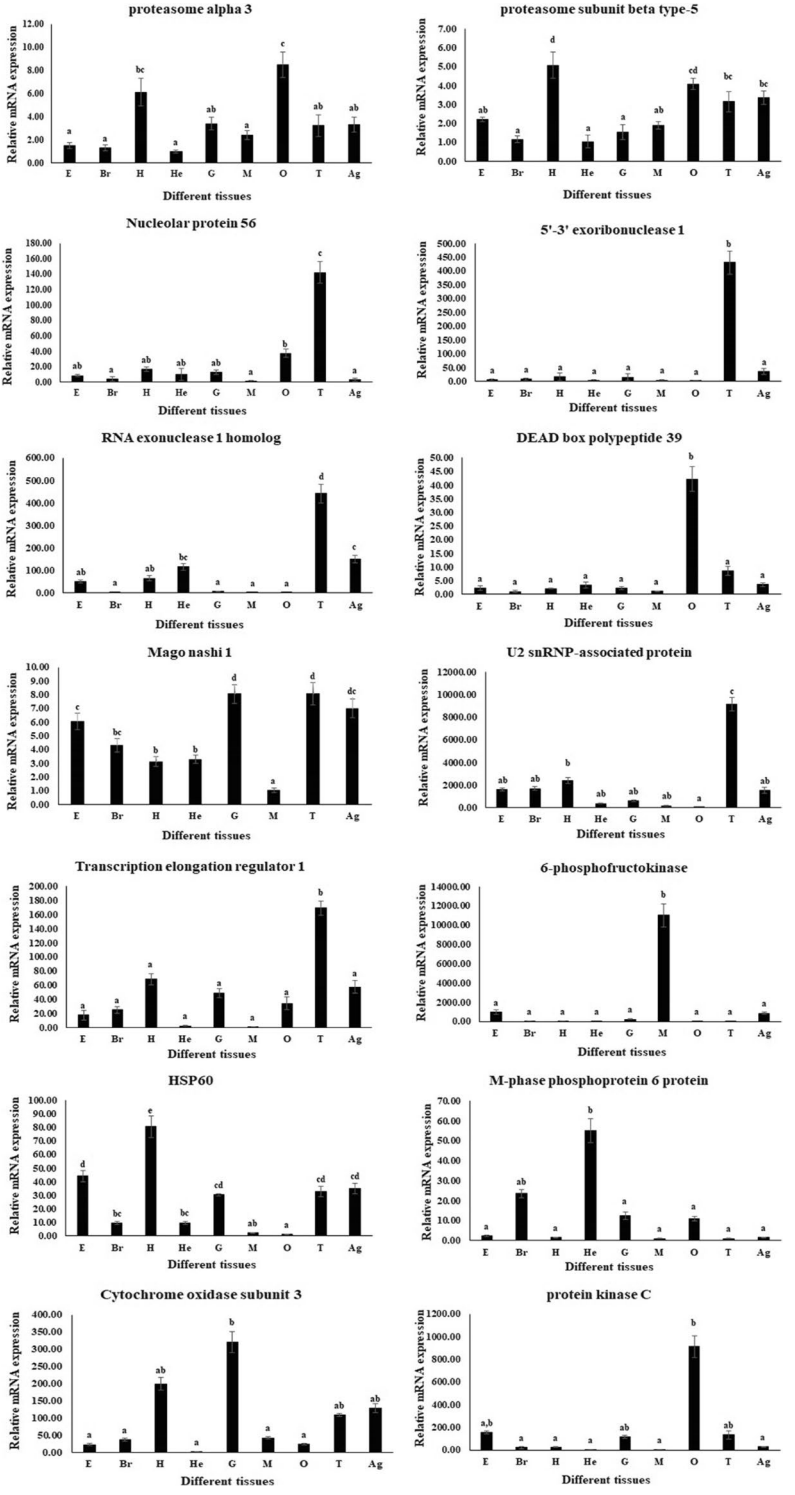

protein kinase C
Relative mRNA expression
7
6
5
4
3
2
1
0
b
a
Ag15
Ag28
Different reproductive cycle of androgenic gland

Fig. 7 Identification of the expressions of 14 DEGs in different tissues by qPCR. The amounts of DEGs ▶ expression were normalized to the EIF transcript level. Data are shown as mean ± SD (standard deviation) of tissues in three separate individuals. Lowercase indicate expression difference of DEGs in different tissues

glands of young leaves of Gossypium (Bell et al. 1978). Saxagliptin was reported to be involved in the treatment of diabetes (Defronzo et al. 2009; Rosenstock et al. 2009).

In this study, a total of 36,717 non-redundant transcripts were assembled, providing valuable data for further studies of male sexual development in *M. nipponense*. Cellular process, cell, cell part, and binding were the main functional groups in GO analysis, and general function prediction only, signal transduction mechanisms, secondary metabolite synthesis, transport and catabolism, and function unknown were the main functional groups identified in COG analysis, which were consistent with previous studies on male sexual development in *M. nipponense* (Jin et al. 2020, 2021b, c). This indicates that these functional groups may consist of male sexual development-related genes, promoting further studies on male sexual development in *M. nipponense*. A total of 9734 unigenes were differentially expressed in the androgenic gland between the reproductive *vs.* non-reproductive seasons. Phagosome, spliceosome, ribosome biogenesis in eukaryotes, RNA degradation, and oxidative phosphorylation were the main enriched metabolic pathways of DEGs. Phagosome, spliceosome, and oxidative phosphorylation were also reported to be the main enriched metabolic pathways of DEGs in the testis between the reproductive *vs.* non-reproductive seasons (Jin et al. 2020). Glycerophospholipid metabolism and sphingolipid metabolism were also the main enriched metabolic pathways of DEMs in both the androgenic gland and testis between the reproductive *vs.* non-reproductive seasons. These results imply that the development of the androgenic gland has some regulatory relationship with testis development in *M. nipponense*, and this is consistent with previous reports which showed that the androgenic gland and its secreted hormones promote the development of male secondary characterization in crustacean species (Rosen et al. 2010; Ventura et al. 2009, 2011). The expression differences of 14 DEGs between the reproductive *vs.* non-reproductive seasons were verified by qPCR and showed the same expression pattern with those of RNA-Seq, indicating the accuracy of RNA-Seq.

Spliceosome and phagosome were the most enriched metabolic pathways, and RNA degradation was a main metabolic pathway in the transcriptome profiling analysis of the androgenic gland between the reproductive *vs.* non-reproductive seasons in *M. nipponense*. Spliceosome plays essential roles in the splicing of the coding regions of precursor messenger RNA (pre-mRNA). Spliceosome plays functions by combining distant regions of pre-mRNA with spliceosomal snRNAs and catalytic proteins (Will and Lührmann 2011). Phagocytosis plays essential roles in the tissue remodeling, inflammation, and defense against infectious agents. Phagocytosis is the process where large particles are absorbed by a cell (Fu and Tom 1990). A phagosome is formed through combination of the specific receptors on the phagocyte surface and ligands on the particle surface. Most bacteria will be killed and degraded into fragments by toxic products, released through the fusion of phagosomes and lysosomes (Hampton et al. 1998). RNA degradation plays essential roles in the degradation of redundant RNA in animals. It is widely acknowledged that cells transcribe more RNA than they accumulate. This implies that RNA degradation systems exist. RNA is degraded at the end of its useful life. This process is dramatically long for a ribosomal RNA, but very short when the introns are excised. The surveillance mechanisms are rapidly active to degrade the RNA molecules with defects in processing, folding, or


proteasome alpha 3
Relative mRNA expression
Different tissues
E Br H He G M O T Ag
proteasome subunit beta type-5
Nucleolar protein 56
5'-3' exoribonuclease 1
RNA exonuclease 1 homolog
DEAD box polypeptide 39
Mago nashi 1
U2 snRNP-associated protein
Transcription elongation regulator 1
6-phosphofructokinase
HSP60
M-phase phosphoprotein 6 protein
Cytochrome oxidase subunit 3
protein kinase C

assembly with proteins. RNA degradation must be carefully controlled to accurately recognize target RNAs, as it is ubiquitous in all cells (Houseley and Tollervey 2009; Lacava et al. 2005). A reasonable explanation for the main enrichment of DEGs in the immune-related metabolic pathways is that the development of the androgenic gland is more active in the reproductive season than that of non-reproductive season. Thus, the aged or redundant cells need to be digested, and the new cells need to be differentiated, in order to maintain normal gonad development. Many important DEGs were selected from the metabolic pathways of spliceosome, and RNA degradation, based on the gene annotation and fold changes of the gene expression, which may play essential roles in promoting the reproductive capacity in the reproductive season. U2 snRNP-associated protein and *TCERG1* were vital DEGs from the metabolic pathway of spliceosome, which were upregulated in the reproductive season. The excision of introns from nuclear pre-mRNA requires assembly of the spliceosome from small nuclear ribonucleoprotein particles (snRNPs) (Guthrie 1991). A large number of the snRNPs have been identified to be required for pre-mRNA splicing in yeast through biochemical and molecular analyses (Beggs 1993; Ruby and Abelson 1991; Vijayraghavan et al. 1989). These snRNPs included U1, U2, U4, U5, and U6. The mutually exclusive U2-U6 snRNA interaction is necessary for catalytic activation. *TCERG1* was reported to play essential roles in transcriptional elongation and alternative splicing of pre-mRNAs (Goldstrohm et al. 2001; Sánchez-Alvarez et al. 2006). Increasing evidence indicates that *TCERG1* has potential roles in the coupling between transcription and splicing. The process of alternative pre-mRNA splicing of several genes was proven to be affected by *TCERG1*, including β-globin, β-tropomyosin, CD44, and fibronectin splicing reporters (Cheng et al. 2007; Lin et al. 2004; Pearson et al. 2008; Sánchez-Alvarez et al. 2010). *TCERG1* also has regulatory effects on the alternative splicing of putative cellular targets, as seen through microarray analysis following TCERG1 knockdown (Pearson et al. 2008). *TCERG1* regulates HIV-1 transcription by increasing the rate of RNAPII elongation through the phosphorylation of serine 2 (Coiras et al. 2013). 6-phosphofructokinase is a vital DEG from the metabolic pathway of RNA degradation, which was upregulated in the reproductive season. 6-phosphofructokinase plays essential roles in controlling glycolysis and respiration in plants (Dixon and ap Rees 1980; Kobr and Beevers 1971; Ruffner and Hawker 1977). Citrate inhibits the activities of phosphofructokinase and could be responsible for the decreased rate of glycolysis in different animals across the animal kingdom.

Ribosomes play vital roles in making proteins and are responsible for the ribosomal biogenesis of the production and correct assembly of four rRNAs and 80 ribosomal proteins in eukaryotes. Ribosomal biogenesis requires hundreds of factors, which were not present in the mature particles. Ribosome biogenesis is stalled, and cell growth is terminated even under optimal growth conditions, when these proteins are absent. *NOL56*, 5′-3′ exoribonuclease 1, and RNA exonuclease 1 homolog are the most important DEGs from the metabolic pathway of ribosome biogenesis in eukaryotes. Nucleolar proteins have a broad range of basic biological processes, including organ morphogenesis, growth, differentiation, homeostasis, and neoplasia. The biological functions of several nucleolar proteins have been identified. Rbm19 is a nucleolar protein, which is essential for the production of the 18S ribosomal RNA during ribosome biogenesis in *C. tentans* and *C. elegans* (Bjork et al. 2002; Jin et al. 2002; Saijou et al. 2004). The nucleolar protein 4-like gene is expressed in multiple organs in zebrafish embryos, implying that it plays important roles in the process of embryogenesis in zebrafish (Supriya et al. 2015). RNA exonuclease is an exonuclease of ribonucleic acid (RNA), which is an enzyme, playing essential roles in degrading RNA and removing nucleotides at the 5′ or 3′ end (Moser et al. 1998). 5′-3′ exoribonuclease 1 was reported to promote higher amounts of essentially full-length mRNAs.

5′-3′ exoribonuclease 1 lacking 5′-3′ exoribonuclease 1 in yeast cells showed poly(A) deficient and partially lacked the 5′ cap structure (Hsu and Stevens 1993). RNA exonuclease 1 homolog plays vital roles in the maintenance of stress response signaling and electrotaxis behavior in many animals (Taylor et al. 2021). In addition, in vitro culture revealed that the expression of RNA exonuclease homolog 1 transcript was primarily restricted in undifferentiated epiblasts (Blomberg et al. 2007).

Adenosine-triphosphate (ATP) is a high-energy compound, which is used as an energy source in nearly all metabolic activities, including male sexual development, especially that for the testis development. Many previous studies indicated that rapid gonad development has negative effects on the growth performance of aquatic species. Oxidative phosphorylation was the main enriched metabolic pathway in this study. Interestingly, a previous study also reported that oxidative phosphorylation was the main enriched metabolic pathway in the transcriptome profiling analysis of testis between the reproductive *vs.* non-reproductive seasons (Jin et al. 2020). Oxidative phosphorylation occurs in the cytoplasm of prokaryotes or in the inner membrane of the mitochondria of eukaryotic cells. The energy released from the oxidation of substances in vivo, which has positive effects on the coupling reaction between inorganic phosphate and adenosine diphosphate. ATP is synthesized through the respiratory chain (Dimroth et al. 2000). A reasonable explanation for this is that oxidative phosphorylation and glycolysis/gluconeogenesis provides energy for the sexual development of male *M. nipponense* during the reproductive season. Cytochrome oxidase subunit 3 and protein kinase C were selected from the metabolic pathway of oxidative phosphorylation. Cytochrome oxidase has significant regulatory roles in aerobic life, which blends the properties of several other metalloproteins, playing essential roles in the functions of either transport or redox. The preparations of cytochrome oxidase normally contain a variety of phospholipids, as well as some copper and iron (Lunt et al. 2019; Sunnucks and Hales 1996). Protein kinase C (*PKC*) is a family of serine/threonine protein kinases. PKCs have functions on their substrates at serine or threonine residues through phosphorylation. PKCs were found to play essential roles in controlling the function of other proteins and in several signal transduction cascades. PKC enzymes are activated by the increase of the concentration of DAG or calcium ions (Ca^{2+}) (Good et al. 1998; Inoguchi et al. 2000).

The qPCR analysis in different mature tissues revealed that 6 out of 14 DEGs showed the highest expression levels in the testis and/or androgenic gland in *M. nipponense*. These 6 DEGs included *NOL56*, 5′-3′ exoribonuclease 1, RNA exonuclease 1 homolog, Mago nashi 1, U2 snRNP-associated protein, and *TCERG1*. Among these 6 DEGs, the expression levels of *NOL56*, 5′-3′ exoribonuclease 1, U2 snRNP-associated protein, and *TCERG1* were highest in the testis, while RNA exonuclease 1 homolog and Mago nashi 1 showed high expression in both the testis and androgenic gland. This indicated that these 6 DEGs can be considered as strong candidate sex-related genes, which may be involved in the mechanism of male sexual differentiation and development in *M. nipponense*. In addition, *HSP60* and cytochrome oxidase subunit 3 also showed dramatically high expression levels in the testis and androgenic gland, suggesting their potential roles in male sexual differentiation and development.

In conclusion, the present study identified the metabolites and genes which may play essential roles in sexual differentiation and development in male *M. nipponense*. This was done the integrated analysis of transcriptomic and metabolomic analysis of the androgenic gland between the reproductive *vs.* non-reproductive seasons. Glycerophospholipid metabolism and sphingolipid metabolism were the main metabolic pathways of DEMs, and phagosome, spliceosome, and oxidative phosphorylation represented the main enriched metabolic pathways of DEGs in this study, which was consistent with that of metabolomic

analysis in the testis during the non-reproductive *vs.* reproductive seasons. This suggested that the androgenic gland plays a regulatory role in testis development. Eight genes were identified as strong candidate of sex-related genes, promoting the process of male sexual differentiation and development of *M. nipponense*, according to qPCR analysis in various mature tissues, which showed the highest expression levels in testis and/or androgenic gland. Overall, the important metabolites and genes were selected from the androgenic gland of *M. nipponense*, providing valuable evidences for further studies on the mechanism of male sexual development in *M. nipponense* and other crustacean species.

Author contribution S.J. designed and wrote the manuscript. W.Z. performed the qPCR analysis. S.J. and Y.X. provided the experimental prawns. H.Q. selected the differentially expressed metabolites. Y.G. selected the differentially expressed genes. Y.W. performed the histological observations. H.F. supervised the experiment.

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Data availability The reads of *M. nipponense* transcriptome were submitted to NCBI with the accession number of SRX5805440-SRX5805445. The data of *M. nipponense* were submitted to MetaboLights with the accession number of MTBLS1025.

Code availability Not applicable.

Declarations

Ethics approval Permission was obtained from the Tai Lake Fishery Management Council and the committee of Freshwater Fisheries Research Center during the experimental programs. All experiments were performed in accordance with relevant guidelines and regulations.

Conflict of interest The authors declare no competing interests.

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