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iTRAQ-based quantitative proteomic analysis of the androgenic glands of the oriental river prawn, *Macrobrachium nipponense*, during nonreproductive and reproductive seasons

Shubo Jin, Hongtuo Fu*, Shengming Sun, Sufei Jiang, Yiwei Xiong, Yongsheng Gong, Hui Qiao, Wenyi Zhang, Yan Wu

Key Laboratory of Freshwater Fisheries and Germplasm Resources Utilization, Ministry of Agriculture, Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences, Wuxi 214081, PR China

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ABSTRACT

The androgenic gland produces hormones that play crucial roles in driving male sexual development. In this study, we investigated the regulatory proteins and pathways affecting male sexual development of *M. nipponense*, by analyzing the proteomes of their androgenic glands during their reproductive and nonreproductive seasons using isobaric tags for relative and absolute quantitation (iTRAQ). A total of 13 sex-related protein homologs out of 999 total proteins were identified in the proteome of *M. nipponense* based on comparisons with the published literature. A total of 32 proteins were differentially expressed in the androgenic gland between the reproductive and nonreproductive seasons of *M. nipponense*. However, only 10 differentially expressed proteins (DEPs) were annotated in the Nr, COG, GO, and KEGG databases. Other non-annotated DEPs may also play vital roles in the male sexual development of *M. nipponense*. The qPCR analysis indicated that the mRNA expression patterns of the 10 annotated DEPs were consistent with that determined by iTRAQ analysis. Additional qPCR analysis of the 10 DEPs in testes, ovaries, and androgenic glands showed that Gem, Ferritin, and Rev3 were highly expressed in the androgenic gland, implying importance in male sexual development, given the important role of the androgenic gland in male differentiation and development in many crustacean species. Our study provided valuable information about DEPs in androgenic glands between reproductive and nonreproductive seasons for *M. nipponense* and identified their potential roles in male sexual development, which will improve our understanding of the development process in both *M. nipponense* and other crustaceans.

1. Introduction

The oriental river prawn, *Macrobrachium nipponense* (Crustacea; Decapoda; Palaemonidae), is a commercially important species with an annual aquaculture production of 205,010 tons (Bureau of Fishery, Ministry of Agriculture, P.R.C., 2016). *M. nipponense* are widely distributed in freshwater and low-salinity estuarine regions of China and other Asian countries (Yu and Miyake, 1972; Cai and Shokita, 2006; Grave and Ghane, 2006; Salman et al., 2006; Ma et al., 2011). Like many other members of the *Macrobrachium* genus, male prawns of *M. nipponense* grow faster than their female counterparts (Yu and Miyake, 1972; Ma et al., 2011). Thus, the farming of all male populations is expected for *M. nipponense* aquaculture. Therefore, it is of great importance to establish artificial sex differentiation techniques that can be used to produce all male progeny on a commercial scale. In order to accomplish this goal, a full understanding of the sex differentiation and determination mechanisms of *M. nipponense* is needed.

The androgenic gland in most crustaceans produces hormones that play a crucial role in driving male sexual differentiation, the development of the testes, and the establishment of male sexual characteristics (Sagi et al., 1990). In *Macrobrachium rosenbergii*, male prawns undergo sex reversal to a female phenotype after ablation of the androgenic gland. All male progeny was generated when the “neo-females” were mated with normal male *M. rosenbergii* (Sagi et al., 1986; Sagi et al., 1990; Sagi and Cohen, 1990). Ablation or implantation of the androgenic gland at specific stages of development can result in sex reversal to either male or female (Sagi et al., 1990), and therefore, studies on crustacean androgenic glands have received much attention in recent years. A full understanding of the proteins in the androgenic gland, especially those playing essential roles in male differentiation and development, is urgently needed. The androgenic gland transcriptome and miRNA library have both been constructed for *M. nipponense* (Jin

* Corresponding author at: Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences, 9 Shanshui East Road, Wuxi 214081, Jiangsu Province, PR China.
E-mail address: fuht@ffrc.cn (H. Fu).

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et al., 2013; Jin et al., 2014), while other non-androgenic gland transcriptomes and miRNA libraries have also been constructed in *M. nipponense*. A series of sex-related genes and miRNAs were identified from these transcriptomes and miRNA libraries (Wu et al., 2009; Ma et al., 2012; Qiao et al., 2012). However, to the best of our knowledge, no previous studies were focused on the analysis of the androgenic gland proteome changes in any crustacean species.

Isobaric tagging for relative and absolute quantitation (iTRAQ) profiling is a reliable and accurate method of measuring protein expression, which is especially useful in annotated, related species. The iTRAQ uses a variety of isotopic reagents to label the N-terminus of a protein or peptide, as well as the groups of lysine side chains and can be used to simultaneously examine the proteomic profiles of different samples (Pierce et al., 2008) via high precision mass spectrometry (Ross et al., 2004). Only a small amount of protein is required for quantification. This new approach avoids some of the drawbacks of 2-dimensional gel electrophoresis (2-DE), including the identification of relatively fewer proteins, difficulties in quantifying less abundant proteins, and relatively complicated procedures (Gao et al., 2017). The iTRAQ has been increasingly used for proteomic analyses in recent years (Brewis and Brennan, 2010; Unwin, 2010; Cha et al., 2012; Ren et al., 2016; Wang et al., 2016; Gao et al., 2017).

In this study, the proteomic changes in androgenic glands between the *M. nipponense* reproductive season and nonreproductive season were analyzed by iTRAQ. These data provided valuable evidence about the proteomic changes, regulatory proteins, and pathways that promote male sexual development in *M. nipponense*.

2. Materials and methods

2.1. Ethics statement

We got the permission from the Tai Lake Fishery Management Council. *M. nipponense* is not an endangered species in China, thus it can be used for experimental purpose. All of the experimental programs involved in this study were approved by the committee of Freshwater Fisheries Research Center, and followed the experimental principles. Tissues from each prawn individuals were sheared under MS222 anesthesia, and efforts were made to minimize stress.

2.2. Sample collection

Male specimens of oriental river prawn at nonreproductive season and reproductive season (body weights, 2.67–5.37 g) were respectively collected from a wild population in Tai Lake, Wuxi, China (120°13′44″E, 31°28′ 22″N) in winter and summer. All the samples were transferred to a 500 L tank and maintained in aerated freshwater. The specimens of nonreproductive season were stayed at 15 °C, and the specimens of reproductive season were stayed at 28 °C. The androgenic gland of nonreproductive season and reproductive season were respectively collected and immediately preserved in liquid nitrogen until used for protein extraction. A total of 30 specimens were used for each proteomic analysis, in order to ensure the sufficient amount of protein. The androgenic gland, testis and ovaries were collected at the reproductive season (28 °C) for Quantitative Real-time PCR (qPCR) analysis.

2.3. Protein preparation and iTRAQ labeling

We analyzed the protein expression profiles of androgenic gland between nonreproductive season and reproductive season in *M. nipponense*. At least 180 androgenic glands were dissected from each reproductive season. A total of 60 androgenic glands were pooled to formed one biological replicate to eliminate the effects of individual differences, and three biological replicates were performed for each reproductive season. The dissected androgenic glands were immediately placed into liquid nitrogen and stored at 80 °C for further analysis. Total proteins were extracted respectively from the three biological replicates of androgenic glands from nonreproductive season and reproductive season using a phenol extraction procedure (Brewis and Brennan, 2010; Unwin, 2010). In a typical procedure, frozen androgenic glands were ground well to powder with a cold mortar and pestle, and kept in a liquid nitrogen bath in a styrofoam box during the entire procedure. The protein concentrations were determined using the Bradford colorimetric method (Treitz et al., 2015). A total of 100 μg of protein from each sample was lyophilized using a speed vacuum system (Martin Christ, Germany).

iTRAQ labeling was performed using an iTRAQ Reagent 8-Plex kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol (Gao et al., 2017; Hou et al., 2016; Jeswin et al., 2016). A total of 100 μg extracted protein from each sample was dissolved in 40 μL 0.5 M triethylammonium bicarbonate, incubated with 10 mL 1 mg/mL trypsin (Promega, Madison, WI, USA) at 37 °C for 16 h, and labeled using iTRAQ Reagent 8-Plex kit.

Table 1
Primers used in this study.

Protein	Sequence
Pre-F1	CCTAACTCTGCTCTCGCCTTT
Pre-R1	TTCAATGCAGGCCCGTGAATA
Root-F1	CTCTGAAGCAAAGCATGCCG
Root-R1	ACTCCTGACTACGACCCACA
Npa-F1	CGGGGTTCACTTACTGCTGA
Npa-R1	TCTCCCGCAACTCATCAACC
Sur-F1	AGGATTCATCAGCCTCCCCTA
Sur-R1	GCACTGTGCGTTGCATCTTTA
Gem-F1	ATGCCCAGTGGCTAGAAGTG
Gem-R1	GCAGAATCCAGCGATGCTCT
Dna-F1	ATTCCTCCACACCTGAAGCC
Dna-R1	GCTGACGCAGTAGCAACATC
Gen-F1	ATGCGACAGAAATGGGGTGA
Gen-R1	CCACTTGCTGGGACATGTGA
Fer-F1	CCGAAATCCGCCAGAACTAC
Fer-R1	GCTTATCGGCATGCTCTCTC
Tro-F1	TCCTCAACACGTTAGGGCAG
Tro-R1	CCATCGCCTCCTTTGTCGTA
Rev3-F1	AGTGACAGCAACGCTAGTGG
Rev3-R1	GGCCAAACAACTCTGTCAGC

2.4. Strong cation exchange chromatography (SCX) fractionation

The iTRAQ-labeled samples were then pooled and purified using a SCX column (Phenomenex, Torrance, CA, USA), and followed by separating using a liquid chromatography-20AB (LC-20AB) HPLC system (Shimadzu, Kyoto, Japan). The peptide mixtures were reconstituted with 4 mL buffer A (25 mM NaH_2PO_4 in 25% ACN, pH 2.7). The reconstituted peptide mixtures were loaded onto a 4.6 × 250 mm Ultremex SCX column with 5 μm particles, and eluted at a flow rate of 1 mL/min with elution buffer B (25% v/v acetonitrile, 25 mM NaH_2PO_4, 1 M KCl, pH 2.7) for 7 min, 5%–60% buffer B for 20 min, and finally 60%–100% buffer B for 2 min, and maintained for 1 min. The absorbance at 214 nm was monitored and 12 fractions were collected. Samples of each fraction were dried and desalted before LC-electrospray ionization-MS/MS analysis (Liu et al., 2015).

2.5. LC-MS/MS analysis

Each dried sample was reconstituted to an average of 0.5 μg/μL using buffer A (5% ACN, 0.1% FA), and followed by centrifuging at 20,000 × *g* for 10 min to remove impurities. A total of 2.5 μg protein was separated using a LC-20AD HPLC system (Shimadzu, Kyoto, Japan). A flow rate of 8 μL/min was used to load the sample onto a Trap column at within 4 min, and then transferred to an analysis column at a