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Molecular cloning and expression pattern of oriental river prawn (*Macrobrachium nipponense*) nitric oxide synthase

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ABSTRACT. Nitric oxide synthase (NOS) produces nitric oxide (NO) by catalyzing the conversion of L-arginine to L-citrulline, with the concomitant oxidation of nicotinamide adenine dinucleotide phosphate. Recently, various studies have verified the importance of NOS invertebrates and invertebrates. However, the *NOS* gene family in the oriental river prawn *Macrobrachium nipponense* is poorly understood. In this study, we cloned the full-length *NOS* complementary DNA from *M. nipponense* (*MnNOS*) and characterized its expression pattern in different tissues and at different developmental stages.

Real-time quantitative polymerase chain reaction (RT-qPCR) showed the *MnNOS* gene to be expressed in all investigated tissues, with the highest levels observed in the androgenic gland ($P < 0.05$). Our results revealed that the *MnNOS* gene may play a key role in *M. nipponense* male sexual differentiation. Moreover, RT-qPCR revealed that *MnNOS* mRNA expression was significantly increased in post-larvae 10 days after metamorphosis ($P < 0.05$). The expression of this gene in various tissues indicates that it may perform versatile biological functions in *M. nipponense*.

Key words: *Macrobrachium nipponense*; Nitric oxide synthase; Cloning; Expression pattern; RT-qPCR; Post-larvae

INTRODUCTION

Nitric oxide synthase (NOS) catalyzes nitric oxide (NO) biosynthesis via the conversion of L-arginine to L-citrulline, with the concomitant oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) (Moncada et al., 1991). Kubes et al. (1991) described the multiple roles of NOS, including in the regulation of vasomotor tone, cell adhesion to the endothelium, inhibition of platelet aggregation (Radomski et al., 1991), and vascular smooth muscle cell proliferation. In response to pathological stimuli, NOS stimulates macrophages, Kupffer cells, neutrophils, fibroblasts, vascular smooth muscle, and endothelial cells (Eddy, 2005). Arginine-derived NO synthesis has been observed in mammals, fish, birds, invertebrates, plants, and bacteria (Liu and Gross, 1996). Mammalian NOS (EC 1.14.13.39) has been extensively studied, and is known to consist of three isoforms: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS; Knowles and Moncada, 1994). These isoforms differ in their structure, occurrence, and domain architecture, thus each displays distinctive features and performs specific functions *in vivo* (Knowles and Moncada, 1994). However, with the remarkable exception of Gram-negative bacteria, bacterial NOS enzymes lack reductase domains and require a supply of suitable reductants to produce NO (Sudhamsu and Crane, 2009). NOS has been comprehensively studied in vertebrates (Bogdan, 2001). To date, few crustacean NOS proteins have been biochemically characterized (Wu et al., 2013), although some investigations of the multiple functions of these enzymes in invertebrates have been published. In vertebrates, NOS is involved in several disparate processes, and this may also be the case in invertebrates (Labbé et al., 2009). NOS has been cloned and characterized in crab (McDonald et al., 2011), shrimp (Yao et al., 2010), and lobster (Rodríguez-Ramos et al., 2010). An earlier study showed that NOS in rats is a mediator in the female and male reproductive tracts (Rosselli et al., 1995). In rat males, androgen influences NOS activity in any tissue (Garban et al., 1995). Whereas, in female rat NO plays an important role in inducing ovulation and in causing luteolysis (McCann and Rettori, 1996). In addition, Buhimschi et al. (1996) suggested that NO appears to have an important role in the maintenance of sperm motility. Thus, it may be useful as another indicator of fertility potential. However, no reports yet exist concerning the function and specific role of NOS in the formation of invertebrate sex organs in general, and in *Macrobrachium nipponense* in particular. Most studies of crustacean NOS have focused on its relationship to the immune system. Therefore, in the present study, we investigated *NOS* in *M. nipponense* to assess its influence on sex determination and reproductive mechanisms.

The androgenic gland (AG) is an endocrine organ exclusive to male crustaceans and present in most such species, including the blue swimming crab *Callinectes sapidus* (Cronin, 1947). The successful removal of gonads and implantation of those of the opposite sex, resulting in the feminization or masculinization of the manipulated animal was demonstrated in amphipods in the 1950s. Subsequently, the AG has been confirmed as having a key role in crustacean sexual differentiation, determining male primary and secondary sexual characteristics (Abdu et al., 2002).

M. nipponense (Crustacea; Decapoda; Palaemonidae), more commonly known as the oriental river prawn, is a commercial freshwater prawn broadly distributed across different countries especially Asian ones, including China (Cai and Ng, 2002). *M. nipponense* is one of the main species cultured by Chinese inland fisheries and is of significant economic importance (Fu et al., 2012). The annual production of farmed oriental river prawn approximates 20 billion tons (Bureau of Fisheries, Ministry of Agriculture, 2014). Although previous studies have examined *M. nipponense* testes complementary DNA (cDNA) libraries and the AG transcriptome (Qiao et al., 2012; Jin et al., 2013), insufficient information regarding sex determination and differentiation-related genes is available for this species. It is known that males grow faster and accumulate more weight by the time of harvesting compared to females in many *Macrobrachium* species, including *M. nipponense* (Jin et al., 2013). In addition, sexual precocity (i.e., early sexual maturity) has become problem at cultured stocks, resulting in a reduction in market value (Qiao et al., 2012). Therefore, establishing effective ways to produce all-male populations through elucidation of the genetic mechanism behind *M. nipponense* development is vital to close the aquaculture industry production gap. Currently, male sexual differentiation-related genes in this organism are poorly understood and require further study to clarify the means by which *M. nipponense* sex is determined. Jin et al. (2013) identified *NOS* as a novel candidate gene and significantly expressed in the AG. Moreover, these authors suggested that it may play an important role in the sex-determination mechanism of the oriental river prawn. No reports have yet been published describing the cloning and expression profiling of AG *NOS* for the investigation of its potential role in *M. nipponense* sex determination. Therefore, in the present study, we cloned the full-length open reading frame (ORF) of *NOS* from the *M. nipponense* AG transcriptome, and characterized its expression profile in various tissues and at different developmental stages using real-time quantitative polymerase chain reaction (RT-qPCR). The highest level of *NOS* expression was found in the AG, and at 10 days post-larvae (PL), the critical organ formation stage. The findings presented here provide insight into our fundamental understanding of *NOS* as a sex differentiation gene in *M. nipponense* and other crustaceans, and will be useful in developing a strategy for sex control in *M. nipponense* aquaculture.

MATERIAL AND METHODS

Experimental animals and tissue collection

Healthy prawns (3.5-4.5 g) were purchased from Lake Tai in Wuxi, China (120°13'44"E, 31°28'22"N). Adults were kept under laboratory breeding conditions at 23°C and acclimated to the laboratory facility for 3 days in a 500-L tank containing aerated freshwater. For *NOS* cloning and tissue-specific expression measurements, various adult tissues, including those of the intestine, hepatopancreas, muscle, brain, gill, heart, eyestalk, testis, ovary, nerve cord, and AG were collected from at least 10 pieces of prawn, each with a wet weight of 2.3-

4.6g, and immediately preserved in liquid nitrogen before being stored at -80°C prior to RNA extraction. Prawns at different developmental phases, including five embryonic, four larval, and four PL stages, were collected from the genetic breeding laboratory of the Freshwater Fisheries Research Center breeding room. After the spawning of gravid females, prawns of each embryonic stage (cleavage, blastula, gastrula pre-nauplius, pre-zoea), four larval stages (larvae were collected every 3 days), and four PL stages (PL were collected every 5 days from day 1-15) were collected and examined using an Olympus (Tokyo, Japan) SZX16 microscope. Developmental stage was determined based on morphological characters and the criteria of Chen et al. (2012). Samples were washed with 1X 0.01 M phosphate-buffered saline, placed in liquid nitrogen, and stored at -80°C until processing to preserve quality.

RNA extraction and cDNA synthesis

Total RNA was extracted from prawns of each developmental stage and the tissues of at least 10 adults using TRIzol reagent (Invitrogen, Waltham, MA, USA), and treated with RNase-free DNase I (TaKaRa, Dalian, China) following the manufacturer protocol, to denature any genomic DNA. Total RNA concentration and quality were determined by ultraviolet light absorbance at 260 nm (A_{260}), the A_{260}/A_{280} ratio, and denaturing agarose gel electrophoresis. First-strand cDNA was synthesized using a Moloney murine leukemia virus reverse transcriptase kit (TaKaRa, Kusatsu, Japan). The reverse transcribed cDNA was stored at -20°C until needed for RT-qPCR (Qiao et al., 2012).

NOS gene 5'- and 3'-random amplification of cDNA ends (RACE)

The *NOS* expressed sequence tag obtained from the *M. nipponense* AG transcriptome (Jin et al., 2013) was used to design four gene-specific primers (Table 1). The cloning of *M. nipponense NOS* (*MnNOS*) cDNA was achieved by 5' and 3'-RACE using the 5'- and 3' Full RACE Kit (TaKaRa) following the manufacturer protocols. The 5'-RACE primer sets consisted of the two gene-specific primers GSP1 and GSP2, and the universal primers 5' RACE OUT and 5' RACE INN (Table 1). For 3'-RACE, the two gene-specific primers GSP3 and GSP4, and the universal primers 3' RACE OUT and 3' RACE INN were used (Table 1). PCR fragments were subjected to electrophoresis on 1.2% agarose gels to compare product lengths based on the *de novo* transcriptome library sequences. Amplified cDNA fragments were inserted into the pMD18-T vector for cloning, and sequenced using M13 forward and reverse primers. The resulting sequences were confirmed and subjected to cluster analysis using BLASTn from National Center for Biotechnology Information (NCBI) tool.

Analyses of nucleotide and deduced amino acid sequences

Based on the BLASTnx algorithm, the sequence from the *M. nipponense* AG transcriptome was found to be highly homologous to members of the *NOS* gene family in *Penaeus monodon*, *Litopenaeus vannamei*, and *Marsupenaeus japonicus*. The ORF of this sequence was identified using the Translate tool (http://web.expasy.org/translate/) and the NCBI BLASTn and BLASTnx programs (http://www.ncbi.nlm.nih.gov/BLAST). Molecular weights (MWs) and isoelectric points (pIs) of deduced amino acid sequences were analyzed with the Compute pI/Mw tool (http://web.expasy.org/compute_pi/), while functional domain

analysis was performed using Genomic SMART (http://smart.embl.de/smart/set_mode.cgi?GENOMIC=1). Multiple sequence alignment was conducted with DNAMAN and MEGA 5.1 (Tamura et al., 2011), was used to generate a neighbor-joining (NJ) phylogenetic tree based on vertebrate and invertebrate amino acid sequences (Table 2).

Table 1. Nucleotide sequences of primers used for *Macrobrachium nipponense* nitric oxide synthase gene (*MnNOS*) cloning and expression analysis.

	Sequence (5' to 3')	Name
Primers for 5'-RACE PCR		
MnNOS 5' GSP primer 1	TCTTCTGGCACATCTTGGTGAA	GSP1
MnNOS 5' GSP primer 2	TTCAGATTCAGCTCCTCGAACC	GSP2
Primers for 3'-RACE PCR		
MnNOS 3' GSP primer 1	CGAGACAAGATCGAGCTGTTCGT	GSP3
MnNOS 3' GSP primer 2	GAAGATGTCCTCTATCTCTCAAG	GSP4
Full RACE Kit primers		
3' RACE OUT	ATCCGACGAAGACAAACTCTACC	
3' RACE IN	CGCGGATCCTCCACTAGTGATTTCACTATAGG	
5' RACE OUT	CTCCACAATGGGCTTTTTCATCC	
5' RACE IN	CGCGGATCCACAGCCTACTGATGATCAGTCGATG	
Primers for RT-qPCR analysis		
MnNOS F primer	TCACATGGAACAAACTGCATGTG	RT-F1
MnNOS R primer	CGTACCCGGCGAAGGCGATCAGC	RT-R1
β-Actin 5' primer	TATGCACTTCCTCATGCCATC	β-ActinF
β-Actin 3' primer	AGGAGGCGGCAGTGGTCAT	β-ActinR

RACE = random amplification of complementary DNA ends; PCR = polymerase chain reaction; RT-qPCR = real-time quantitative PCR; F = forward; R = reverse.

Table 2. Nitric oxide synthase (NOS) sequences from the National Center for Biotechnology Information used for multiple alignment, homology assessment, and phylogenetic analysis.

Species	Name/accession No.	Order/Family	Identity (%)
Limulus polyphemus	iNOS/XP_013781426.1	Xiphosura/Limulidae	34
Homo sapiens	nNOS/NP_001191147.1 eNOS/BAA05652.1 iNOS/NP_000616.3	Primates/Hominidae	30 33 34
Mus musculus	nNOS/NP_032738.1 eNOS/NP_032739.3 iNOS/NP_035057.1	Rodentia/Muridae	27 32 33
Danio rerio	nNOS/XP_005165110.1	Cypriniformes/Cyprinidae	29
Chrysochloris asiatica	iNOS/XP_006874299.1	Afrosoricida/Chrysochloridae	35
Myotis davidii	iNOS/ELK24792.1	Chiroptera/Vespertilionidae	32
Sus scrofa	iNOS/NP_001137162.1	Artiodactyla/Suidae	41
Physeter catodon	iNOS/XP_007109277.1	Cetartiodactyla/Physeteridae	30
Orcinus orca	iNOS/XP_012387409.1	Cetartiodactyla/Delphinidae	33
Carcinus maenas	NOS/ACY56317.1	Decapoda/Portunidae	41
Scylla paramamosain	NOS/CCC18661.1	Decapoda/Portunidae	44
Gecarcinus lateralis	NOS/AAT46681.1	Decapoda/Gecarcinidae	43
Panulirus argus	NOS/ACZ60615.1	Decapoda/Palinuridae	42
Marsupenaeus japonicus	NOS/BAI67609.1	Decapoda/Penaeidae	45
Litopenaeus vannamei	NOS/ADD63793.1	Decapoda/Penaeidae	45
Penaeus monodon	NOS/ACJ54486.1	Decapoda/Penaeidae	45
Fenneropenaeus chinensis	NOS/AFJ74715.1	Decapoda/Penaeidae	44
Halyomorpha halys	NOS/XP_014272116.1	Hemiptera/Pentatomidae	42
Drosophila melanogaster	NOS/NP_523541.2	Diptera/Drosophilidae	38
Apis mellifera	NOS/NP_001012980.1	Hymenoptera/Apidae	40
Bombus impatiens	NOS/XP_003485294.1	Hymenoptera/Apidae	39
Monomorium pharaonis	NOS/XP_012524269.1	Hymenoptera/Formicidae	40
Harpegnathos saltator	NOS/EFN84471.1	Hymenoptera/Formicidae	38
Macrobrachiumnipponense	NOS/KU382528	Decapoda/Palaemonidae	100

iNOS = inducible NOS; nNOS = neuronal NOS; eNOS = endothelial NOS.

Expression of *MnNOS* in different adult tissues and developmental stages

Transcription of the *MnNOS* gene in various embryonic and PL stages and several adult tissues was measured by SYBR Green RT-qPCR analysis using an iCycler iQ5 Real-Time System (Bio-Rad, Hercules, CA, USA), with the β-actin gene as an internal control. The specificity of the *MnNOS* and β-actin primers (Table 1) was verified by sequencing the PCR products. Three samples were collected for each developmental stage and tissue type, and each sample was analyzed in triplicate. Each 25-µL PCR comprised 1µL cDNA (50 ng), 10 µL SsoFast EvaGreen Supermix (Bio-Rad), 0.5 µL 10 µM gene-speciðc forward and reverse primers (Table 1), and 13 µL diethyl pyrocarbonate-treated water. The reaction mixture was initially incubated for 30 s at 95°C to activate the Hot Start *Taq* DNA polymerase, before 40 cycles of 10-s denaturation at 95°C and 10-s extensions at 60°C. Melting curve analysis was performed from 65°-95°C in 10-s0. 5°C increments to verify the amplification of a single product (Zhang et al., 2013).

Statistical analysis

The comparative threshold cycle (Ct) method ($2^{-\Delta\Delta Ct}$; Livak and Schmittgen, 2001) was used to analyze *MnNOS* expression, which is reported as mean relative mRNA level ± standard error. Data were subjected to one-way analysis of variance, and P values smaller than 0.05 were considered statistically significant.

RESULTS

Characterization of full-length *MnNOS*

The sequence obtained by 5'- and 3'-RACE was 3716 bp in length, representing the full-length *MnNOS* cDNA. It contained an ORF of 3447 bp, encoding a polypeptide of 1148 amino acids. The corresponding protein had an estimated MW of 129.209 kDa and a pI of 7.12. Untranslated regions of 130 and 139 bp were identified at the 5'- and 3'-end, respectively, and a complete poly (A) tail was present. The ORF begins with an ATG start codon and contains a TGA stop codon at its 3'-end (Figure 1). The cDNA sequence incorporating the full length *MnNOS* ORF has been deposited in GenBank under accession No. KU382528 (Figure 1). Homology searches using the predicted MnNOS amino acid sequence were performed using the BLASTp algorithm on the NCBI web site.

Prediction of MnNOS domains by SMART based on the deduced amino acid sequence revealed an NOS domain at positions 62 to 424. In addition, a flavodoxin 1 domain was found at positions 474 to 680, a flavin adenine dinucleotide (FAD)-binding domain at positions 776 to 945, and an NAD-binding domain at positions 977 to 1054 (Figure 1). The complete domain structure was compared with those of other NOS enzymes from different species to determine the degree of similarity across evolutionary distance. These domains were highly conserved among NOS homologs, from crustaceans to vertebrates, including humans and fish (Figure 2).

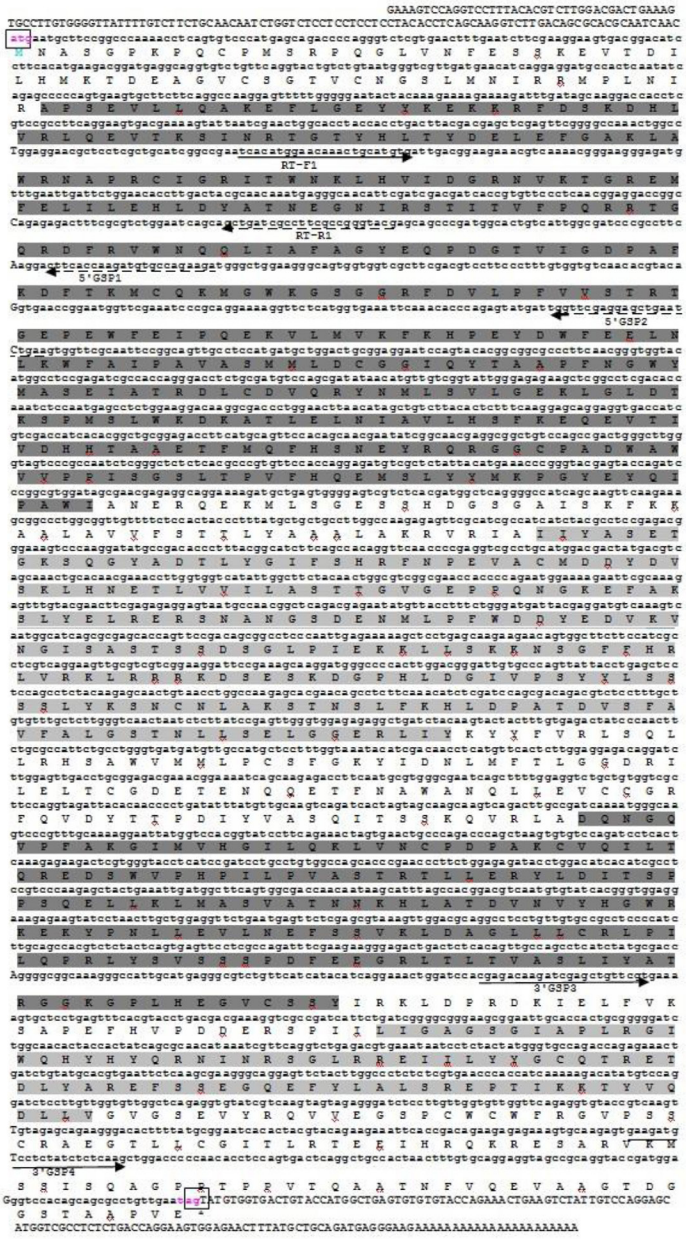

Figure 1. Complete nucleotide and deduced amino acid sequence of *Macrobrachium nipponense* nitric oxide synthase (NOS). Bold letters in boxes indicate start (**atg**) and stop (**tag**) codons and the polyadenylation signal sequence. NOS (amino acids 62-424), flavodoxin 1 (474-680), flavin adenine dinucleotide-binding (776-945), and nicotinamide adenine dinucleotide-binding (977-1054) domains are indicated (light gray highlighting). Primers used for real-time quantitative polymerase chain reaction are represented using broken lines and bold letters. Solid-line arrows and bold letters signify the locations and directions of the sequence-specific nested primers used for 5'- and 3'-RACE. This sequence has been deposited in GenBank under accession No. KU382528.

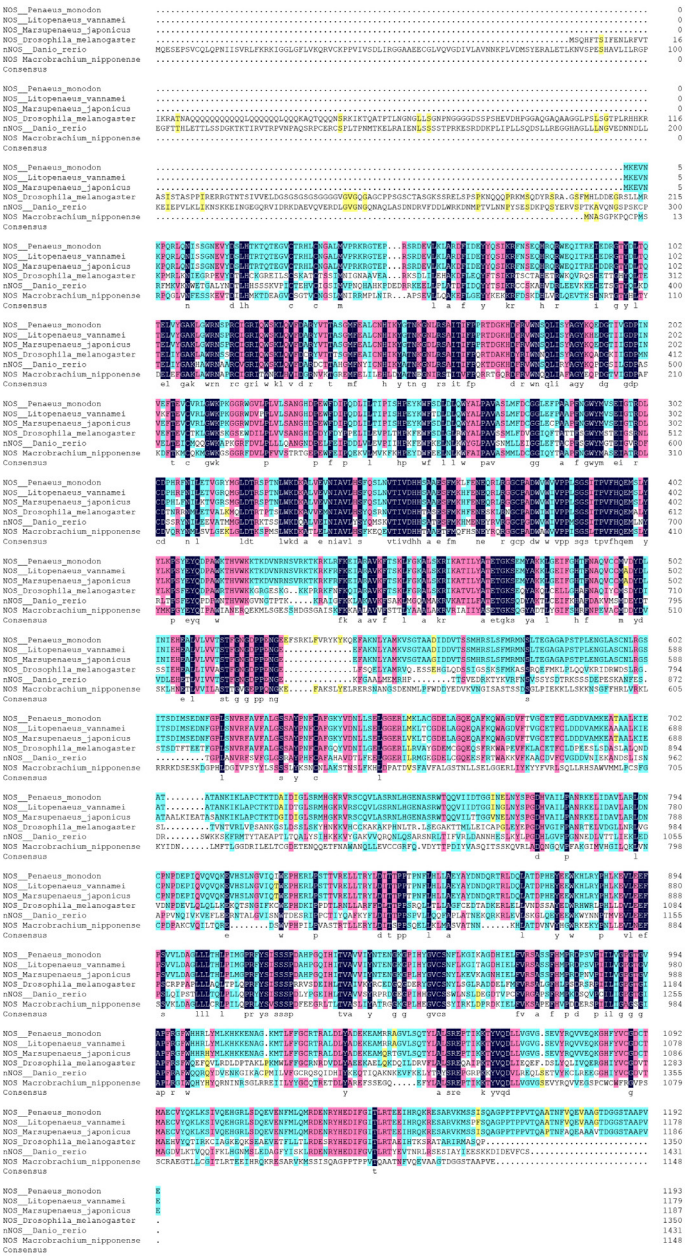

Figure 2. Alignment of the deduced nitric oxide synthase (NOS) amino acid sequence of *Macrobrachium nipponense* (accession No. KU382528) with those of other species, details of which are summarized in Table 2. Identical residues are highlighted using a dark background, while similar amino acids are indicated in pink. Insertions (…) were added to maximize sequence identity. NOS enzymes from *Penaeus monodon* (accession No. ACJ54486.1), *Litopenaeus vannamei* (ADD63793.1), *Marsupenaeus japonicus* (BAI67609.1), *Drosophila melanogaster* (NP_523541.2), and *Danio rerio* (XP_005165110.1) are included. Sequences were aligned using DNAMAN (Lynnon Biosoft, San Ramon, CA, USA).

Phylogenetic analysis of MnNOS

Twenty-eight NOS sequences from crustaceans, fish, and mammals were used to construct a phylogenetic tree. The deduced NOS amino acid sequence from *M. nipponense* demonstrated the closest phylogenetic relationship with those of other crustacean species. Inferred protein sequences of three types of invertebrate and vertebrate (nNOS, eNOS, and iNOS) were compared (Figure 3).

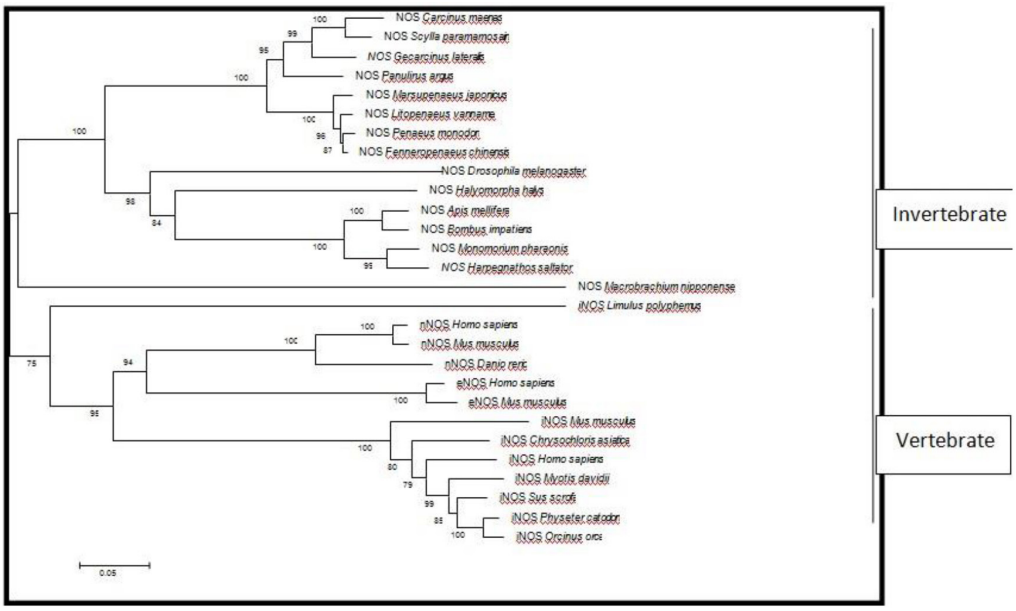

Figure 3. Phylogenetic tree of 28 nitric oxide synthase (NOS) proteins from crustaceans, fish, insects, and mammals. Complete amino acid sequences were aligned using DNAMAN, and a neighbor-joining tree was constructed with MEGA 4.1. Bootstrap analysis was performed using 1000 replicates to test relative support for each cluster. Names and accession Nos. are as follows: *Limulus polyphemus* (iNOS, XP_013781426.1), *Homo sapiens* (nNOS, NP_001191147.1; iNOS, NP_000616.3; eNOS, BAA05652.1), *Mus musculus* (nNOS, NP_032738.1; iNOS, NP_035057.1; eNOS, NP_032739.3), *Danio rerio* (nNOS, XP_005165110.1), *Chrysochloris asiatica* (iNOS, XP_006874299.1), *Myotis davidii* (iNOS, ELK24792.1), *Sus scrofa* (iNOS, NP_001137162.1), *Physeter catodon* (iNOS, XP_007109277.1), *Orcinus orca* (iNOS, XP_012387409.1), *Carcinus maenas* (NOS, ACY56317.1), *Scylla paramamosain* (NOS,CCC18661.1), *Gecarcinus lateralis* (NOS, AAT46681.1), *Panuli rusargus* (NOS, ACZ60615.1), *Marsupenaeus japonicus* (NOS, BAI67609.1), *Litopenaeus vannamei* (NOS, ADD63793.1), *Penaeus monodon* (NOS, ACJ54486.1), *Fenneropenaeus chinensis* (NOS, AFJ74715.1), *Drosophila melanogaster* (NOS, NP_523541.2), *Halyomorpha halys* (NOS, XP_014272116.1), *Apis mellifera* (NOS,NP_001012980.1), *Bombus impatiens* (NOS, XP_003485294.1), *Monomorium pharaonis* (NOS,XP_012524269.1), *Harpegnathos saltator* (NOS, EFN84471.1), and *Macrobrachium nipponense* (NOS, KU382528). Information regarding these sequences is summarized in Table 2. iNOS = inducible NOS; nNOS = neuronal NOS; eNOS = endothelial NOS.

Tissue expression of *MnNOS* mRNA

The expression of MnNOS in various tissues was examined by RT-qPCR. MnNOS transcription was detected in gill, muscle, brain, intestine, eyestalk, testis, ovary, heart, hepatopancreas, AG, and nerve cord samples. *MnNOS* was relatively abundant in the

hepatopancreas and muscle, whereas only trace levels were detectable in the heart, gonads, and nerve cord. The highest expression was observed in the AG ($P < 0.05$; Figure 4).

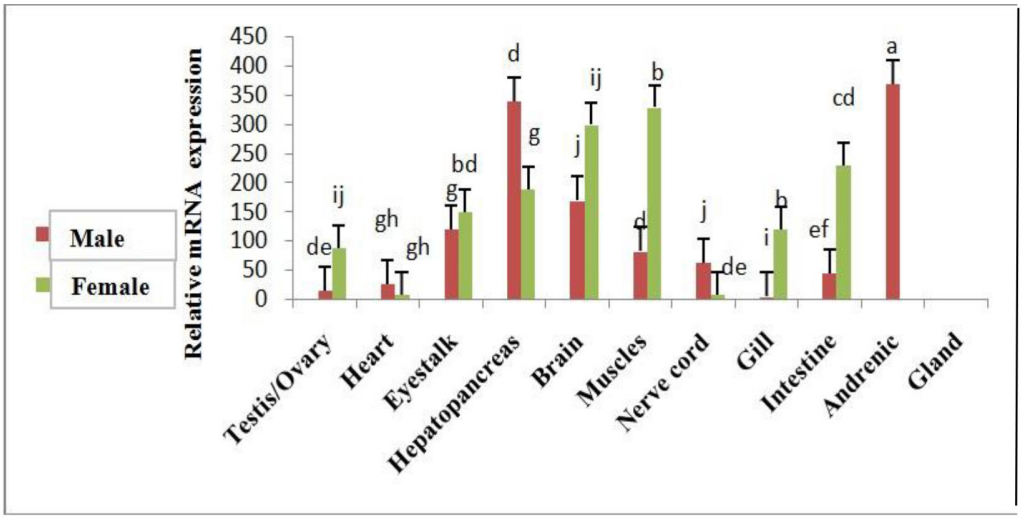

Figure 4. Distribution of nitric oxide synthase mRNA in the different tissues of adult male and female *Macrobrachium nipponense*. Data are reported as mean fold changes (means ± standard errors of the mean; N = 3). Statistical significance was calculated by one-way analysis of variance and multiple comparison tests.

Expression of *MnNOS* mRNA during different developmental stages of prawn embryos, larvae, and PL

RT-qPCR was applied to investigate the expression of *MnNOS* throughout development. *MnNOS* mRNA could be detected at all stages, and its transcription underwent two increases during certain larva land PL phases. The first occurred at larval stage L13 after hatching and the second at 10 days PL (PL10), at which point *MnNOS* expression was at its highest ($P < 0.01$; Figure 5).

DISCUSSION

NOS is an important enzyme due its specific role in synthesizing NO by catalyzing the conversion of L-arginine to L-citrulline, accompanied by the oxidation of NADPH (Yao et al., 2010). Consequently, it is crucial to the physiological functioning of NO. Many studies have confirmed that NOS has beneficial and versatile roles in the microvascular, neural, and immune systems (Figueroa et al., 2001). NOS sequences have been examined in several crustaceans (Rodríguez-Ramos et al., 2010; Yao et al., 2010; McDonald et al., 2011), but that of *M. nipponense* remains unknown. In the current study, we cloned *NOS* and demonstrated its elevated expression in the *M. nipponense* AG, suggesting that it is of great importance in male sexual differentiation in this species.

The full-length *MnNOS* cloned in this investigation comprised a complete ORF of 3447 bp, encoding a 1148-amino acid protein with an estimated MW of 129.209 kDa and a pI

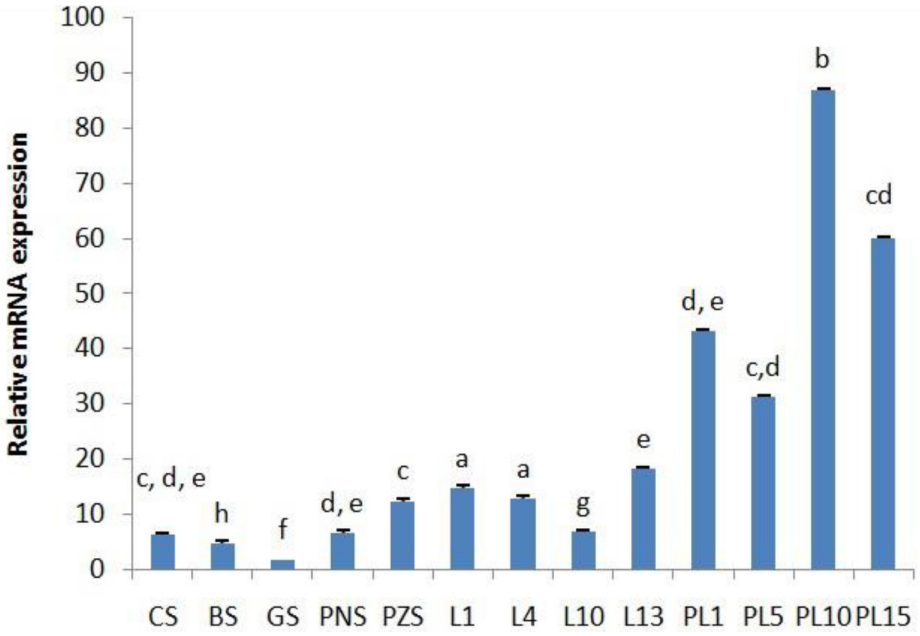

Figure 5. Expression of the *Macrobrachium nipponense* nitric oxide synthase gene at different development stages. Data are reported as means ± standard errors of the mean, based on three samples of each embryonic, larval (L), and post-larval (PL) stage. Bars with different letters are significantly different ($P < 0.05$). CS = cleavage stage; BS = blastula stage; GS = gastrula stage; PNS = pre-nauplius stage; PZS = pre-zoea stage; L1 = first-day larvae after hatching; PL1 = first-day post-larvae after metamorphosis.

of 7.12. Untranslated regions of 130 and 139 bp were detected at the 5'- and 3'-end, respectively, in addition to a complete poly(A) tail (Figure 1). Structural analysis of the MnNOS amino acid sequence using the SMART program revealed its organization to be remarkably similar to that of other crustacean NOS proteins. It includes four principal domains: a highly conserved N-terminal NOS domain from position 62 to 424, vital for the production of NO; a flavodoxin 1 domain between positions 474 and 680; a FAD-binding domain at position 776 to 945; and a C-terminal NAD-binding domain homolog encompassing residues 977 to 1054 that is functionally important in various electrontransport systems (Figure 1). Furthermore, multiple sequence alignment showed that the deduced MnNOS amino acid sequence is very similar to those of vertebrate (Wang et al., 2001) and invertebrate (Hoskins et al., 2007; McDonald et al., 2011) NOS enzymes (Figure 2). Considering such homology, we speculate that NOS function in *M. nipponense* is typical of this enzyme type, and as in other organisms, MnNOS may affect multiple roles.

In the present study, we constructed an NJ phylogenetic tree based on the amino acid sequences of 28 NOS enzymes (including nNOS, eNOS, and iNOS) from invertebrates (crustaceans and arthropods) and vertebrates (fish and mammals). MnNOS formed a distinct cluster with other invertebrate NOS proteins, demonstrating their close taxonomic relationship compared with vertebrate sequences, and indicating that NOS is highly conserved across these organisms. The alignment also showed that MnNOS shared 45% identity with the *P. monodon*, *L. vannamei*, and *M. japonicus* NOS protein family, but only low identity with *Mus musculus*

sequences (27% with murine nNOS). Thus, compared to vertebrate enzymes, the MnNOS sequence exhibited a relatively high level of identity with crustacean NOSs (Figure 3). This evolutionary relationship implies that, with the exception of iNOS produced by the arthropod *Limulus polyphemus*, different isoforms of this enzyme may not be found in invertebrates, and this lineage may have inherited only on eNOS type, as in mollusks (Andreakis et al., 2011), the mud crab *Scylla paramamosain* (Li et al., 2012), and the Zhikong scallop *Chlamys farreri* (Jiang et al., 2013). Furthermore, the observed connections between these groups might reflect taxonomic relationships, and appear to show that although MnNOS clustered with invertebrate NOS enzymes, it was not closely related to that of any species examined. Given the lack of similarity to other crustacean sequences, this NOS may be exclusive to *M. nipponense* and may display unique features (Figure 3). The activity of this enzyme needs further investigation.

In the present study, *NOS* was expressed in all of the tissues tested, consistent with previous studies in which this gene has been shown to be widely expressed in insect, mammalian, and crustacean tissues, and to regulate a variety of physiological processes (Kim et al., 2004). Furthermore, most prior reports have confirmed the involvement of NO in metabolism, growth, cell proliferation, development, and neurogenesis in vertebrates, insects, and mollusks, verifying the broad role of NOSs in the microvascular, neural, and immune systems (Kuzin et al., 1996; Andreakis et al., 2011; Hampton et al., 2015). Furthermore, NO in rat has additional functions as it appears to be found in all parts of the male reproductive system as well and seems to play a role in testicular, epididymal and vas deferens function. Moreover, in female reproductive tract, it relaxes uterine muscle via cGMP and contracts it via prostaglandins, and it is expression was increased in the cervix, and decreased in the uterus, during labour and preterm labour (Buhimschi et al., 1996). NO also seems to be involved in pre-eclamptic conditions and pregnancy-related hypertension (Buhimschi et al., 1996). These findings are in consistency with our RT-qPCR result which revealed that *MnNOS* level was expressed in all detected tissues including gonads (testis and ovary), hepatopancreas, gill, eyestalk, brain, intestine, muscles, nerve cord and AG which represent the significantly higher expression in *M. nipponense*. Additionally, with the result in scallop *C. farreri*, the highest expression of *NOS* was found in the gonads (Jiang et al., 2013). Kim et al. (2004) found *NOS* to be expressed in nerve ganglia, Y-organ, gill and gonad of the tropical land crab, *Gecarcinus lateralis*. And McDonald et al. (2011) found high expression of NOS in Y-organs of the green shore crab *Carcinus maenas*. In the Pacific white shrimp *L. vannamei* and the mud crab *S. paramamosain*, expression has been detected in the hepatopancreas (Yao et al., 2010; Li et al., 2012), whereas in the kuruma shrimp *M. japonicus*, it has been observed in the gills and brain (Inada et al., 2010). In addition, iNOS is expressed in hemocytes of the spiny lobster *Panulirus argus* (Rodríguez-Ramos et al., 2010). Identification of the expression of this interesting molecule in the AG may provide insights into the genetic framework behind sex determination in *M. nipponense*. Moreover, these findings collectively expand our current understanding of *M. nipponense* and crustacean sexual differentiation. Consequently, *MnNOS* may prove significant in efforts to manipulate the *M. nipponense* reproductive system to produce all-male populations. Here, the *NOS* gene was expressed in all tested tissues, indicating that it may perform versatile biological functions in this organism. Furthermore, the AG could play an important role in synthesizing NOS in *M. nipponense*. A more detailed assessment of *MnNOS* activity is needed.

Interestingly, *MnNOS* was expressed throughout development, from early embryonic

to late PL stages. Expression levels in the blastula and gastrula are crucial, as these are key stages of cell differentiation and organ formation (Zhang et al., 2010). An earlier finding of Herrero et al. (1997) demonstrated that the presence of NOS in spermatazoa induces sperm motility, which implies its multiple role to promote reproduction throughout all levels in the organism, therefore, NO considered as a sexual gas (McCann and Rettori, 1996). In this study, the expression of *NOS* decreased during the initial larval stages, reaching its lowest level at L10. Metamorphosis takes place at this point, during which the transcription of most genes decreases, including *NOS*. RT-qPCR measured the highest expression at stage PL10. In our previous study, we found that *M. nipponense* primordial germ cells (PGCs) appear between stages PL10 and PL15, together with external sexual characteristics (Zhang et al., 2013). Thus, the current results are consistent with these observations, suggesting that *MnNOS* may be involved in organ formation. Significant *MnNOS* transcription during PL10 may coincide with the development of PGCs and external sexual attributes in *M. nipponense*. Based on these findings and the result of Herrero et al. (1997) and McCann and Rettori (1996), we speculated that this gene may be involved in sex differentiation in this organism. Furthermore, the expression of *MnNOS* across different stages of development might indicate that it also plays an important role in this process as well as in the developmental stages.

In conclusion, we generated a full-length clone of the MnNOS gene, a novel NOS sequence, and investigated its transcriptional patterns in different tissues and developmental stages of *M. nipponense*. From this, we can infer that the presence of MnNOS during embryonic and PL stages may have an important influence on external sexual development and determination of male sex in this organism. Therefore, MnNOS could serve an important purpose in producing all-male cultures of these prawns. Moreover, the protein encoded by this gene might fulfill varied biological roles in *M. nipponense*. Further detailed investigations should be carried out to elucidate the functions of MnNOS.

Conflicts of interest

The authors declare no conflict of interest.

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