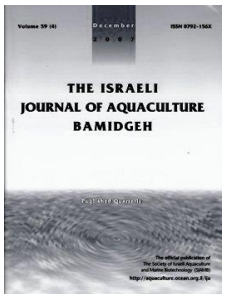
The Israeli Journal of Aquaculture - Bamidgeh, IJA_69.2017.1374, 11 pages



RNA Interference Studies on the Sex- Linked Genes *transformer-2*and *sex-lethal* in the Oriental River Prawn *Macrobrachium nipponense*

Suhua Chen[1], Hongtuo Fu[1,2,*], Hui Qiao[2], Shengming Sun[2], Wenyi Zhang[2], Shubo Jin[2], Yongsheng Gong[2], Sufei Jiang[2], Yiwei Xiong[2], Yan Wu[2]

[1] *Wuxi Fisheries College, Nanjing Agricultural University, Wuxi 214081, PR China*

[2] *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*

Keywords: *Macrobrachium nipponense*; transformer-2; sex-lethal; RNA interference; sexual regulation

Abstract

The *sex-lethal (sxl)* and *transformer-2 (tra-2)* genes play key roles in the regulation of the sex differentiation pathway in insects. This study investigated the transcriptional patterns of *Mntra-2* and *Mnsxl* in different tissues in the oriental river prawn *Macrobrachium nipponense*. The qPCR results showed that the highest level of *Mntra-2* expression was observed in the muscle, and the highest level of *Mnsxl* expression was observed in the hepatopancreas. RNA interference with *Mntra-2*/*Mnsxl* dsRNA effectively resulted in a decrease in the expression of *Mntra-2*/*Mnsxl* in the muscle, hepatopancreas and gonads in both males and females. *Mntra-2* dsRNA had a significant inhibitory effect on *Mnsxl* mRNA expression in the testis and ovaries, but *Mnsxl* dsRNA did not affect *Mntra-2* mRNA expression in either organ. Thus, *Mntra-2* may be an upstream gene in the sex determination pathway in *M. nipponense*, and the regulatory mechanisms may be different from those observed in *Drosophila melanogaster*, even though these genes are homologous to the Drosophila *tra-2* and *sxl* sex-determination genes. Thus, the present results are preliminary and the sex determination pathway in *M. nipponense* needs further in-depth research.

* Corresponding author. e-mail: fuht@ffrc.cn

Introduction

The sex determination pathway in insects is well characterized in the fruit fly *Drosophila melanogaster*. A schematic overview of the sex determination cascade for *D. melanogaster* has been reported (Zhang et al. 2014). The master switch gene at the top of the cascade is *Sex-lethal* (*sxl*), which regulates female-specific splicing of the pre-mRNA of the *transformer* (*tra*) gene (Bell et al. 1988; Penalva and Sanchez 2003). The functional *tra* protein interacts with the protein product of the *transformer-2*(*tra-2*) gene, which results in the female-specific splicing of the mRNA of the bottom gene in the cascade, *doublesex* (*dsx*) (Sievert et al. 1997). *Sxl*, besides regulating the female-specific splicing of its own pre-mRNA, also regulates the female-specific splicing of the pre-mRNA of *tra*, resulting in the production of the functional *tra* protein only in females (Evans and Cline 2013). The *tra-2* protein contains an RNA recognition motif domain and serine/arginine-rich regions; Moreover, it heterodimerizes with *tra* and binds to exonic splicing enhancer sequences present in the fourth exon (female-specific exon) of the *dsx* pre-mRNA (Lynch and Maniatis 1996). However, genes that are homologous to the sex-determining genes of *Drosophila* have not been found in all insect species (Kageyama et al. 2012; Schutt and Nothiger 2010; Heimpel et al. 2008).

In crustaceans, the molecular mechanisms regulating the expression of sex-specific and sex-determining genes have received significant attention in recent years. Several homologs of *D. melanogaster* sex-determining genes have been observed in *Daphnia magna* (*tra* and *dsx*), *Tribolium castaneum* (*tra-2*), *Penaeus monodon* (sex-related genes in the testis), *Fenneropenaeus chinensis* (*tra-2*) and *Eriocheir sinensis* (*Dmrt* and *sxl*) (Kato et al. 2010; Kato et al. 2011; Shukla and Palli 2013; Leelatanawit et al. 2009; Li et al. 2012; Zhang and Qiu 2010; Shen et al. 2014). Most of these genes in crustaceans exhibit high sequence similarity with their homologs in insects, but their functional consequences and sex determination pathways have not been predicted or are unclear.

The oriental river prawn *Macrobrachium nipponense* is an important commercial species in China. Male *Macrobrachium* prawns grow faster and gain more weight at harvest time than females, which makes the male prawns more economically important. Therefore, it is important to study the sex mechanism of this species, as this would help make genetic improvements and improve its production. In our previous study, we cloned two *Drosophila* homologous genes—*tra-2* and *sxl*—present in *M. nipponense* and examined the expression patterns of these two genes (Zhang et al. 2013a, 2013b). In this study, we have further studied these two genes by analyzing their regulatory effects on each other via RNA interference (RNAi) experiments.

Materials and methods

Experimental animals and sampling. Adult healthy *M. nipponense* individuals weighing between 1.5 and 4.65 g (female prawns, 1.5–2.5 g; male prawns, 3.5–4.65 g) were collected from Tai lake in Wuxi. The prawns were maintained in a re-circulating freshwater aquarium system at a constant temperature of 25°C ± 1°C and fed paludina twice a day. A variety of organs, including the eyestalk, hepatopancreas, gill, heart, muscle, testis and ovary, were collected from mature male and female prawns. All tissues were dissected and washed in 1× phosphate-buffered saline (0.01 M), frozen directly in liquid nitrogen, and stored at -80°C until analysis.

RNAi experiments. RNA interference has been used successfully in our laboratory (Bai et al. 2015; Qiao et al. 2015; Li et al. 2015). dsRNA of *Mntra-2* and *Mnsxl* were synthesized in vitro using the Transcript AidTM T7 High Yield Transcription kit (Fermentas Inc., USA) according to the manufacturer's instructions. Specific primers containing the T7 promoter site of the interfering RNA were designed using Snap Dragon (http://www.flyrnai.org/cgibin/RNAifind_primers.pl). The *tra-2* and *sxl* dsRNA synthesis primers are shown in Table 1. The purity and integrity of dsRNA were examined by standard agarose gel electrophoresis. The concentration of dsRNA was measured at 260 nm with a BioPhotometer (Eppendorf, Hamburg, Germany), and then maintained at -20°C until use.

tra-2 dsRNA was injected into the pericardial cavity of 100 healthy mature female *M. nipponense* and 100 male *M. nipponense* individuals. The ovarian cycle of *M. nipponense* was classified into five stages based on previous results (Gao et al. 2006) and ovary

color: Stage I, undeveloped stage characterized by oogonium proliferation in which the ovary appears transparent; Stage II, developing stage characterized by primary vitellogenesis in which the ovary appears yellow; Stage III, nearly ripe stage characterized by secondary vitellogenesis in which the ovary appears light green; Stage IV, ripe stage characterized by vitellogenesis termination and a dark green colored ovary; and Stage V, spent stage in which the ovary appears gray. The female prawns were selected in the proliferation stage (stage I) according to ovary color to ensure that development of the ovaries was synchronous, and they were equally distributed into two groups. In the experimental treatment group (N = 50), *Mntra-2* dsRNA was injected into the pericardial cavity membrane of the carapace at a dose of 4 μg/g of body weight (BW). In the experimental control group (N = 50), H_2O was injected at an equal dose. Male prawn of similar size were selected and also distributed equally between two treatment groups: in the experimental treatment group (N = 50), *tra-2* dsRNA was injected into the pericardial cavity membrane at a dose of 4 μg/g, and the experimental control group (N = 50) was injected with an equal dose of H_2O. Four prawns from each group were randomly collected on the 1st, 4th, 7th, 10th and 13th day after injection. The muscle, hepatopancreas and gonad were dissected, frozen immediately in liquid nitrogen, and stored at -80°C until analysis.

The protocol for the *Sxl* dsRNA injection experiment was the same as described above. The hepatopancreas, muscle and gonad were collected, frozen immediately in liquid nitrogen, and stored at -80°C until use.

Total RNA isolation and cDNA synthesis. Total RNA was isolated using the RNAiso Plus Reagent (TaKaRa, Japan) according to manufacturer's protocols. For all RNA samples, the A260/A280 ratio was in the range of 1.9–2.1, and RNA integrity was verified on a 1.2% agarose gel. Total RNA was treated with RNase-free DNase I (Sangon, China) to eliminate any possible genomic DNA contamination. The concentration of RNA was quantified with BioPhotometer (Eppendorf). Then, 1 μg of total RNA was reverse transcribed with the iScript™ cDNA Synthesis Kit (BIO-RAD, USA) following manufacturer's instruction.

Expression analysis by quantitative real-time PCR. The expression patterns of these two genes were evaluated using a quantitative real-time PCR assay on a Bio-Rad iCycler iQ5 Real-Time PCR System (Bio-Rad, USA). The qPCR amplification was carried out in a total volume of 25 μL that contained 1 μL of cDNA (50 ng), 10 μl of SsoFast™ EvaGreen Supermix (Bio-Rad, USA), 0.5 μL of 10 μM specific forward and reverse primers (Table 1), and 13 μL of nuclear water.

Table 1. Primers of sequence used

Primer Name	*Sequence(5′→3′)*	*Description*
Tra-2-dsF	TAATACGACTCACTATAGGGAAGCAGAGAGGACCCTAGCC	Primer for Mntra 2 dsRNA
Tra-2-dsR	TAATACGACTCACTATAGGGTCCTCCATATCCACTGGACC	Primer for Mntra 2 dsRNA
sxl-dsF	TAATACGACTCACTATAGGGCCACAGACGCTAACTGACCA	Primer for Mnsxl dsRNA
sxl-dsR	TAATACGACTCACTATAGGGAGTCCTGCATGATACCCAGC	Primer for Mnsxl dsRNA
tra 2-RTF	GCCCCAGTACGTGTTTAGGT	primer for qRT- PCR
tra 2-RTR	TCCTGCGGCCATCTATTTCC	primer for qRT- PCR
sxl-RTF	GCTGCTAAGGCAATTCTTCAGTT	primer for qRT- PCR
sxl-RTR	CAAACCCGACTCCTCTAGGTAAC	primer for qRT- PCR
β-actinF	TATGCACTTCCTCATGCCATC	primer for qRT- PCR
β-actinR	AGGAGGCGGCAGTGGTCAT	primer for qRT- PCR

The reaction mixture was initially incubated at 95°C for 30 s to activate Hot Start Taq DNA polymerase; this was followed by 40 cycles of 95°C for 10 s and 60°C for 10 s. Melting curve analysis was performed at the end of the qPCR reaction at 65–95°C (at 0.5° increases) for 10 s. Four replicate qPCRs were performed per sample of each four prawns, and β-actin was used as the internal control. To ensure that only one PCR

product was amplified and detected, dissociation curve analysis of the amplification products was performed at the end of each PCR analysis. Significant differences in expression were assumed at *P* values of <0.05. The relative copy number was calculated according to the $2^{-\Delta\Delta CT}$ comparative CT method (Livak and Schmittgen 2001).

Statistical analysis. Quantitative data are expressed as mean ± SD values (n = 4). Statistical analysis was performed using SPSS 20.0. Statistical differences were estimated by one-way ANOVA followed by Duncan's multiple range tests. Significance was set at $P < 0.05$.

Results

Tissue distribution of Mntra-2 and Mnsxl. As shown in Fig. 1, *Mntra-2* mRNA was expressed in all the examined tissues of the adult prawn, with the highest mRNA levels observed in the muscles of both female and male individuals. In males, the *Mntra-2* mRNA expression level is the second highest in the testis. In females, *Mntra-2* mRNA expression in the ovary was lower than that in the muscle, hepatopancreas and heart.

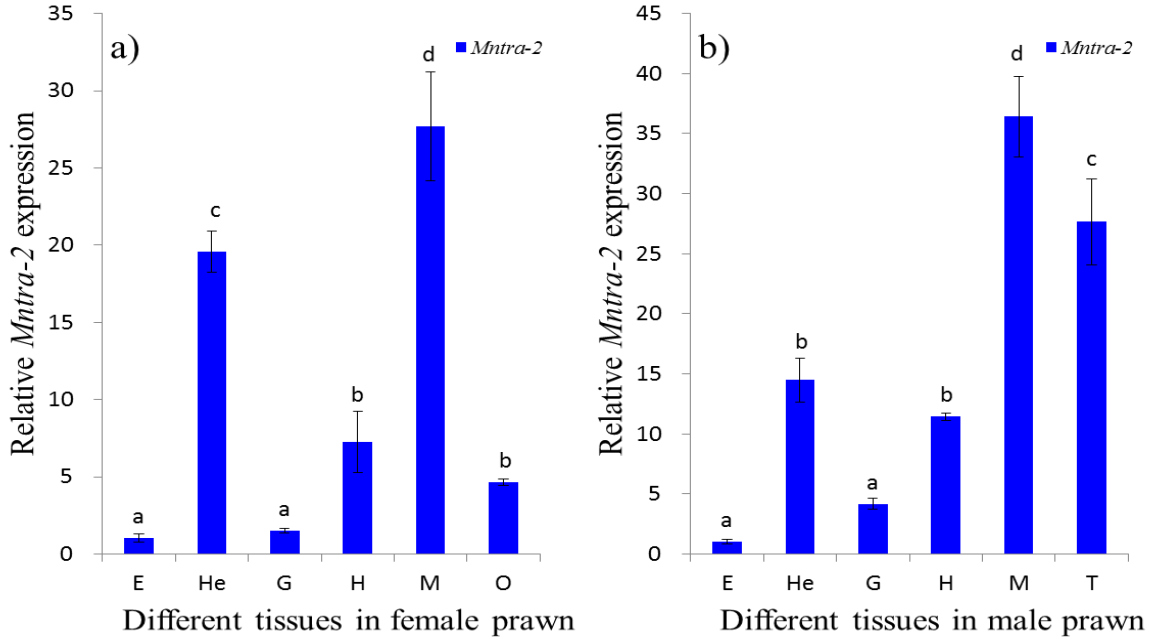


Fig. 1. The expression of *Mntra-2* was analyzed by quantitative real-time PCR in different tissues. The mRNA level of the two genes was normalized to the β-actin transcript level. Data are shown as the mean ± SD values (n = 4). Bars marked with different letters are considered to indicate significance at $P < 0.05$. E: eyestalk, He: hepatopancreas, G: gill, H: heart, M: muscle, O: ovary, T: testis. Statistical analyses were performed with one-way ANOVA.

Mnsxl showed the highest expression in the hepatopancreas of both female and male individuals (Fig. 2), but its expression in the gonads was lower than that in the hepatopancreas as well as the heart.

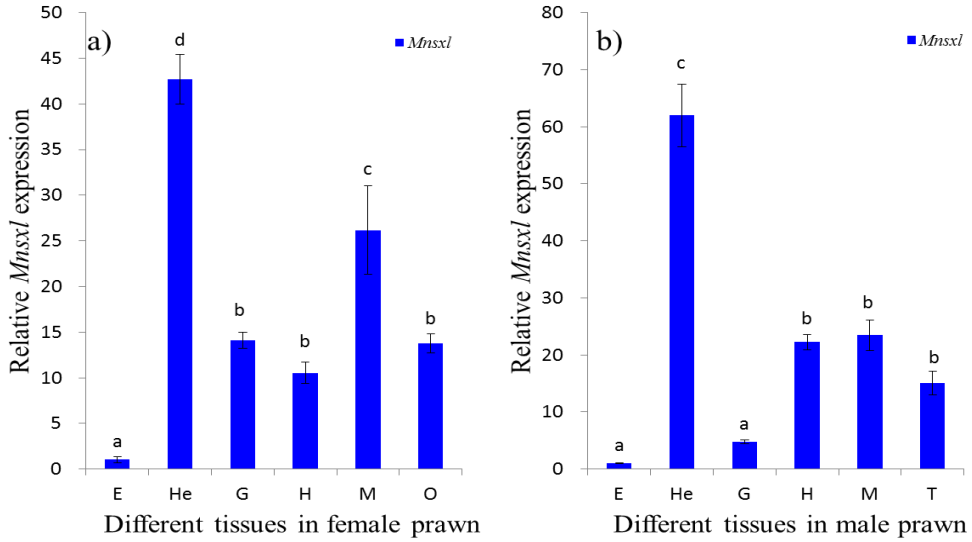


Fig. 2. The expression of *Mnsxl* in different tissues was analyzed by quantitative real-time PCR. The mRNA expression level of two genes was normalized to the *β-actin* transcript level. Data are shown as means ± SD values (n=4). Bars marked with different letters are considered significant at $P<0.05$. E: eyestalk, He: hepatopancreas, G: gill, H: heart, M: muscle, O: ovary, T: testis. Statistical analyses were performed with one-way ANOVA.

Effect of RNAi with Mntra-2 dsRNA on Mntra-2 expression. In the female muscle tissue (Fig. 3a), injection of *Mntra-2* dsRNA resulted in downregulation of *Mntra-2* expression compared with the control group. The expression of *Mntra-2* began to decrease on the 1st day and showed a 70% decrease from the 4th day to the 10th day. The expression recovered slightly on the last day. In the ovary (Fig. 3b), the mRNA level of *Mntra-2* decreased and was the lowest on the 4th day, and it reached a 67.4% decrease on the 7th day.

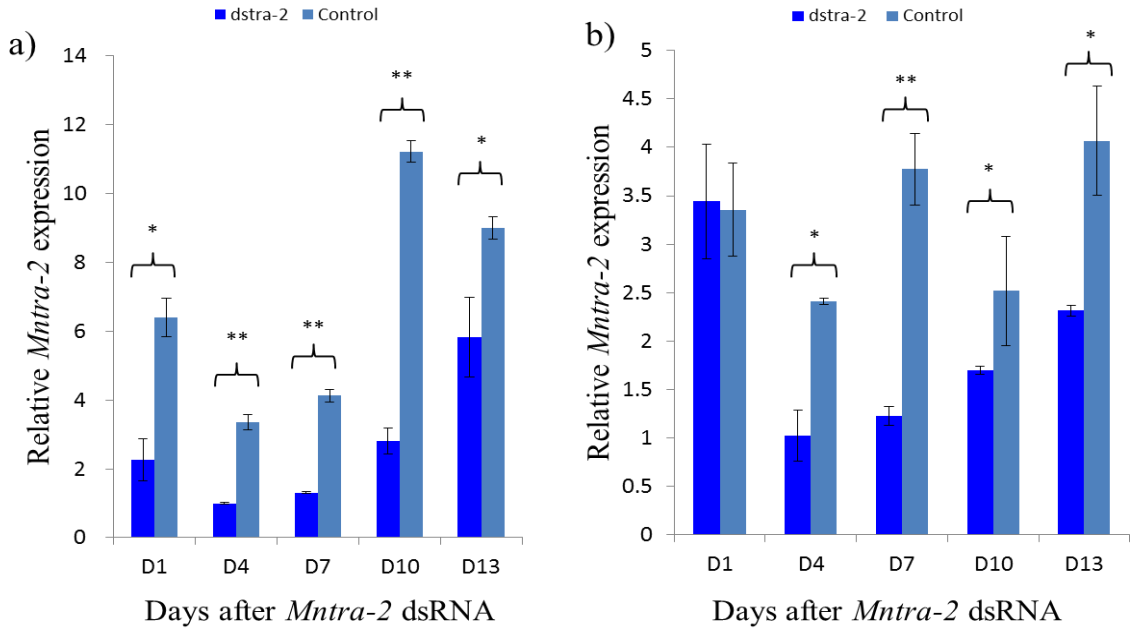


Fig. 3. Real-time PCR analysis of the effect of the *tra-2* dsRNA (4 μg/g) injection in female prawns. (a) represents the relative *Mntra-2* expression level in the muscle after RNA interference; (b) represents the relative *Mntra-2* expression level in the ovary after RNA interference. The asterisk indicates significant difference between the experimental group and the control group (*$P < 0.05$, **$P < 0.01$)

In males, qPCR analysis indicated that *Mntra-2* dsRNA injection resulted in an 86.8% decrease in *Mntra-2* expression in the muscle (Fig. 4a) and a 92% decrease in *Mntra-2* expression in the testis on the 7th day (Fig. 4b). On later experimental days, the mRNA levels increased gradually.

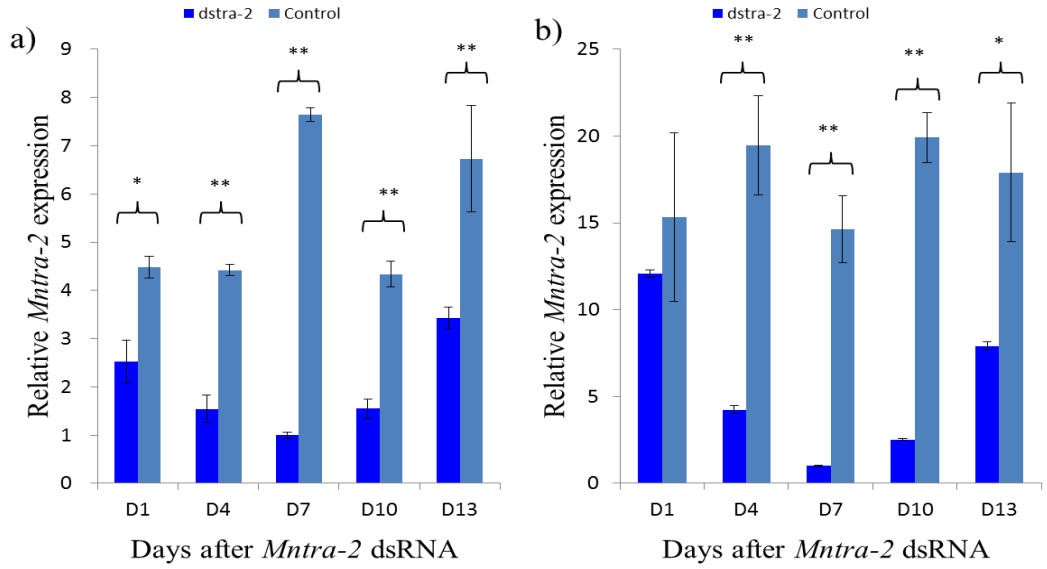


Fig.4. Real-time PCR analysis of the effect of the *tra-2* dsRNA (4 μg/g) injection in male prawns. (a) represents the relative *Mntra-2* expression level in the muscle after RNA interference; (b) represents the relative *Mntra-2* expression level in the testis after RNA interference. The asterisk indicates significant difference between the experimental group and the control group (*$P < 0.05$, **$P < 0.01$).

Effect of RNAi with Mnsxl dsRNA on Mnsxl expression. In the hepatopancreas and ovary of *Mnsxl* dsRNA-injected female prawn, the expression of *Mnsxl* mRNA decreased by 80% and 90%, respectively, in the knockdown group relative to the control group on the 4th day (Fig. 5a and b).

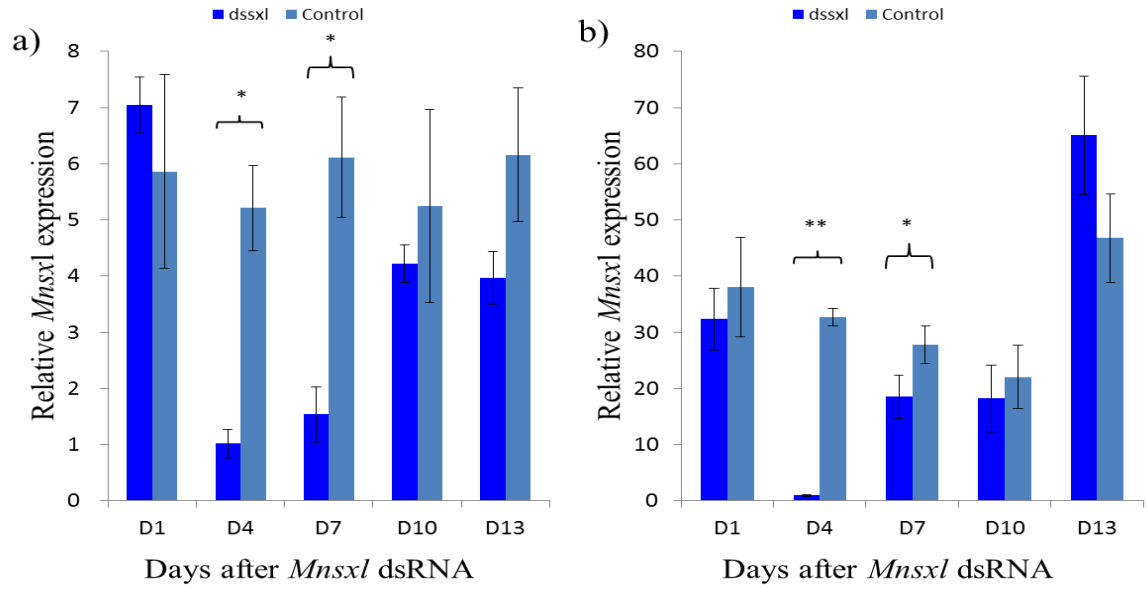


Fig. 5. Real-time PCR analysis of the effect of *sxl* dsRNA (4 μg/g) injection in female prawns. (a) represents the relative *Mnsxl* expression levels in the *hepatopancreas* after RNA inference; (b) represents the relative *Mnsxl* expression levels in the ovary after RNA inference. The asterisk indicates significant difference between the experimental group and control group (*$P<0.05$, ** $P<0.01$).

In the male hepatopancreas, the *Mnsxl* mRNA level reduced to 90% on the 4th day (Fig. 6a). Further, the transcript level in the testis declined by 85% on the 7th day (Fig. 6b).

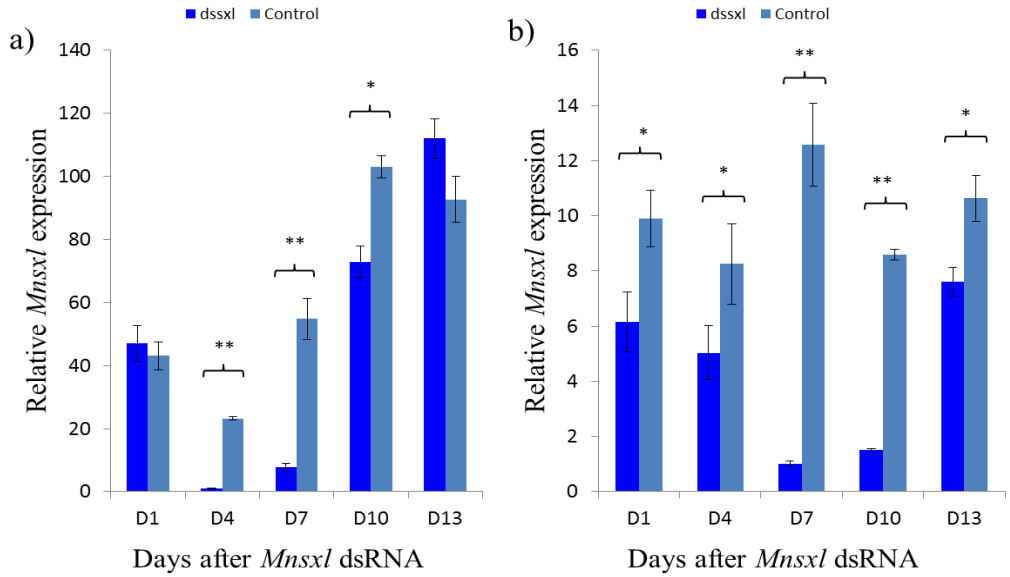


Fig. 6. Real-time PCR analysis of the effect of *sxl* dsRNA (4 μg/g) injection in male prawns.
(a) represents the relative *Mnsxl* expression level in the *hepatopancreas* after RNA inference;
(b) represents the relative *Mnsxl* expression level in testis after RNA inference.
The asterisk indicates significant difference between the experimental group and control group (**P*<0.05, ***P*<0.01).

Influence of Mntra-2 silencing on Mnsxl gene transcription. In the *Mntra-2* dsRNA-injected prawn, the expression of *Mnsxl* in the ovary reduced significantly from the 1st day to the 7th day (decreased by 70% on the 7th day). The difference in expression compared to the control group on the 10th day and 13th day was not significant (Fig. 7a). The knockdown of *Mntra-2* in the testis was successful and resulted in down-regulation of the *Mnsxl* gene compared with the control animals throughout the duration of the experiment (*Mnsxl* expression decreased by 55% on 10th day) (Fig. 7b).

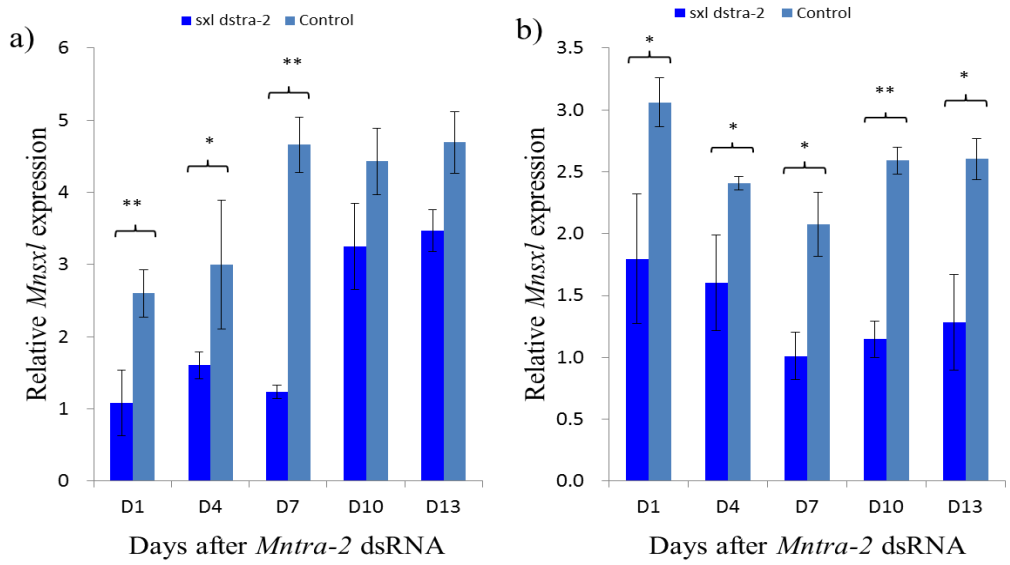


Fig. 7. Effects of *Mntra-2*-dsRNA-injection on *Mnsxl* in (a) the ovary and (b) the testis. *Mnsxl* mRNA levels were analyzed by qPCR. mRNA levels were normalized to β-actin mRNA levels. Data from qPCR are shown as means ± SD values (**P*<0.05, ***P*<0.01).

Influence of silencing Mnsxl on Mntra-2 gene transcription. After injection of *Mnsxl* dsRNA, the mRNA levels of *Mntra-2* in both the ovary and testis were similar in the treated and control groups (Fig. 8a and b). Although the mRNA levels in the ovary in the treated group were slightly below those in the control group, the difference in expression between these two groups was not significant ($P > 0.05$). The results confirmed that silencing of *Mnsxl* clearly did not affect *Mntra-2* expression.

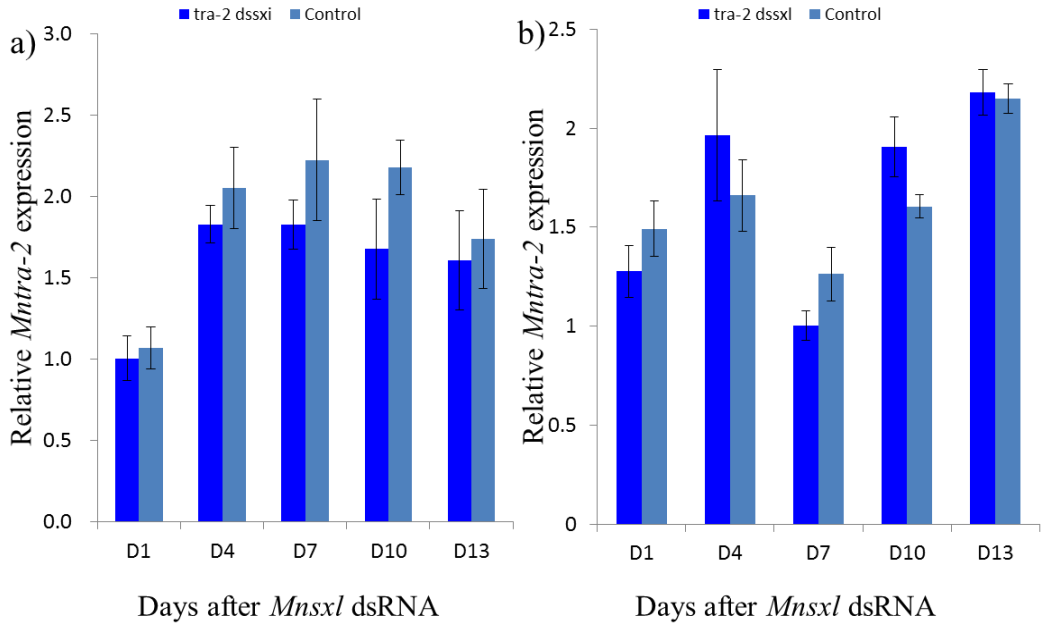


Fig. 8. Effects of *Mnsxl*-dsRNA-injection on *Mntra-2* in (a) the ovary and (b) the testis. *Mntra-2* mRNA levels were analyzed by qPCR. mRNA levels were normalized to β-actin mRNA levels. Data from qPCR are shown as means ± SD values (*P<0.05, **P<0.01).

Discussion

This study demonstrates, for the first time, the regulatory effects of the *Mntra-2* gene on expression of the *Mnsxl* gene in *M. nipponense*. These findings lay the foundation for further studies on the genes involved in sex determination in this species, as these two genes are homologous to two genes that are involved in the sex determination cascade in *D. melanogaster*.

Mntra-2 mRNA was expressed in all examined tissues of the adult prawn, and that the mRNA levels were the highest in the muscle and second highest in the testis (Zhang, 2013ba). These results were similar to those of our present study. Further, the expression level of *Mntra-2* mRNA in the testis was significantly higher than that in the ovary (four times higher). The *Mnsxl* mRNA expression level was the highest in the hepatopancreas, followed by the muscle, but its expression in the gonads was lower than that in the hepatopancreas and muscle (Zhang et al. 2013b). In our study, too, the tissue distribution of *Mnsxl* was similar to that reported by Zhang et al. (2013b). The *Mntra-2* mRNA level was the highest in muscle tissue, and the *Mnsxl* mRNA level was the highest in the hepatopancreas. Therefore, the muscle tissue and the hepatopancreas, and the gonads were used for the remaining experiments.

In previous RNAi studies, we have shown that 4 μg of dsRNA per gram of shrimp is effective to suppress the expression of several genes (Bai et al. 2015; Qiao et al. 2015). In this study, injection of dsRNA successfully reduced the transcriptional levels of the target genes (*Mntra-2* and *Mnsxl*) in the examined tissues at a dose of 4 μg per gram BW. In particular, *Mntra-2* dsRNA inhibited the *Mntra-2* mRNA level in the testis more effectively: the expression of *Mntra-2* in the testis in the control group was more than 15 times that in the treatment group on the 7th day. Moreover, the expression of *Mnsxl* in the ovary in the control group was more 30 times that in the experimental group on the 4th day. In *D. melanogaster*, *tra-2* is a key gene that acts on the cascades regulating both somatic sexual differentiation and male fertility (Belote and Baker 1983). *sxl* functions as the master switch gene for sex determination in *D. melanogaster* (Bell et al. 1988; Zhang et al. 2014; Gempe and Beye 2011). Based on these reports, we believe

that these two genes (*Mntra-2* and *Mnsxl*) may be related to sex determination in *M. nipponense*.

The *Mnsxl* mRNA levels in the testis and ovary were significantly reduced by injection of *Mntra-2* dsRNA, but the *Mntra-2* mRNA levels were not reduced by injection of *Mnsxl* dsRNA. These results indicate that *Mntra-2* has a positive regulatory effect on *Mnsxl* at the mRNA level in the gonads, which means that Mntra-2 may be an upstream gene in the sex determination pathway in *M. nipponense*. This is different from the relationship between *sxl* and *tra-2* in *D. melanogaster* (Zhang et al. 2014). In *Bombyx mori*, too, the chromosomal sex determination mechanism is distinct from that in *D. melanogaster*; moreover, female sex in *B. mori* is determined by the presence of a dominant feminizing factor (*fem*) and not *sxl*, which plays a sex-independent role in *B. mori* (Niimi et al. 2006; Suzuki 2010). A *tra* homolog has not been found in the *Bombyx* genome, but *Bmtra-2* is reportedly required for normal testis development (Suzuki et al. 2012; Sakai et al. 2014). Thus, genes that are homologous to the *Drosophila* sex-determining genes may have different functions in other insect species and should therefore be investigated in depth in other species too.

There is still much research lacking on sex determination in crustaceans. For example, in the freshwater prawn species *Macrobrachium rosenbergii* and *F. chinensis*, in which sex is determined chromosomally, with the females being heterogamous (ZW) and the males being homogamous (ZZ), the sex determination pathway is currently unclear (Malecha et al. 1992; Li et al. 2003). Further, the genotype of *M. nipponense* has not been determined, and several sex-specific and sex-determining genes have not been found or researched in depth. This study is still in the preliminary stage of exploring the sex regulatory mechanism of *M. nipponense*.

Conclusion

We examined the tissue distribution of *Mntra-2* and *Mnsxl* in *M. nipponense*. Injection of dsRNA resulted in a successful decrease in the transcriptional levels of the target genes. The RNAi results along with other evidence from the literature indicate that *Mntra-2* and *Mnsxl* play important roles in sex differentiation in *M. nipponense*. However, sex regulation of these two genes was different from that in *D. melanogaster*. Our findings indicate that *Mntra-2* may be an upstream gene involved in the expression of *Mnsxl* in *M. nipponense*, but further research is required to completely understand the sex-determination pathway in this species.

Acknowledgements

This work was supported by the National Natural Science Foundation of China [grant number 31572617]; the Freshwater Fisheries Research Center, China Central Governmental Research Institutional Basic Special Research Project from the Public Welfare Fund [grant number 2015JBFM05]; the Fund of Independent Innovation of Agricultural Sciences of Jiangsu province [grant number CX (15)1012-4]; the Three Aquatic Projects of Jiangsu Province [grant number D2015-16]; and the Science and Technology Development Fund of Wuxi [grant number CLE02N1514].

References

Bai, H.K., Qiao, H., Li, F.J., Fu, H.T., Sun, S.M., Zhang, W.Y., Jin, S.B., Gong, Y.S., Jiang, S.F., and Y.W. Xiong, 2015. Molecular characterization and developmental expression of vitellogenin in the oriental river prawn *Macrobrachium nipponense* and the effects of RNA interference and eyestalk ablation on ovarian maturation. *Gene*, 562: 22-31.

Baker, B.S., and J.M. Belote, 1983. Sex determination and dosage compensation in *Drosophila melanogaster*. *Annu. Rev. Genet*., 17: 345-393.

Bell, L.R., Maine, E.M., Schedl, P., and T.W. Cline, 1988. *Sex-lethal*, a *Drosophila* sex determination switch gene, exhibits sex-specific RNA splicing and sequence similarity to RNA binding proteins. *Cell*, 55: 1037-1046.

Evans, D.S. and T.W. Cline, 2013. *Drosophila* switch gene *Sex-lethal* can bypass its switch-gene target transformer to regulate aspects of female behavior. *Proc. Natl. Acad. Sci.*, 110: e4474-e4481.
Gao, X.G., Liu, H., Xu, J.N., and S.L. Cai, 2006. Study on Site of Vitellogenin Synthesis in the Freshwater Prawn *Macrobrachium Nipponese*. *Biotechnology Bulletin*, supplement 438-444. (In Chinese)
Gempe, T. and M. Beye, 2011. Function and evolution of sex determination mechanisms, genes and pathways in insects. *Bioessays*, 33: 52-60.
Gempe, T., Hasselmann, M., Schiøtt, M., Hause, G., Otte, M., and M. Beye, 2009. Sex determination in honeybees: two separate mechanisms induce and maintain the female pathway. *Plos Biol.*, 7: e1000222.
Heimpel, G.E. and J.G. de Boer, 2008. Sex determination in the Hymenoptera. *Annu. Rev. Entomol*., 53: 209-230.
Kageyama, D., Narita, S., and M. Watanabe, 2012. Insect sex determination manipulated by their endosymbionts: incidences, mechanisms and implications. *Insects*, 3: 161-199.
Kato, Y., Kobayashi, K., Oda, S., Tatarazako, N., Watanabe, H., and T. Iguchi, 2010. Sequence divergence and expression of a transformer gene in the branchiopod crustacean, *Daphnia magna*. *Genomics*, 95: 160-165.
Kato, Y., Kobayashi, K., Watanabe, H., and T. Iguchi, 2011. Environmental sex determination in the branchiopod crustacean *Daphnia magna*: deep conservation of a *Doublesex* gene in the sex-determining pathway. *Plos Genet.*, 7: e1001345.
Leelatanawit, R., Sittikankeaw, K., and P. Yocawibun, 2009. Identification, characterization and expression of sex-related genes in testes of the giant tiger shrimp *Penaeus monodon*. *Comp. Biochem. Phys. A*, 152: 66-76.
Li, F.J., Bai, H.K., Xiong, Y.W., Fu, H.T., Jiang, S.F., Jiang, F.W., Jin, S.B., Sun, S.M., Qiao, H., and W.Y. Zhang, 2015. Molecular characterization of insulin-like androgenic gland hormone-binding protein gene from the oriental river prawn *Macrobrachium nipponense* and investigation of its transcriptional relationship with the insulin-like androgenic gland hormone gene. *Gen. comp. endocrinology*, 216: 152-160.
Li, F., Xiang, J., Zhang, X., Zhou, L., Zhang, C., and C. Wu, 2003. Gonad development characteristics and sex ratio in triploid Chinese shrimp (*Fenneropenaeus chinensis*). *Mar. Biotechnol*., 5: 528-535.
Li, S., Li, F., Wen, R., and J. Xiang, 2012. Identification and characterization of the sex-determiner *transformer-2* homologue in Chinese shrimp, *Fenneropenaeus chinensis*. *Sex. Dev.*, 6: 267-278.
Livak, K.J. and T.D. Schmittgen, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the Method. *Methods*, 25: 402-408.
Lynch, K.W. and T.Maniatis, 1996. Assembly of specific SR protein complexes on distinct regulatory elements of the *Drosophila doublesex* splicing enhancer. *Genes dev*., 10: 2089-2101.
Malecha, S.R., Nevin, P.A., Ha, P., Barck, L.E, Lamadrid-Rose, Y., Masuno, S., and D. Hedgecock, 1992. Sex-ratios and sex-determination in progeny from crosses of surgically sex-reversed freshwater prawns, *Macrobrachium rosenbergii*. *Aquaculture*, 105: 201-218.
Niimi, T., Sahara, K., Oshima, H., Yasukochi, Y., Ikeo, K., and W. Traut, 2006. Molecular cloning and chromosomal localization of the *Bombyx Sex-lethal* gene. *Genome*, 49: 263-268.
Penalva, L. O. and Sanchez, L. 2003. RNA binding protein *Sex-lethal* (*Sxl*) and control of *Drosophila* sex determination and dosage compensation. *Microbiol. Mol. Biol. Rev.*, 67: 343-359.
Qiao, H., Xiong, Y.W., Zhang, W.Y., Fu, H.T., Jiang, S.F., Sun, S.M., Bai, H.K., Jin, S.B., and Y.S. Gong, 2015. Characterization, expression, and function analysis of gonad-inhibiting hormone in Oriental River prawn, *Macrobrachium nipponense* and its induced expression by temperature. *Comp. Biochem. Phys. A*, 185: 1-8.
Sakai, H., Aoki, F., and M.G. Suzuki, 2014. Identification of the key stages for sex determination in the silkworm, *Bombyx mori*. *Dev. Genes. Evol.*, 224: 119-123.

Schutt, C. and R. Nothiger, 2000. Structure, function and evolution of sex-determining systems in Dipteran insects. *Development*, 127: 667-677.
Shen, H., Hu, Y., and X. Zhou, 2014. *Sex-lethal* gene of the Chinese mitten crab *Eriocheir sinensis*: cDNA cloning, induction by eyestalk ablation, and expression of two splice variants in males and females. *Dev. Genes. evol.*, 224: 97-105.
Shukla, J.N. and S.R. Palli, 2013. *Tribolium castaneum Transformer-2* regulates sex determination and development in both males and females. *Insect biochem. Molec.*, 43: 1125-1132.
Sievert, V., Kuhn, S., and W. Traut, 1997. Expression of the sex determining cascade genes *Sex-lethal* and *doublesex* in the phorid fly *Megaselia scalaris*. *Genome*, 40: 211-214.
Suzuki, M.G., Imanishi, S., Dohmae, N., Asanuma, M., and S. Matsumoto, 2010. Identification of a male-specific RNA binding protein that regulates sex-specific splicing of *Bmdsx* by increasing RNA binding activity of *BmPSI*. *Mol. Cell. Boil.*, 30: 5776-5786.
Suzuki, M.G., Suzuki, K., Aoki, F., and M. Ajimura, 2012. Effect of RNAi-mediated knockdown of the *Bombyx mori transformer-2*gene on the sex-specific splicing of *Bmdsx* pre-mRNA. *Int. J. Dev. Biol.*, 56: 693-699.
Zhang, E.F. and G.F. Qiu, 2010. A novel *Dmrt* gene is specifically expressed in the testis of Chinese mitten crab, *Eriocheir sinensis*. *Dev. genes evol*., 220: 151-159.
Zhang, Y.P., Fu, H.T., Qiao, H., Jin, S.B., Jiang, S.F., Xiong, Y.W., and X. Zhang, 2013a. Molecular Cloning and Expression Analysis of *Transformer-2* Gene During Development in *Macrobrachium nipponense* (de Haan 1849). Journal of the world Aquaculture Society 44: 338-349.
Zhang, Z., Klein, J., and Nei, M. 2014. Evolution of the *Sex-lethal* gene in insects and origin of the Sex-determination system in *Drosophila*. *J. world. Aquacult. Soc*., 78: 50-65.
Zhang, Y.P., Qiao, H., Zhang, W.Y., Sun, S.M., Jiang, S.F., Gong, Y.S., and H.T. Fu, 2013b. Molecular cloning and expression analysis of two sex-lethal homolog genes during development in the oriental river prawn, *Macrobrachium nipponense*. *Genet. Mol. Res.*, 12: 4698-4711.