RNA interference analysis of potential functions of cyclin A in the reproductive development of male oriental river prawns (*Macrobrachium nipponense*)

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Cyclin A (CycA) plays essential roles in regulating multiple steps of the cell cycle, and it affects gonad development in mammals and invertebrates. Previous RNA interference (RNAi) analysis revealed that knocking-down the expression of CycA in female oriental river prawns (*Macrobrachium nipponense*) inhibited ovarian development. CycA was also predicted to have regulatory roles in reproductive development of male *M. nipponense* based on significant changes of Mn-CycA expression after eyestalk ablation. The goal of this study was to investigate the potential functions of CycA in the reproductive development of male *M. nipponense* using RNAi and histological observations. Quantitative real-time PCR analysis revealed that both single-side and double-side eyestalk ablation stimulated the expressions of Mn-CycA, and the expression was higher in prawns with double-side eyestalk ablation (*p* < 0.05). Mn-CycA expression was significantly higher in the testis and androgenic gland during the reproductive season than during the non-reproductive season (*p* < 0.05). In the RNAi analysis, Mn-CycA expression significantly decreased after prawns were injected with dsCycA, and the expression of insulin-like androgenic gland hormone (Mn-IAG) also decreased as Mn-CycA expression decreased. This result indicated that CycA positively regulated the expression of IAG in *M. nipponense*. Histological observations revealed that the number of sperm decreased dramatically to <5% of the total cells in the testis of the dsCycA-treated group compared to that of control group on day 14, indicating that knockdown of Mn-CycA expression inhibited testis development by affecting the expression of Mn-IAG in *M. nipponense*. These results highlighted the functions of CycA in male reproductive development of *M. nipponense*, which can be applied to future studies of male reproduction in other crustacean species.

**KEYWORDS**

*Macrobrachium nipponense*, cyclin A, RNA interference, insulin-like androgenic gland hormone, male reproduction
Introduction

The oriental river prawn (Macrobrachium nipponense) (Crustacea; Decapoda; Palaeomonidae) is widely distributed in China and other Asian countries, where it inhabits freshwater and low salinity estuarine regions (Jin S. B. et al., 2021). It is an economically important freshwater prawn in China, with annual aquaculture production of 225,321 metric tons in 2019 (Zhang et al., 2020). Previous histological observations revealed that newly hatched male and female M. nipponense can reach sexual maturity within 40 days after hatching during the reproductive season (Jin et al., 2016). The rapid gonad development of hatchlings leads to inbreeding between young prawns, resulting in mating and propagation of multiple generations in the same ponds. This leads to prawns with smaller market size, thereby restricting the sustainable development of the M. nipponense industry (Jin et al., 2022). Thus, the long-term goal of genetic improvements in M. nipponense is to regulate the process of gonad development.

Jin S. B. et al. (2021) previously reported that the expression of Mn-Cyclin A (Mn-CycA) increased after the ablation of eyestalks from male M. nipponense. They also found that eyestalk ablation promoted testis development by stimulating the expression of insulin-like androgenic gland hormone (IAG) (Jin S. B. et al., 2021; Jin et al., 2021b). The negative regulatory relationship between CycA and eyestalks suggested that CycA may be involved in regulating male reproductive development in M. nipponense.

Cyclins are synthesized and degraded in a cell cycle-dependent fashion. They are involved in cell cycle regulation through the formation of integral regulatory subunits of protein kinase complexes with cyclin-dependent kinases (CDKs). CycA is involved in the regulation of multiple steps in the cell cycle, including the S and G2/M phases of Saccharomyces cerevisiae and animal cells (Tarn and Lai, 2011). CycA binds with CDK2 during G1/S conversion to promote replication of meiotic chromosomes and to enhance transcriptional activity of genes encoding estrogen and progesterone receptors (Rogatsky et al., 1986; Narayanan et al., 2005). CycA also forms a complex with CDK1 during the late S phase, and the compound activates and stabilizes cyclin B and CDK1 (Minshull et al., 1990; Lees and Harlow, 1993; Li et al., 2004).

The full-length cDNA sequence of Mn-CycA has been submitted to NCBI with the accession number of MT802360.1. In a previous study of M. nipponense, CycA expression was highest in the ovary and then testis, and the values were significantly higher than those of the other tested tissues, indicating the CycA may play regulatory roles in gonad development in M. nipponense (Zhou Z. Y. et al., 2021). RNA interference (RNAi) analysis in female M. nipponense revealed that knockdown of CycA expression inhibited ovarian development (Zhou Z. Y. et al., 2021). However, the regulatory roles of CycA on the reproductive development of male M. nipponense still needs to be investigated.

The goal of this study was to analyze the potential functions of CycA in male reproductive development of M. nipponense using quantitative real-time PCR (qPCR), in situ hybridization, RNAi, and histological observations. The results of this study highlighted the functions of CycA in M. nipponense, providing a basis for further studies of the mechanism of male reproduction in other crustacean species.

Materials and methods

Ethics statement

We obtained permission from the Institutional Animal Care and Use Ethics Committee of the Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences (Wuxi, China) to conduct all experiments involving M. nipponense.

The animal study was reviewed and approved by We obtained the permission from the Institutional Animal Care and Use Ethics Committee of the Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences (Wuxi, China) for all experiments involving M. nipponense. Written informed consent was obtained from the owners for the participation of their animals in this study.

The qPCR analysis

The qPCR was used to measure the relative mRNA expression of Mn-CycA in the testis after the ablation of eyestalks from male M. nipponense and the expression in the testis and androgenic gland of male prawns collected during the reproductive and non-reproductive seasons. Three hundred healthy male M. nipponense (body weight, 3.47–3.89 g) were collected from the Dapu M. nipponense Breeding Base in Wuxi, China (120°13′E, 31°28′N). The prawns were randomly divided into three groups: control (CG), single-side eyestalk ablation (SS), and double-side eyestalk ablation (DS). They were transferred into three 500 L tanks and maintained in aerated freshwater for 3 days before eyestalk ablation. The testes were collected from prawns in each group on days 1, 4, and 7 and immediately preserved in liquid nitrogen until used for qPCR analysis. Testes from five different prawns were pooled to form a biological replicate, and three biological replicates were analyzed for each time point.

For the temporal comparison, testis and androgenic gland were collected from male M. nipponense during the reproductive season in July, when the water temperature was 30 ± 2 °C and the illumination time was ≥16 h. Tissues
TABLE 1 universal and specific primers used in this study.

| Primer name   | Nucleotide sequence (5’→3’)                                      | Purpose                      |
|--------------|-----------------------------------------------------------------|------------------------------|
| CycA-RF      | TGCCCAAACCGAAGTCATTTC                                          | FWD primer for Ferritin expression |
| CycA-RTR     | TATGCTTGAGCCGATGCTTCAAA                                       | RVS primer for Ferritin expression |
| CycA anti-sense Probe | CCGAGCAGGAAAGGAGGGAGTCAGCATGAAAC | Probe for Ferritin ISH analysis |
| CycA sense Probe | GTTTGATACCTACACTATAGGGAAATGTTTTGCGCCAAACCAGA              | Probe for Ferritin ISH analysis |
| CycA RNAi-F  | TAATAGCCTACTATAGGGAAATGTTTTGCGCCAAACCAGA                         | RVS primer for RNAi analysis |
| CycA RNAi-R  | TAATAGCCTACTATAGGGAAATGTTTTGCGCCAAACCAGA                         | FWD primer for RNAi analysis |
| EIF-F        | CATGATGTACAATTGATGAAAC                                          | RVS primer for EIF expression |
| EIF-R        | CGTCAGGAGAAGGTGGTCCTTCA                                        | FWD primer for EIF expression |

In situ hybridization

In situ hybridization was used to determine the mRNA locations of Mn-CycA in the testis and androgenic gland in shrimp collected during the reproductive season in July. The tissues were fixed in paraformaldehyde (4%) (Sangon, Shanghai, China), and then they were embedded in paraffin and sliced into 4 μm thick sections for the analysis. Details about the primer design and in situ hybridization can be found in previous reports (Jin et al., 2018; Li et al., 2018; Jin et al., 2022). Briefly, the specific anti-sense (experimental group) and sense (control group) probes were designed using Primer5 software based on the cDNA sequence of Mn-CycA. The specific primers with a digoxigenin (DIG) tag were synthesized by Shanghai Sangon Biotech Company (Table 1). The Zytostat PLUS CISH Implementation Kit (Zyto Vision GMBH, Bremerhaven, Germany) was used to perform the in situ hybridization analysis of the sectioned tissues following the manufacturer’s instructions. Slides were examined under a light microscope (Olympus Corporation, Tokyo, Japan).

RNAi analysis

The potential function of CycA in reproductive development of male *M. nipponense* was investigated by RNAi. Snap Dragon tools (http://www.flyrnai.org/cgi-bin/RNAifind_primers.pl) was used to design the specific RNAi primer with a T7 promoter site based on the open reading frame of Mn-CycA (Table 1). The Mn-CycA dsRNA (dsCycA) was synthesized using the Transcript Aid™ T7 High Yield Transcription kit (Fermentas, Waltham, MA, United States) following the manufacturer’s protocol. The green fluorescent protein dsRNA (dsGFP) was also synthesized and used as the negative control (Zhang et al., 2016).

Six hundred male *M. nipponense* were collected from the Dapu *M. nipponense* Breeding Base approximately 5 months after hatching (body weight, 3.15–4.37 g) and randomly divided into the dsCycA group (RNAi) and dsGFP group (control). The injected dose of dsCycA and dsGFP was 4 μg/g (Jiang et al., 2014; Li et al., 2018). Seven days after the first injection, 4 μg/g of dsCycA or dsGFP were injected again. Androgenic gland samples were collected from both the control group and the RNAi group on days 1, 7, and 14 after dsGFP or dsCycA injection. Androgenic gland samples from five different prawns at each time point were collected and pooled to form a biological replicate, and three biological replicates were tested. The Mn-CycA mRNA expression in the samples was measured by qPCR in order to confirm the silencing efficiency. The mRNA expression of *Mn-IAG* was also measured using the same cDNA templates to...
evaluate the regulatory relationship between CycA and IAG in *M. nipponense*.

**Haematoxylin and eosin (HE) staining**

Morphological differences in the testis between prawns in the control and RNAi groups were assessed by HE staining following previously reported procedures (ShangGuan et al., 1991; Ma et al., 2006). Briefly, the tissues were dehydrated in different concentrations of ethanol and then embedded in xylene and wax. The embedded tissues were sectioned to a thickness of 5 µm using a microtome (Leica, Wetzlar, Germany). The sectioned tissues were placed on a slide and stained with HE for 3–8 min. The slides were observed under an Olympus SZX16 microscope (Olympus Corporation, Tokyo, Japan).

**Statistical analysis**

All statistical analyses were conducted using SPSS Statistics 23.0 (IBM, Armonk, NY, United States). The independent samples t-test was used to evaluate differences between the control and RNAi groups on the same day. Statistical differences were also identified by analysis of variance followed by least significant difference and Duncan’s multiple range tests. Quantitative data were expressed as mean ± standard deviation. A *p*-value < 0.05 was considered to be statistically significant.

**Results**

The qPCR analysis

The physiological functions of a gene can be preliminary reflected by tissues distribution. The mRNA expression of *Mn-CycA* did not differ significantly in the testes between different days in control prawns (*p* > 0.05) (Figure 1). However, both single-side and double-side eyestalk ablation stimulated the mRNA expression of *Mn-CycA*. Expression continuously increased from day 1 to day 7, when the expression levels after single-side and double-side eyestalk ablation were 5.12-fold and 8.17-fold higher than that of day 1 in control prawns, respectively (*p* < 0.05). Furthermore, *Mn-CycA* expression in the prawns with double-side ablation was significantly higher than those of the control and single-side ablation groups on the same day after eyestalk ablation (*p* < 0.05). The qPCR analysis also showed that *Mn-CycA* expression in the testes and androgenic gland were 4.12-fold and 2.98-fold higher, respectively, during the reproductive season than during the non-reproductive season (*p* < 0.05) (Figure 2).
In situ hybridization

In the in situ hybridization assay, HE staining revealed that the testis was composed of spermatogonia, spermatocytes, and sperm, and the androgenic gland includes the ejaculatory bulb and androgenic gland cells (Figure 3). No signal was directly observed in the androgenic gland cells, whereas DIG signals were observed in the ejaculatory bulb surrounding the androgenic gland cells. In addition, strong DIG signals were observed in the spermatogonia of the testis, whereas no signal was observed in the spermatocytes and sperm (Figure 3).

RNAi analysis

In the RNAi experiment, the mRNA expression of Mn-CycA in the androgenic gland did not differ significantly between different days after the dsGFP treatment, and this result was verified by qPCR analysis (p > 0.05). However, a decrease of >90% was detected on days 7 and 14 after dsCycA treatment compared to that of the dsGFP-injected group on the same day (p < 0.01) (Figure 4A). The expression of Mn-IAG did not differ significantly on day 1 after dsCycA injection compared to that of the dsGFP group (p > 0.05). However, it decreased by >80% on days 7 and 14 in the dsCycA-injected group compared to the dsGFP group on the same day (p < 0.01) (Figure 4B).

Histological observations

Histological observations revealed that the cell types did not differ on different days in the control group, and over 50% of the cells were sperm in the testis of dsGFP-treated prawns.
contrast, knockdown of the expression of Mn-CycA significantly decreased the number of sperm in the testis, and <5% cells were sperm on day 14 in the dsCycA-treated prawns (Figure 5).

Discussion

Tarn and Lai (2011) reported that CycA regulates multiple steps of the cell cycle and plays a critical role in the S and G2/M phases in animal cells. A previous study of CycA in M. nipponense revealed that knockdown of CycA expression resulted in delayed ovarian development in M. nipponense (Zhou Z. Y. et al., 2021). In addition, Zhou Z. Y. et al. (2021) found that CycA expression was highest in the ovary and then the testis, and Jin S. B. et al. (2021) reported that its expression significantly increased in the androgenic gland at 7 days after eyestalk ablation from male M. nipponense. These results suggested that CycA was involved in the regulation of reproductive development in male M. nipponense, and in this study we further investigated the potential roles of CycA in this process.

To the best of our knowledge, we are the first to analyse the regulatory relationship between the eyestalk and CycA expression in a crustacean species. The qPCR analysis revealed that the expression of Mn-CycA significantly increased over time after both single-side and double-side eyestalk ablation, and expression was higher in the double-side eyestalk ablation group. This result indicated that double-side eyestalk ablation had a greater effect on promoting male reproductive development than single-side eyestalk ablation, which is consistent with results of previous studies (Jin S. B. et al., 2021; Jin et al., 2021b). The X-organ–SG complex is located in the eyestalk and is a principal neuroendocrine gland that stores and releases many neurosecretory hormones (Hopkins, 2012), including ion transport peptides, gonad-inhibiting hormone (GIH), crustacean hyperglycemic hormone (CHH), molt inhibiting hormone (MIH), and mandibular organ-inhibiting hormone. These hormones play essential roles in many biological functions of crustacean species (Santos et al., 1997; Almeida et al., 2004; Tiu and Chan, 2007; Sainz-Hernandez et al., 2008; Treerattrakool et al., 2011; Diarte-Plata et al., 2012; Pamuru et al., 2012; Salma et al., 2012; Revathi et al., 2013; Shen et al., 2013; Treerattrakool et al., 2013). In M. nipponense, CHH and MIH were reported to be involved in regulation of testis development and molting, respectively (Jin et al., 2013; Qiao et al., 2018). Additionally, the neurosecretory hormones secreted by eyestalks were found to have negative regulatory effects on both testis and ovarian development in M. nipponense. Qiao et al. (2015) showed that RNAi of GIH significantly promoted ovarian development in M. nipponense. Ablation of eyestalks from male M. nipponense stimulated the expression of Mn-IAG (Jin S. B. et al., 2021), with promoted testis development (Jin et al., 2021b). In the current study, the continuous increase of Mn-CycA expression after eyestalk ablation from male M. nipponense indicated that CycA may positively regulate reproductive development of male prawns. qPCR analysis also revealed higher Mn-CycA expression in the testis and androgenic gland during the reproductive season than during the non-reproductive season, which further supported the role of CycA in regulating reproductive development of male M. nipponense.

Prior to this study, in situ hybridization analysis of CycA had only been reported for female M. nipponense. Zhou Z. Y. et al. (2021) detected DIG signals of Mn-CycA throughout ovarian development, and they were mainly located in oogonia and oocytes. In male crustaceans, the androgenic gland and testis are the main reproductive organs (Sagi et al., 1986; Sagi and Cohen, 1990; Qiu et al., 1995). In the current study, DIG signals were observed in the ejaculatory bulb that surrounds the androgenic gland but not in androgenic gland cells, indicating that CycA did not directly involved in process of sex determination and differentiation in M. nipponense, while it played essential roles in the formation and maintenance of the normal structure of the androgenic gland (Jin et al., 2018; JinC et al., 2021; Jin et al., 2022). In the testis, DIG signals were only observed in the spermatogonia, indicating that CycA was involved in the process of spermatogenesis in M. nipponense, especially that of the spermatogonia proliferation (JinC et al., 2021; Jin et al., 2022).

The expression of CycA is known to affect the process of testis and ovarian development in many mammals and invertebrates.
Liu et al. (1998) reported that targeted deletion of CycA1 from male mice blocked spermatogenesis development before the first meiosis, resulting in male infertility. Female fruit flies (Drosophila) can lay eggs after knockdown of the expressions of CycA, but the eggs cannot hatch. In addition, low expression of CycA can result in delayed rupture of the meiotic nuclear membrane and mislocalization of the middle of the spindle at the end of mitosis (Bergman et al., 2015; Bourouh et al., 2016). Sandra et al. (2012) found that the development of germinal vesicle rupture and G2/M was inhibited during meiotic phase I after injection of CycA antibody into mouse oocytes during meiosis. Knockdown of CycA2 expression in oocytes at meiotic stage II inhibited the separation of sister chromatids. In the silk moth Bombyx mori, ovarian development was blocked in the G1 phase after knockdown of the expression of CycA in ovarian cells, which effectively inhibited cell proliferation (Liu et al., 2015). Knockdown of the expression of CycA in female M. nipponense by RNAi resulted in delayed ovarian development (Zhou Z. Y. et al., 2021). In the present study, RNAi was also employed to investigate the potential functions of CycA in the reproduction of male M. nipponense. The injection of dsCycA resulted in a significant decrease of Mn-CycA and Mn-IAG expressions on days 7 and 14, indicating that the synthesized dsCycA efficiently knocked-down the expression of these genes and that CycA has a positive regulatory role in the expression of IAG in M. nipponense. IAG is the main gene expressed in the androgenic gland, and its regulatory effects on male differentiation and reproduction have been well studied in Macrobrachium rosenbergii, especially those related to spermatogenesis (Sagi et al., 1986; Sagi and Cohen, 1990; Ventura et al., 2012). Similar functions of IAG have been reported in many other crustacean species (Li et al., 2012; Huang et al., 2014; Ma et al., 2016; Zhou T. T. et al., 2021; Liu, 2021). Histological observations revealed that sperm were the main cell type in the testis of dsGFP-injected prawns but that they accounted for <5% of cells on day 14 after dsCycA injection. This result indicated that RNAi of Mn-CycA had a significant inhibitory role on testis development of M. nipponense, which confirmed the positive regulatory roles of CycA in the regulation of testis development in this prawn.

In conclusion, eyestalk ablation from male M. nipponense continuously stimulated the expression of Mn-CycA, and Mn-CycA expression was higher in the testis and androgenic gland during the reproductive season than during the non-reproductive season. Knockdown of the expression of Mn-CycA by RNAi in male M. nipponense revealed that CycA had a positive regulatory effect on testis development of this prawn, which was verified by histological observations of testis samples from dsGFP-injected and dsCycA-injected prawns. These results showed that CycA had an important role in the reproductive development of male M. nipponense, which can be applied to the development of techniques to regulate testis development in M. nipponense.

**Data availability statement**

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

**Ethics statement**

The animal study was reviewed and approved by the Institutional Animal Care and Use Ethics Committee of the Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences (Wuxi, China) for all experiments involving M. nipponense. Written informed consent was obtained from the owners for the participation of their animals in this study.

**Author contributions**

WZ wrote the manuscript. YX provided the experimental prawns. PW performed the qPCR analysis. TC synthesized the dsRNA. SJ performed the in situ hybridization analysis. HQ performed the histological observations. YG ablated the eyestalk from male M. nipponense. YW extracted RNA for this study. SJ revised the manuscript. HF supervised the experiment.

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**Conflict of interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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