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Profiles of cyclin B and cdc2 during ovarian and embryonic development in *Exopalaemon carinicauda*

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Abstract

Mitosis-promoting factor (MPF) is a complex formed by cyclin B (cyclin B) and cyclin-dependent kinase (cdc2). To investigate the role of MPF in the reproduction of *Exopalaemon carinicauda*, we cloned its full-length cDNA of the *Ec-cyclin B* and *Ec-cdc2* genes. We analyzed their molecular characteristics and expression profiles during ovarian and embryonic development. The results showed that the *Ec-cyclin B* gene was 1194 bp long and encoded a 397 amino acid (aa) long protein. However, *Ec-cdc2* was 900 bp long, which encoded 299 aa with a conserved cyclin binding motif PSTAIRE. The phylogenetic tree analysis showed that *Ec-cyclin B* had the highest homology with the *cyclin B* of *Macrobrachium rosenbergii* (81.06%). In comparison, *Ec-cdc2* had the highest homology with the *cdc2* of *E. modestus* (96.80%). *Ec-cyclin B* showed the highest expression in the ovary, whereas *Ec-cdc2* was the highest in the hepatopancreas, followed by the ovary. In the five stages of ovarian development, Ec-cyclin B and Ec-cdc2 expression levels reach the highest at stage Ⅴ($p < 0.05$). Overall, the expression of these two genes first increased and then decreased at different embryonic developmental stages. Therefore, these findings suggested that *cyclin B* and *cdc2* played an essential role in the ovarian and embryonic development of *E. carinicauda*.

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Introduction

The maturation of oocytes depends on the interaction of gonadotropin, maturation-inducing hormone, and MPF, which then induces the maturation of oocytes (Zhou et al., 2021). An extensive and elaborate oocyte maturation regulatory network in the organism is formed, centered on the MPF, and involves many signal factors (Wu, 2009; Han et al., 2010). Masui and Markert (1971) first discovered the MPF as an activator, which causes oocytes to resume meiosis, thus inducing nuclear membrane rupture of oocytes in unfertilized eggs of *Xenopus laevis*, while also proving that MPF was a heterodimer comprising regulatory subunit cyclin B and catalytic subunit cyclin-dependent kinase cdc2, which are highly conserved among different species. Cyclin B synthesis varies with the cell cycle and is hydrolyzed upon the completion of its function. Cdc2 kinase functions as a catalytic subunit by combining with its regulatory subunit cyclin B to form then MPF, which then induces the meiotic maturation of oocytes or G2/M phase transition in the eukaryotic cell cycle (Yamashita, 1998). With the recent development of research on the reproductive biology of shrimps and crabs, numerous studies have focused on the two subunits of MPF. Currently, the *cyclin B* gene has been studied in some crustaceans, including *Marsupenaeus japonicus*, *Penaeus monodon*, *Eriocheir sinensis*, *Scylla paramamosain* (Li et al., 2013; Wu, 2009), whereas the *cdc2* gene has been studied in *Penaeus monodon*, *Eriocheir sinensis, Cherax quadricarinatus* and *Scylla serrata* (Qiu and Liu, 2009; Wang et al., 2013).

Exopalaemon carinicauda is an economically significant shrimp in China due to its short breeding cycle, environmental solid adaptability, delicious meat, etc. (Gao et al., 2021). These have made it one of the main shrimps cultured in the monoculture and mixed culture ponds in China; it contributes to 30% of the production in polyculture ponds in eastern China (Liang et al., 2020; Wang et al., 2021; Deng et al., 2021). The ovary of *E. carinicauda* can develop several times a year. However, the artificial large-scale breeding technology of *E. carinicauda* has not been resolved yet, due to the ovaries of different individuals developing asynchronously. The juvenile *E. carinicauda* are mainly obtained from natural sea areas (Li et al., 2019; Liang et al., 2020; Zhang et al., 2022a, 2022b). A need exists for a clearer understanding of the reproduction processes in this shrimp to provide the necessary background knowledge to support *E. carinicauda* farming. Therefore, we cloned the *cyclin B* and *cdc2* genes from *E. carinicauda* in this study. *We analyzed their functions in the crustacean ovary and embryo development, thus allowing us to understand its underlying molecular regulatory mechanisms*.

Materials and methods

Animals and culture conditions

Adult *E. carinicauda* (1.93 ± 0.21 g) was obtained using artificial propagation and rearing procedures by culturing them in polyvinyl chloride polymer (PVC) tanks with continuous oxygenation. The seawater salinity was 5 ppt, pH 8.0, and temperature 24 ± 1℃. The water in each tank was replaced daily to remove any contaminants.

Sample preparation

We collected nine healthy tissues, including the stomach, gill, intestine, muscle, hepatopancreas, ovarian tissues, and embryos at different developmental stages, to analyze the expression patterns of *cyclin B* and *cdc2* gene from *E. carinicauda*. Based on the color, external morphology, and histological characteristics of the ovarian tissue, the development of ovary was divided into five stages (Liang et al., 2020; Wang, 1987). Embryo developmental stages were determined according to the literature description (Liang et al., 2020).

Total RNA extraction and cDNA synthesis

The total RNA from each tissue was extracted using the Trizol reagent (Invitrogen, USA). The concentration and purity of RNA were evaluated spectrophotometrically via a NanoDrop 2000 UV spectrophotometer. The RNA integrity was evaluated using a 1.5% agarose gel. The cDNA was obtained by reverse transcription using the HiScript® III RT SuperMix for the qPCR (+gDNA wiper) kit. The cDNAs of 5′ RACE and 3′ RACE were synthesized per the manufacturer's instructions using the SMART™ amplification kit (Clontech, Japan) and stored at -20ºC for further use.

Cloning of Ec-cyclin B and cdc2 full-length cDNA

The degenerate primers of *cyclin B*-F1 and *cyclin B*-R1 were designed using the Primer Premier 5.0 software, based on the conserved domains in *cyclin B* cDNA sequences in *Scylla paramamosain* (GenBank: FJ705251.2), *Eriocheir sinensis* (GenBank: EU622123.1), and *Macrobrachium rosenbergii* (GenBank: HQ395226.1). Specific primers *cyclin B* 5' and *cyclin B* 3' were designed using Primer Premier 5.0 software, with the 5' and 3' sequences of *cyclin B* being amplified according to the SMART™ RACE kit instructions.

The degenerate primers of *cdc2*-F and *cdc2*-R were designed using Primer Premier 5.0 software, based on the conserved domains in *cdc2* cDNA sequences *Scylla paramamosain* (GenBank: FJ015041.1), *Eriocheir sinensis* (GenBank: FJ210468.1), and *Penaeus monodon* (GenBank: EU492538.1). The specific primers *cdc2* 5' and *cdc2* 3' were designed using Primer Premier 5.0 software, with the 5' and 3' sequences of *cdc2* being amplified according to the SMART™ RACE kit instructions.

The cloning procedures for *Ec-cyclin B* and *Ec-cdc2* were conducted in three steps. First, their respective cDNAs were amplified by following their 3′-RACE and 5′-RACE products and also for universal primer (UPM). The PCR reaction procedures were followed as previously described (Liang et al., 2016). To isolate the PCR products, 1% agarose gel was used, and its target band was purified using a PCR purification kit (Promega, USA), followed by ligation to a PMD vector (TaKaRa, Japan), and transformed in *Escherichia coli* TOP10 cells (Invitrogen, USA). Recombinant bacteria were identified using the blue/white screening method and confirmed using PCR (Liang et al., 2016). Three positive clone cell solutions were selected for sequencing. The sequences of four fragments obtained were spliced to the full-length *Ec-cyclin B* and *Ec-cdc2* cDNAs.

Sequence analysis and construction of phylogenetic tree

The ORF Finder software (https://www.ncbi.nlm.nih.gov/orffindr/) was used to obtain the gene open reading frame (ORF). The theoretical isoelectric point and molecular weight of the predicted protein encoded by the gene were calculated using DNASTAR. The BLAST (https://www.ncbi.nlm.nih.gov/) was used to align the homology of amino acids among different species. InterproScan (http://www.ebi.ac.uk/interpro/searc/sequence/) was used to analyze the protein function structure domain, while MEGA 7.0 was used for multiple alignments of amino acid sequences and phylogenetic tree construction using the NJ (neighbour--joining) method.

Gene expression analysis

The quantitative real-time PCR (qRT-PCR) gene primers were designed using the full-length cDNA as the template (**Table 1**), with the 18S rRNA used as the internal reference gene. The cDNA from six different tissues of adult shrimp, the ovarian tissues from different development stages, and embryonic at different developmental stages were used as the templates, with the reaction system and procedures being as per the instructions for the SYBR Premix Ex Taq kit (TaKaRa, Japan) for qRT-PCR amplification. The quantitative results were statistically analyzed using the $2^{-\Delta\Delta CT}$ method (Chen et al., 2017) and then analyzed with the SPSS 19.0

software for ANOVA and multiple comparisons ($p < 0.05$ indicated a significant difference).

Table 1 The sequence of primers used in the study

Gene names	Sequence (5'-3')	Usage
Cyclin B	F-TBGAYAGATWYCTCCAG R-TAYTTYWGYCTYACRGCCTG	intermediate amplification
	5'- ACGGGGTATGTTTCTTTCAACCTGGAG 3'- ATTGCCGCCGCAGCACTCTGTCTTTCC	Race PCR
	F-GGAGCTGTGTTTGTTGGAATAC R-CAGTCGCTGCCATCAGATAA	Real-time RT-PCR
Cdc2	F-GTGGTRTACAARGCSAAGAACCG R-GCTCATCTGTATTCATCTGCTTA	intermediate amplification
	5'-TCCTTAGCAGTTATTCTTCGGGTTGGGTC 3'-GGCCTGGWGTRACRCARCTGCAGGACTAC	Race PCR
	F-TGGGTCCTCCCGATATTCTT R-CTGAATCACCATGGAAGAGAGG	Real-time RT-PCR
M13	F-CGCCAGGGTTTTCCCAGTCACGAC R-AGCGGATAACAATTTCACACAGGA	bacterial suspension PCR
18s	F-GGGGAGGTAGTGACGAAAAAT R-TATACGCTAGTGGAGCTGGAA	Real-time RT-PCR

Results

Cloning and sequence analysis of Ec-cyclin B and Ec-cdc2 genes

The *cyclin B* gene's cDNA sequence being cloned had a length of 2,309 bp, with a 1,194 bp ORF. It encoded a 397 amino acid long protein with a molecular weight of 45.38 k Da and a calculated theoretical isoelectric point of 9.18. The cDNA sequence was registered in GenBank under the accession number KP202169.1. Upon comparing the amino acid sequences of cyclin B from *E. carinicauda* and those from other species, the Ec-cyclin B aa was highly conserved and had a proteinase K site (RRXSK) unique to cell cycle proteins (**Figure 1**).

The cDNA sequence of the *Ec-cdc2* gene was 1,598 bp, with an ORF of 900 bp. It encoded a 299 amino acid long protein with a molecular weight of 34.71 kDa and a calculated theoretical isoelectric point of 6.61. The cDNA sequence has been registered in GenBank with the accession number KP202168.1. And the serine/threonine protein kinase active site (124–136 aa) and the conserved ATP-binding sequence GxGxxGxV are both present in the *E. carinicauda* cdc2. The PSTAIRE helix, the conserved cyclin binding sequence, and the distinct CDK sequence were all present in Ec-cdc2 (**Figure 1**).

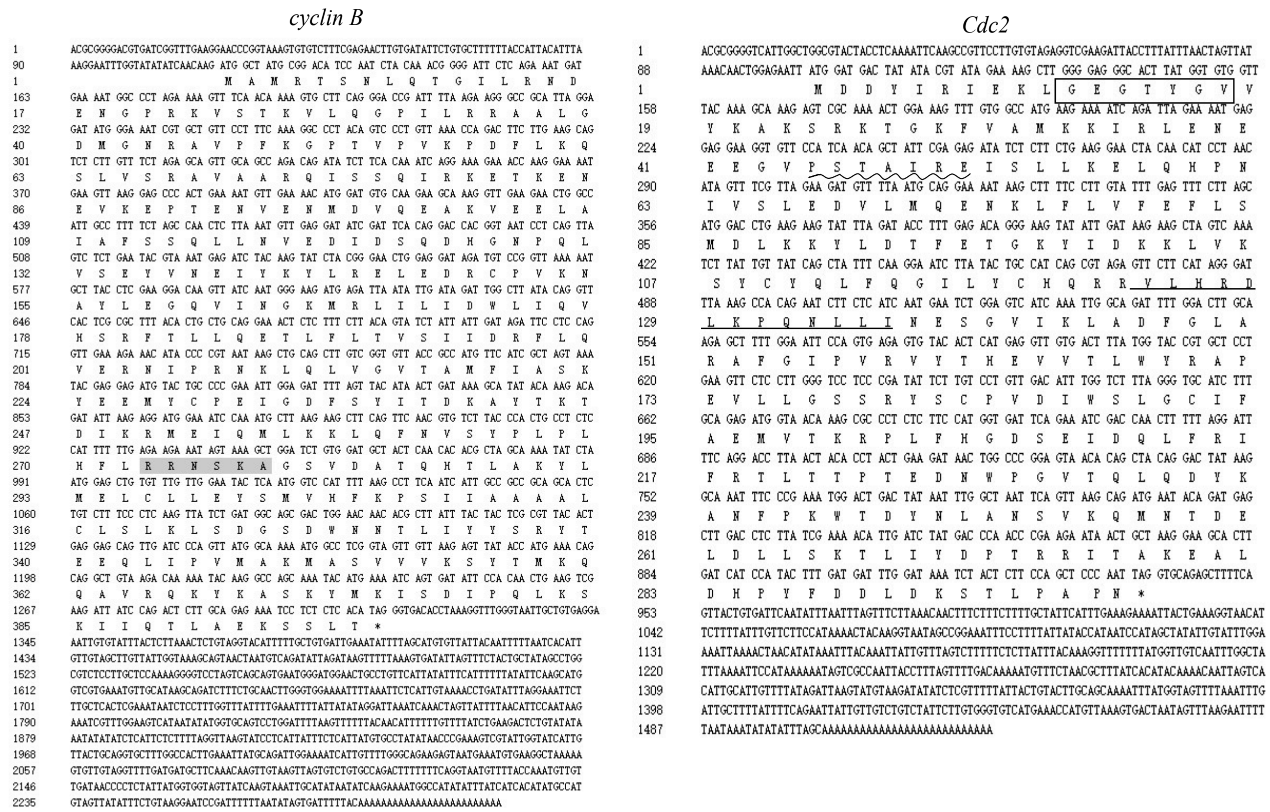

Figure 1 Nucleotide sequence and deduced amino acid sequence of *Ec-cyclin B* and *Ec-cdc2*
Note: "*" represents no translation amino acid; gray shading indicates the sequence of cyclin B characterized proteinase K site (RRXSK); conserved sequence GXGXXGXV related to ATP-binding is box; the underline of curve represented PSTAIRE sequences; the serine/threonine protein kinase active site is underlined.

Homology analysis of the amino acid sequence of Ec-cyclin B and Ec-cdc2

Using BLAST, the amino acid sequences of Ec-Cyclin B and Ec-cdc2 were compared to those of other species. The results showed that Ec-Cyclin B and cyclin B *of M. rosenbergii* shared the greatest similarity (81.06%), while Cyclin B from *M. nipponense* had the second highest similarity (76.67%) (**Table 2**). The cdc2 from *E. carinicauda* and *P. modestus* shared the highest similarity of 96.80%, while that from *M. nipponense* shared the second-highest similarity (**Table 3**).

Table 2 Comparison of amino acid sequences of *Ec-cyclin B* with those of *cyclin B* of other species

Species	Amino acid similarity/%	GenBank ID
Macrobrachium rosenbergii	81.06	HQ395226.1
Macrobrachium nipponense	76.67	FJ919326.1
Marsupenaeus japonicus	72.13	EU707332.1
Penaeus monodon	71.46	KAA0706147.1
Metapenaeus ensis	70.39	HM113467.1
Metapenaeus affinis	69.78	HM113468.1
Litopenaeus vannamei	73.37	FJ228728.1
Fenneropenaeus penicillatus	71.31	FJ228727.1
Scylla paramamosain	67.73	FJ705251.2
Eriocheir sinensis	66.82	EU622123.1

Table 3 Comparison of amino acid sequences of *Ec-cdc2* with those from other species

Species	Amino acid similarity/%	GenBank ID
Palaemon modestus	96.80	MK188896.1
Macrobrachium nipponense	80.69	FJ919328.1
Procambarus clarkii	79.80	KR135176.1
Cherax quadricarinatus	78.75	JQ088282.1
Penaeus monodon cell	78.68	EU492538.1
Fenneropenaeus indicus	77.87	KT218675.1
Eriocheir sinensis	75.17	FJ210468.1
Scylla paramamosain	75.00	FJ015041.1
Haliotis diversicolor	69.49	HM534206.1
Danio rerio	66.82	NM_001003773.1

Construction of phylogenetic tree of Ec-cyclin B and Ec-cdc2

As shown in **Figure 2**, Ec-cyclin B is evolutionarily most closely related to those from *M. rosenbergii* and *M. nipponense*, followed by *E. sinensis* and *S. paramamosain*. However, they were distantly related evolutionarily to those of *L. vannamei* and *F. penicillatus*. The cdc2 is evolutionarily most closely related to that from *M. rosenbergii* and was found clustered with *M. nipponense* then with *P. monodon*, *P. clarkii*, and finally with *Haliotis diversicolor supertax*. The longest evolutionary distance was with that from *Danio rerio*.

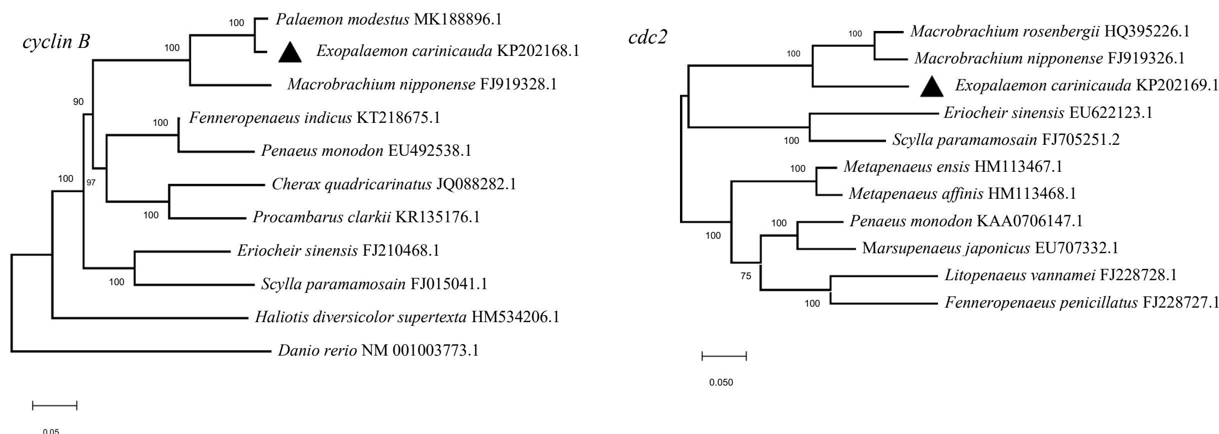

Figure 2 Phylogenetic tree of Ec-cyclin B and Ec-cdc2

Tissue expression pattern of Ec-cyclin B and Ec-cdc2 mRNA

Both *Ec-cyclin B* and *Ec-cdc2* were expressed in all tissues of *E. carinicauda* (**Figure 3**). The highest *cyclin B* expression was seen in the ovary, followed by the hepatopancreas, whereas it decreased in other tissues. In contrast, *cdc2* expression showed the highest in the hepatopancreas, followed by the ovary, and the lowest in muscle.

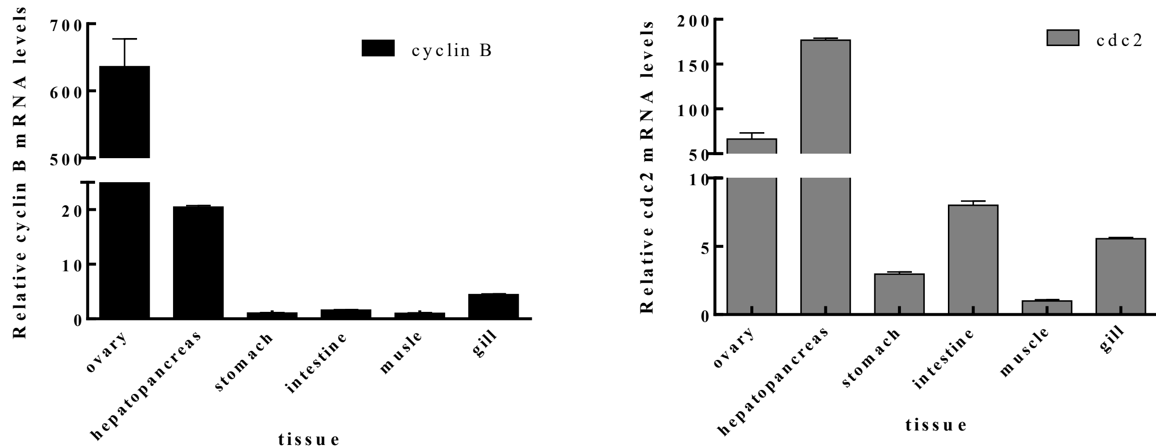

Figure 3 Relative expression of *Ec-cyclin B* and *Ec-cdc2* genes in different tissues

Expression analysis of Ec-cyclin B and Ec -cdc2 in ovarian and embryonic development

The expression of *Ec-cyclin B* tended first to increase and then decrease with ovarian development (**Figure 4**), while its expression peaked at the recovery stage ($p < 0.05$). With ovarian development, the *Ec-cdc2* expression tended to decrease gradually but maximized at the recovery phase, which was significantly higher than in other developmental stages ($p < 0.05$).

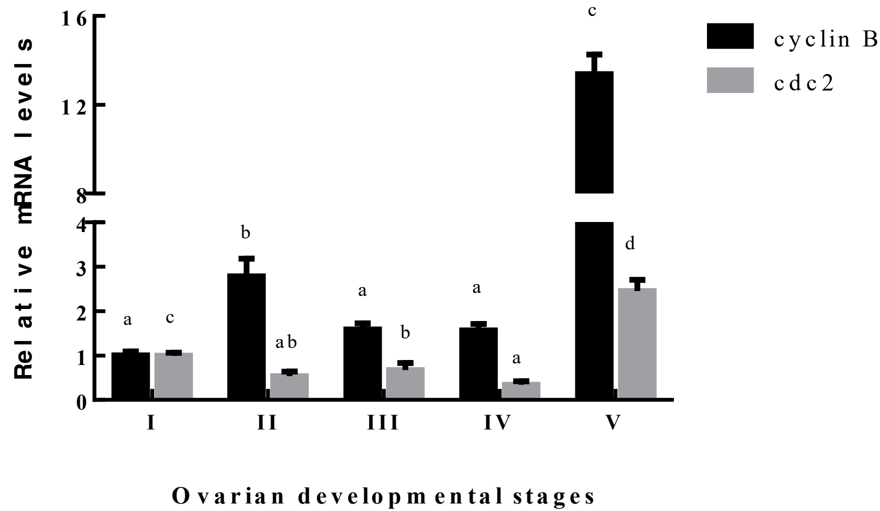

Figure 4 Relative expression levels of *Ec-cyclin B* and *Ec-cdc2* genes at different ovarian developmental stages
Note: Stage I proliferative phase; Stage II minor growth phase; Stage III major growth phase; Stage IV mature phase; Stage V postpartum recovery phase.

With embryonic development, the *Ec-cyclin B* expression showed a trend of increasing and then decreasing, with the highest expression seen at the blastula stage and then gradually decreasing (**Figure 5**). Therefore, the expression trend of *Ec-cdc2* during embryonic development was consistent with that of *Ec-cyclin B*.

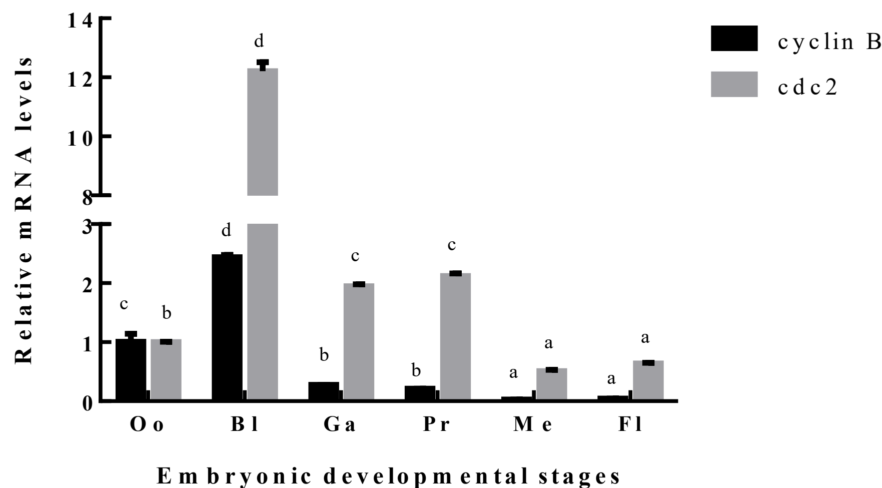

Figure 5 Relative expression levels of *Ec-cyclin B* and *Ec-cdc2* genes at different embryonic developmental stages
Note: Oo: oosperm stage; Bl: blastula stage; Ga: gastrula stage; Pr: protozoea stage; Me: metazoea stage; Fl: first larvae stage.

Discussion

In this study, we obtained the full-length cDNAs of *Ec-cyclin B* and *Ec-cdc2*. The putative amino acid sequence of Ec-cyclin B showed high similarity (66.82–81.06%) to those from other GenBank-registered shrimps and crabs in terms of cyclin family features, including a proteinase K site (RRXSK). The putative amino acid sequence of Ec-cdc2 also showed a high similarity (75.00–99.80%) to other GenBank-registered amino acid sequences of shrimp and crab cdc, in terms of characteristics of CDK molecules, including serine/threonine protein kinase active sites, ATP binding-related conserved sequence (GxGxxGxV), and conserved cyclin binding sequence (PSTAIRE region).

MPF is a vital protein kinase in mitosis and meiotic G2/M transition in eukaryotic cells and is important in oocyte maturation (Xi et al., 2002). Cyclin B, a regulatory subunit of MPF, is crucial in mitosis and meiosis (Dalby and Glover, 1992). According to previous studies, the association between cyclin B and ovarian development has been explored in *M. nipponense*, *P. monodon*, *E. sinensis*, *M. rosenbergii*, etc. (Wu, 2009; Qiu et al., 2007; Feng et al., 2020). This study found that *Ec-cyclin B* expression was highest in the ovary. The quantitative *Ec-cyclin B* expression results in different ovary developmental stages showed that it was significantly greater in stage II than in stages I, III, and IV ($p < 0.05$). The highest expression was seen in stage V, significantly greater than in the other stages ($p < 0.05$). The *cyclin B* mRNA expression was the highest in the ovaries of *P. montanus*, with the Pm-cyclin B protein being localized to the cytoplasm of prophase oocytes at stage II and enriched in the nuclei of pro-metaphase oocytes at stages III and IV (Zhao et al., 2015; Qiu et al., 2007). In *M. nipponense*, *cyclin B* was highly expressed in the ovary and played an essential role in ovarian development (Zhang et al., 2022c). However, a previous study cloned the *E. sinensis cyclin B* gene and found it mainly expressed in the ovary and testis. That study also found that although *cyclin B* mRNA was expressed at high levels during the previtellogenesis and late vitellogenesis stages, it was low during the early and middle vitellogenesis (Fang and Qiu, 2009), which was different from our study results. The proliferative process of oocytes mainly happens in the early stages of ovarian development in *E. carinicauda* (Wang, 1987). The high expression of *Ec-cyclin B* in the ovarian minor growth phase and postpartum recovery phase found in *E. carinicauda*, suggests that it is closely associated with the proliferation of oogonia cells, involved in the meiosis and mitosis of oocytes and is vital in the maturation of oocytes.

Cdc2 kinase is the first member of the family of cell cyclin-dependent kinases (CDKs), and is also known as p34cdc2 or Cdk1 kinase. The cdc2 kinase is the catalytic subunit of MPF, a central factor in the induction of meiotic maturation in oocytes (Katsu et al., 1993). The cdc2 protein of *E. sinensis* was found to be localized only in the cytoplasm of the oocyte before vitellogenesis, then re-localized in the nucleus during vitellogenesis, and finally accumulated on the meiotic spindle during oocyte maturation, thus indicating that cdc2 kinase plays an important function in the maturation of the oocyte of *E. sinensis* (Qiu and Liu, 2009). This study found that the highest *Ec-cdc2* expression was in the hepatopancreas, followed by the ovary, and the lowest in the muscle tissue. Although the *Ec-cdc2* expression level in the ovaries gradually decreased with ovarian development, it maximized at the recovery stage, significantly greater than in other developmental stages. The *Ec-cdc2* expression levels were similar to *Ec-cyclin B* at different developmental stages in the ovaries. The results indicate that cdc2 kinase, a component of MPF, was vital during ovary development in *E. carinicauda*.

Cell cycle proteins play an important role in early mammalian embryonic development. The accumulation and degradation of cyclin B is essential for driving the cell cycle via the activation and inactivation of CDK1 (Polański et al., 2012). Cytosolic protein B was predominantly cytoplasmic and only localized in the nucleus during the late prophase of the *Drosophila* early embryonic cycles; increased cyclin B dosage also boosted the activity of cyclin B-Cdk1 (Stiffler et al., 1999). During 0 h to 15 h of *Artemia sinica,* embryonic cells may undergo cell division, protein synthesis, and other life-essential activities. Therefore, *As-cyclin*

B shows an increasing trend from 15 h to 3 d, the main life activity experienced was cell differentiation, and the expression of the *As-cyclin B* gene decreased (Chu, 2014). In our study, the *Ec-cyclin B* expression was the highest in the blastocyst stage, significantly higher than in other periods ($p < 0.05$), gradually decreasing with embryonic development. Maybe due to the enhanced cell cycle regulation activities brought on by the continual division of embryonic cells, the expression of genes involved in cell cycle regulation tends to rise as oosperm develops into nauplius (Zhang et al., 2022a). The expression of *Mn-cyclin B* mRNA was higher in the embryonic development stage than in the larval and post-larval development stages and the highest in the cleavage stage, suggesting that cyclin B played an essential role in the embryogenesis of *M. nipponense* (Zhang et al., 2022c). These results indicated that cyclin B plays an essential role in embryogenesis. Previous studies have shown that using zebrafish embryos as a model to explore the role of cdk1 in regulating cell cycle and embryonic development and found that cdk1 interacts with cyclin A2 to regulate cell S phase progression and with cyclin B1 to regulate cell M phase progression (Jin et al., 2021). Furthermore, a study cloned the complete cdc2 kinase in *C. quadricarinatus* and found that its transcriptional level peaked at the gastrula stage and then decreased after the nauplius stage (Wang et al., 2013). Our study found that the expressions of *Ec-cdc2* showed the same trend with *Ec-cyclin B* at different stages of embryonic development. It was the highest at the blastocyst stage, and then it decreased after the gastrula stage; however, it was higher than the zoea stage. This may be due to the division of many mesodermal and ectodermal cells before nodal larva. The cell division was slow after nauplius (Zhang et al., 2022a). Thus, the present study suggests that Ec-cyclin B and Ec-cdc2 were jointly involved in regulating the embryonic development of *E. carinicauda*.

In this study, the *Ec-cyclin B* expression was the highest in the ovaries. In contrast, *Ec-cdc2* expression was the greatest in the hepatopancreas, followed by the ovaries. Both their expression levels were the highest at stage V of ovarian development, with both genes showing an increasing and later decreasing trend at different stages of embryonic development. This indicated that *cyclin B* and *cdc2* play important roles in ovarian and early embryonic development and are essential genes associated with gonadal development and embryonic development in *E. carinicauda*. Therefore, the findings of this study provide a foundation for understanding the role of MPF in the gonadal and embryonic development of *E. carinicauda*.

Acknowledgments

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Declaration of Competing Interest

The authors have declared that there is no competing interest.

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