

Original Research Articles

Expression analysis of the insulin-like peptides genes (ILPs) in the redclaw crayfish *Cherax quadricarinatus* and new insights into sexual development

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Insulin-like peptides (ILPs) are involved in various physiological processes in metazoans, including metabolism, growth, and reproduction. However, their presence and function in decapods are largely unexplored. To investigate the role of ILPs in the redclaw crayfish, this study characterized two ILP genes (Cq-ILP1 and Cq-ILP2) and analyzed their genomic structure and protein domains. Multiple sequence alignments revealed highly conserved structural features across ILPs from different species. Tissue expression analysis indicated that Cq-ILP1 was exclusively expressed in the ovary, suggesting its involvement in female reproduction. In early juvenile stages, Cq-ILP1 expression was first detected in female individuals at 2 cm body length (BL2), coinciding with the appearance of genital pores. Additionally, Cq-ILP1 expression progressively increased during ovarian development stages from I to V, then decreased to its lowest level at the end of stage VI. These findings suggested that Cq-ILP1 played a pivotal role in sexual development. Moreover, injection of Cq-ILP1-dsRNA resulted in a significant reduction in vg expression. These results provided new insights into the function of non-IAG ILP genes and offered valuable resources for future research on ovarian development in crustaceans.

1. INTRODUCTION

Extensive studies in animals have demonstrated that insulin-like peptides (ILPs) can activate MAPK and PI3K/AKT pathways through the ILP/IGF signaling cascade, regulating a broad range of physiological processes, including growth, development, metabolism, immunity, and reproduction.¹⁻³ Invertebrate models, such as *Drosophila melanogaster* have been extensively studied, revealing the presence of eight 8 ILP genes (*ILP1-8*) and two receptors (*InR* and *Lgr3*).⁴ Notably, *ILP5* produced in the ovaries and malpighian tubule of *D. melanogaster*, suggested a role in ovarian development.⁵ Furthermore, insulin-like receptors (*InR*) have been identified in the ovaries of other Lepidoptera species, and mutations in *InR* can lead to ovarian dysplasia during early vitellogenesis, indicating ILPs' involvement in insect reproduction.² Crustaceans and insects, both belonging to the arthropod phylum, share a close genetic relationship.⁶ Many key genes and signaling pathways are conserved be-

tween these groups, performing similar functions throughout evolution.⁷

To the best of our knowledge, insulin-like androgenic gland (IAG) has been identified as the exclusive androgenic sex hormone for regulating sexual differentiation in crustaceans. However, no other ILPs from decapod crustaceans have been reported in recent decades.^{8,9} With the advent of high-throughput sequencing technology, more ILP genes have been identified and characterized in crustaceans, such as *Sv-ILP1* in *Sagmariasus verreauxi*,¹⁰ insulin-like and relaxin-like peptides in **Procambarus clarkii* (**Veenstra*¹¹), *Mn-ILP* in *Macrobrachium nipponense*,¹² and *ILP1/ILP2* in *Sinonovacula constricta*.¹³ Notably, studies of these ILPs suggested that their roles extend beyond masculinization and testis development, potentially influencing growth and innate immune responses.

The redclaw crayfish, *Cherax quadricarinatus*, native to the tropical regions of northern Australia and southern New Guinea, is renowned for its large size and exceptional taste, making it one of the world's most valuable freshwater

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species.¹⁴ Introduced to China in the 1990s, it quickly gained popularity among domestic breeding enterprises and consumers. Previous studies have established the role of *Cq-ILP1* in regulating masculinization in *C. quadricarinatus*, with sexual reversal observed upon silencing the *Cq-ILP1* gene.⁸ However, research on non-*ILP1* ILPs and the regulatory mechanisms governing feminization maintenance and ovarian development in *C. quadricarinatus* remains limited.

Crustaceans possess unique endocrine systems, with most sexually dimorphic traits regulated by key reproductive hormones or peptides. In these species, insulin-like androgenic gland hormone (IAG) is a male-specific ILP, which is responsible for sexual differentiation and maintains male secondary sex characteristics.^{15,16} However, very little information is available regarding the function of non-*ILP1* ILPs in female reproduction of crustaceans. For this purpose, we focused on the roles of ILPs (*Cq-ILP1* and *Cq-ILP2*) in ovarian development in *C. quadricarinatus*, aiming to elucidate their potential involvement in sex differentiation and development. Relative expression levels were assessed in various tissues and developmental stages of ovaries and juveniles using qRT-PCR. Additionally, the transcriptional expression of *Cq-vg* was analyzed following the silencing of the *Cq-ILP1* gene. These findings could provide deeper insights into the molecular mechanisms by which *Cq-ILP1* regulated ovarian development.

2. MATERIALS AND METHODS

2.1. ANIMALS AND SAMPLES PREPARATION

The redclaw crayfish of both sexes (n=50) used in this study were sourced from a freshwater lobster breeding base in Huzhou, China, and transferred to the laboratory for 1 week of acclimatization. Tissues, including the heart (Hea), hepatopancreas (Hep), muscle (Mu), gill (Gi), eyestalk (Eys), ovary (Ov), testis (Te), and intestines (In), were dissected from six healthy adult crayfish (average body weight: 55±10 g) on ice. Ovaries at different developmental stages (I to VI) were sampled based on biological processes and histological characteristics. Juvenile samples at various body lengths (1 cm, 2 cm, 4 cm, 5 cm, 8 cm, and 10 cm) were collected from pond-cultured crayfish at different time points, and three individuals were sampled per stage. All animal experiments that were conducted in this study were approved by the Committee of Laboratory Animal Experimentation at the Zhejiang Institute of Freshwater Fisheries, Huzhou, China.

2.2. SEQUENCE BIOINFORMATICS ANALYSIS

The genomic structure of intron-exon composition was analyzed based on the published *ILP1* (GenBank accession No. KP006644) and *ILP2* (KP006645) cDNA sequences, as well as the genomic sequence of *C. quadricarinatus*.¹⁷ Multiple amino acid sequence alignments of ILPs were performed using DNAMAN software, comparing *C. quadricarinatus* and *Macrobrachium rosenbergii*. Signal peptides were predicted

using the SignalP 3.0 Server (<http://www.cbs.dtu.dk/services/SignalP/>). Secondary protein domains and three-dimensional (3D) structures were identified using the SMART program (<http://smart.embl-heidelberg.de/>) and I-TASSER software (<http://zhanglab.ccmb.med.umich.edu/I-TASSER/>). A phylogenetic tree was constructed using the neighbor-joining (NJ) method with MEGA 5.0 software, with 1000 bootstrap replicates. Protein sequences and primers used in this study are listed in **Tables 1 and 2**, respectively.

2.3. RNA EXTRACTION AND QRT-PCR PERFORMANCE

Total RNA was extracted from the collected *C. quadricarinatus* samples using TRIzol® Reagent (Total RNA Extractor Kit, Sangon Biotech). RNA quality and concentration were assessed by agarose gel electrophoresis and a nucleic acid analyzer (NanoDrop Technologies Inc., Wilmington, DE, USA). First-strand cDNA was synthesized using the HiFiScript cDNA first-strand synthesis kit (Cwbio, Beijing). The cDNAs were stored at -20°C for subsequent mRNA expression level analysis.

qRT-PCR was employed to quantify the mRNA expression levels. Gene-specific primers were designed using Primer 5 software (**Table 2**). qPCR was performed on a LightCycler 480 System (Roche Switzerland) in a 10 µL reaction volume containing 1 µL cDNA template, 1 µL of each primer (10 µM), 5 µL 2x SYBR Premix Ex Taq™ (TaKaRa, Japan) and 2 µL of water. The cycling conditions were as follows: initial incubation for 30 s at 95°C, followed by 40 cycles of 10 s denaturation at 95°C, and 10 s extension at 60°C. Dissociation curve analysis was performed after the end of amplification to determine specific primer efficiencies. Gene expression levels were normalized to β -actin as an internal control that validated as previously described,¹⁴ and a reaction without cDNA was used as the negative control. Each reaction was performed in triplicate.

2.4. EYESTALK ABLATION AND B-ESTRADIOL (E2) INJECTION ASSAY

In this study, healthy crayfish weighing approximately 40-50 grams weight (n=12) were randomly selected for unilateral eyestalk ablation using sterile surgical scissors. The wounds were cauterized to minimize hemolymph loss, and no mortality was observed during the post-operative monitoring. Four eyestalk-ablated individuals (ESA) and four control crayfish with intact eyestalks were collected at 24 and 96 hours post-ablation, respectively, and placed in an ice bath until anesthetized. Ovarian tissues were then dissected and sampled for RNA extraction and qRT-PCR analysis. For the β -Estradiol *in vivo* injection assay, estradiol (Macklin, China, purity≥98%) was dissolved in ethanol to achieve a final concentration of 50 µg/mL. The injection dosage (0.1 µg/g body weight) and volume (100 µL) followed the protocol from a previous study.¹⁸ In this experiment, the estradiol was injected into the fifth abdominal segment of female individuals (n=12), with a control group receiving the same volume of ethanol. Four crayfish from each treatment group were collected at 24 and 96 hours post-injection.

Table 1. GenBank accession No. of ILPs used for multi-alignment and phylogenetic tree analysis.

Protein	Species	GenBank No.	Protein	Species	GenBank No.
ILP2	<i>Cherax quadricarinatus</i>	KP006645	ILP1	<i>Sinonovacula constricta</i>	KR534869
ILP1	<i>Cherax quadricarinatus</i>	KP006644	ILP2	<i>Sinonovacula constricta</i>	KR534870
ILP2	<i>Tigriopus japonicus</i>	KF951035	ILP3	<i>Sinonovacula constricta</i>	KX069229
ILP1	<i>Tigriopus japonicus</i>	KF951034	Insulin	<i>Danio rerio</i>	CAC20109
ILP1	<i>Macrobrachium rosenbergii</i>	MN614427	ILP2	<i>Sagmariasus verreauxi</i>	KP006646
Insulin	<i>Homo sapiens</i>	AAA59172	ILP1	<i>Sagmariasus verreauxi</i>	KP006643
ILP	<i>Mizuhopecten yessoensis</i>	BAD13420	Insulin	<i>Crassostrea gigas</i>	EKC18433
ILP2	<i>Macrobrachium rosenbergii</i>	MN614428	ILP1	<i>Daphnia pulex</i>	EFX76240.1

Table 2. Primers used for gene expression analysis in *C. quadricarinatus*.

Primer name	Sequences (5'-3')	Purpose
<i>Cq</i> -ILP1-RT-F	ATGATACAGCGGGGCGAGAC	qRT-PCR
<i>Cq</i> -ILP1-RT-R	AGTGGCTGGACTGGTCTGGATG	qRT-PCR
<i>Cq</i> -ILP2-RT-F	CGCTGCCACCACCATCACTG	qRT-PCR
<i>Cq</i> -ILP2-RT-R	CGCCATTCTCCCTGATCGTTG	qRT-PCR
β -actin-RT-F	TCACTGCTCTGGCTCCTGCTAC	qRT-PCR
β -actin-RT-R	ACTCGTCGTA CTCTCTTGGTG	qRT-PCR
vg-RT-F	CAGTCCGAGTATTCGCCAAGAAC	qRT-PCR
vg-RT-R	AACCAGTCGTTACCAACTTCACCATC	qRT-PCR
<i>Cq</i> -ILP1-dsRNA-F	TAATACGACTCACTATAGGGATGCTAGCACTGACTGCCAT	RNAi
<i>Cq</i> -ILP1-dsRNA-R	TAATACGACTCACTATAGGGATGCTAGCACTGACTGCCAT	RNAi

tion, anesthetized on ice, and dissected to obtain ovarian tissues for subsequent expression analysis.

2.5. RNA INTERFERENCE

Approximately 400 bp fragments of *Cq*-ILP1 and *GFP* were subcloned into pUC57 vectors, respectively. Specific primers with a T7 promoter were designed (Table 2), and PCR amplification was performed using plasmids as templates. The PCR products were purified and used as templates for dsRNA synthesis using the T7 High Yield RNA Transcription Kit (Vazyme, China) according to the manufacturer's instructions. For the *in vivo* dsRNA injection experiment, female adult crayfish ($n=30$) were assigned two treatment groups: *Cq*-ILP1 dsRNA ($n=15$) and GFP dsRNA ($n=15$). Each animal was injected with 5 μ g/g body weight, and ovarian tissues were collected 24 hour after injection. Finally, qRT-PCR was conducted to analyze the expression of the target genes, *Cq*-ILP1 and *vg*, in dsRNA-injected crayfish.

2.6. STATISTICAL ANALYSIS

The qRT-PCR data were calculated using $2^{-\Delta\Delta C_t}$ method¹⁹ and expressed as means $\pm$ standard. Statistical differences were estimated by one-way ANOVA followed by LSD and Duncan's multiple range test in tissue distribution, embryonic stages, and ovary cycle. Besides, a two-side t-test was

used to compare expression levels in RNAi experiments. All statistical analyses were performed using SPSS 20.0 (SPSS, Chicago, IL, USA).

3. RESULTS

3.1. CHARACTERIZATION OF THE REDCLAW CRAYFISH ILP GENES

The complete genomic sequences of *Cq*-ILP1 and *Cq*-ILP2 were obtained from the transcriptomic and genomic data of *C. quadricarinatus*. The exonic-intronic structure, consisting of four exons and three introns, was analyzed (Fig 1a). The predicted amino acid sequences of *Cq*-ILP1 and *Cq*-ILP2 revealed the presence of a signal peptide and conserved domains (Fig 1b). Multiple sequence alignment indicated that ILPs from *C. quadricarinatus* and *M. rosenbergii* consisted of three peptide chains and six conserved cysteine residues located within the B and A chains. The three-dimensional structures of *Cq*-ILP1 and *Cq*-ILP2 were predicted using the PDB template, as shown in Fig. 1c and 1d. Phylogenetic tree analysis demonstrated that crustaceans clustered together, with *Cq*-ILP sharing the closest genetic relationship with *Sagmariasus verreauxi* and *M. rosenbergii* among the examined species (Fig 1e).

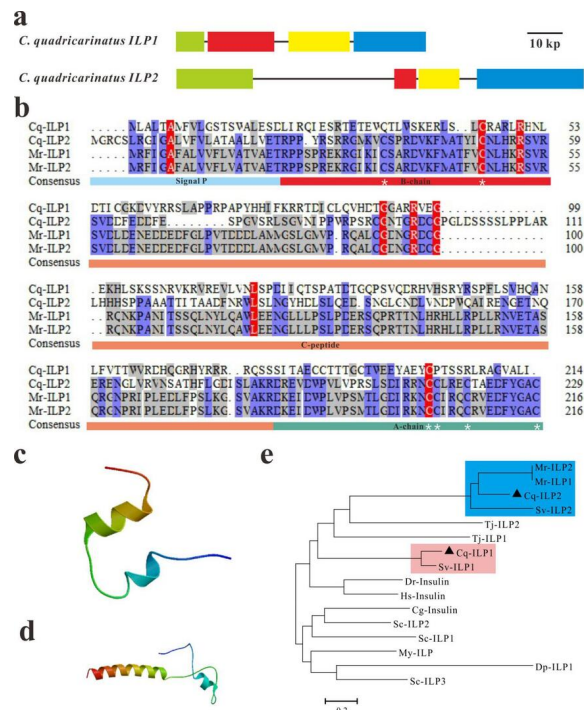


Figure 1. Analysis of the *ILPs* structure and phylogenetic tree in *C. quadricarinatus*.

a. Exon-intron structure of the redclaw crayfish *ILPs* genes. Box and line indicate exon and intron, respectively. Scale bar represents 10 kp bases. b. Multiple alignment of *ILPs* amino acid sequences between *C. quadricarinatus* and *M. rosenbergii*. Domain is predicted using SMART program. Sequence identities are highlighted in red color, and pink depicts the conservative changes. Asterisks denote conservation of cysteines. c, d. 3-D structure modeling of Cq-ILP1 and Cq-ILP2 with SWISS-Model: PDB 5I3m.1A model was used as a template. e. Phylogenetic tree is constructed using MEGA5 program with neighbor-joining method at 1000 bootstrap replicates from protein sequences of *ILPs* among different species.

3.2. THE EXPRESSION PATTERN OF *ILPS* IN VARIOUS TISSUES

qRT-PCR was employed to assess the transcriptional levels of *Cq-ILPs* in various tissues. Tissue distribution analysis revealed that *Cq-ILP1* expression was highest in the ovaries, followed by the gills and hepatopancreas, while *Cq-ILP2* expression was predominantly restricted to the ovaries, albeit at lower levels (Fig 2). These results indicated that *Cq-ILPs* were significantly more expressed in ovarian tissue, suggesting their potential involvement in female reproduction in *C. quadricarinatus*.

3.3. EXPRESSION OF *ILPS* IN DIFFERENT DEVELOPMENTAL STAGES OF THE OVARIES

To investigate the role of *Cq-ILPs* in ovarian development, expression patterns were analyzed across different ovarian developmental stages using qRT-PCR. As shown in Fig. 3, the highest expression level of *Cq-ILP1* occurred at Stage V (ovarian maturation) and decreased to its lowest level at Stage VI (ovarian degeneration). In contrast, *Cq-ILP2* was barely detectable at any stage of the reproductive cycle. These results suggested that *Cq-ILP1* exhibited an ovary-specific expression pattern and is likely involved in ovarian development in *C. quadricarinatus*.

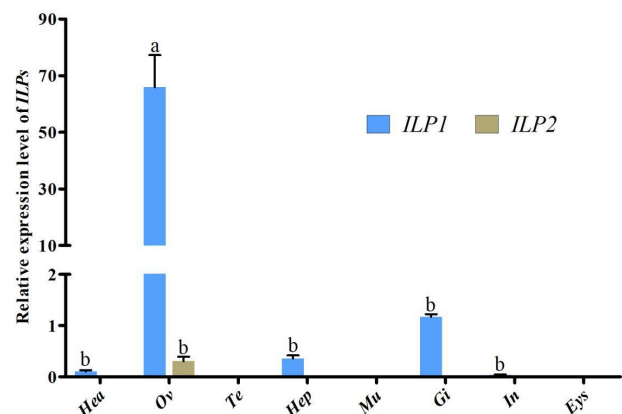


Figure 2. The expression pattern of *ILPs* in various tissues.

Hea: heart, Ov: ovary, Te: Testis, Hep: Hepatopancreas, Mu: muscle, Gi: Gill, In: Intestines, Eys: eyestalk. Statistical analyses are performed with one-way ANOVA analysis. The post-hoc test following ANOVA analysis used LSD. Bars with different letters are considered significant at $P < 0.05$.

3.4. THE EXPRESSION PROFILE OF *ILPS* DURING EMBRYO DEVELOPMENT

Further investigation of *Cq-ILP* expression during early sex differentiation revealed its expression profile at different growth stages. qPCR analysis indicated *Cq-ILP1* was first detectable in female individuals at a body length of 2 cm (BL2♀), with expression levels steadily increasing throughout subsequent developmental stages. Notably, both *Cq-*

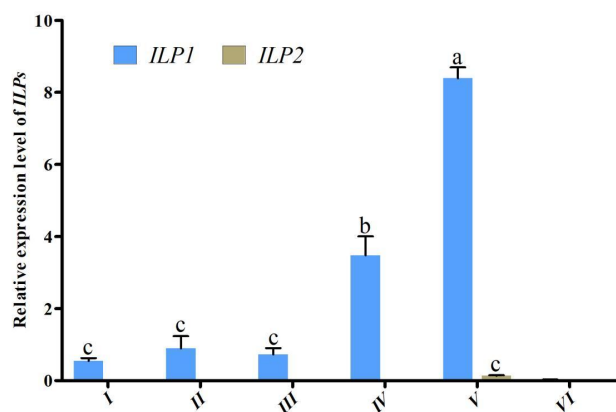


Figure 3. Temporal expression of *Cq*-ILPs during different ovarian developmental stages.

I: undeveloped stage, II-III: developing stage, IV: nearly-ripe stage, V: ripe stage, VI: degeneration stage. Statistical analyses are performed with one-way ANOVA analysis. The post-hoc test following ANOVA analysis used LSD. Bars with different letters are considered significant at $P < 0.05$.

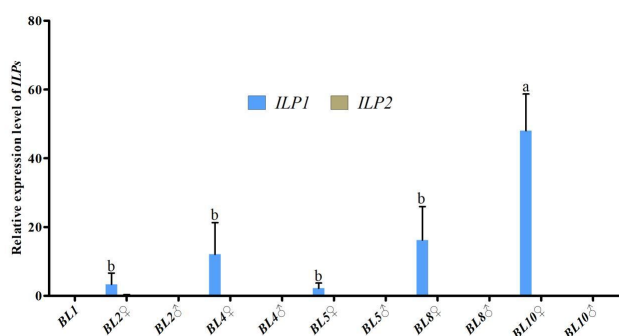


Figure 4. Spatio-temporal expression analysis of *Cq*-ILPs during embryonic development.

BL=body length. β -actin was used as a positive control. Bars with different letters are considered significant at $P < 0.05$.

ILP1 and *Cq-ILP2* exhibited significantly lower expression levels in males at all examined stages (Fig. 4). Collectively, the spatio-temporal expression analysis of *Cq*-ILPs suggested that *Cq-ILP1* played a critical role in ovarian development and sex differentiation in *C. quadricarinatus*.

3.5. THE EXPRESSION DIFFERENCES UNDER EYESTALK ABLATION AND E2 STIMULATION

qRT-PCR results indicated that exogenous E2 injection significantly increased *Cq-ILP1* expression at 96 hours post-treatment ($P < 0.01$), with no significant change at 24 hours (Fig. 5a). For the *vg* gene, exogenous E2 injection elevated expression levels at both 24 and 96 hours (Fig. 5b). Additionally, the expression of *Cq-ILP1* and *vg* in ovaries was assessed following eyestalk ablation (ESA). As anticipated, both *Cq-ILP1* and *vg* expression levels were significantly higher in the ESA group compared to the control group.

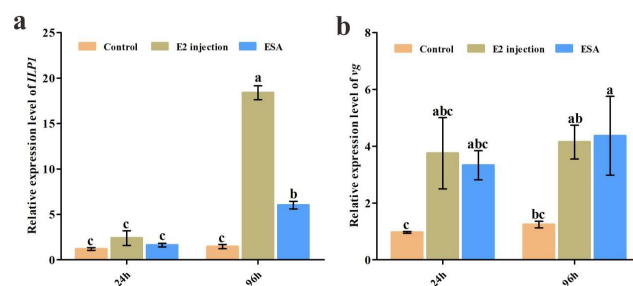


Figure 5. The differences in the expression of *Cq*-ILP1 and *vg* under various conditions.

a. Relative expression of *Cq-ILP1* mRNA in ovaries after ESA and exogenous estrogen. b. Relative expression of *vg* mRNA in ovaries after eyestalk ablation and exogenous estrogen. Brown box indicates the control group. Blue box indicates the eyestalk ablation (ESA) group. Gray box indicates the E2 injection group. Bars with different letters are considered significant at $P < 0.05$.

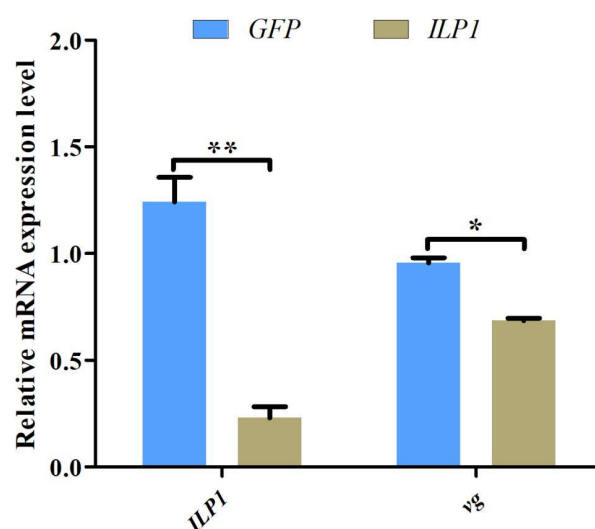


Figure 6. The *Cq*-ILP1 and *vg* expression level after RNAi.

Results are expressed as mean $\pm$ SEM, and significance of comparison is defined as $P < 0.05$ (*) and $P < 0.01$ (**) by Student's *t* tests.

3.6. EFFECTS OF *ILP1* KNOCKDOWN ON *vg* TRANSCRIPTION BY RNAI

Given the potential role of *Cq-ILP1* in ovarian development, RNA interference (RNAi) was used to investigate its function in female crayfish. *Cq-ILP1* expression was reduced by 81.47% following injection with *Cq-ILP1*-dsRNA ($P < 0.01$), confirming the success of the RNAi-mediated knockdown. The effect of *Cq-ILP1* silencing on *vg* transcript was also assessed, and results revealed a decrease in *vg* abundance in the ovaries (Fig. 6). These results demonstrated that RNAi-mediated silencing of *Cq-ILP1* reduced *vg* accumulation in the ovaries of *C. quadricarinatus*.

4. DISCUSSION

In this study, sequence analysis and expression profiling of *Cq-ILP1* and *Cq-ILP2* in the redclaw crayfish were conducted. Previous research has shown that *ILP* genes in invertebrates are not derived from single-copy genes but are encoded by multiple genes.^{20,21} Amino acid sequences encoded by *ILP* genes typically share conserved structural features, including a signal peptide followed by the B, C, and A-chains, which are highly conserved within the *ILP* superfamily.^{22,23} Additionally, *Cq-ILP1* and *Cq-ILP2* contained conserved cysteine residues that form disulfide bonds, essential for protein function through peptide cross-linking.²⁴ Phylogenetic analysis revealed that the *ILPs* from the redclaw crayfish were highly similar to those of *S. verreauxi*.

Insulin superfamily polypeptides regulate a broad range of functions in metazoans, including metabolism, growth, and reproduction.^{25,26} Studies in decapod crustaceans have suggested that *ILPs* play important roles in regulating growth and metabolism.^{12,24,27} However, the identification and role of non-IAG *ILP* members in crustacean reproduction remain poorly understood. Gene expression analysis is often closely linked to biological function,¹² prompting the spatio-temporal expression analysis of *ILPs* to explore their potential roles. In this study, *Cq-ILP1* exhibited high expression levels in the ovary compared to other tissues, suggesting its involvement in sexual differentiation or female reproduction. A similar pattern of *ILP1* expression during ovarian development was observed in *Sinonovacula constricta*.¹³ However, studies on *S. verreauxi* and *Tigriopus japonicus* indicated higher *ILP1* expression in male glands,^{22,27} creating a notable divergence in the redclaw crayfish. Thus, the high expression of *ILP1* in *C. quadricarinatus* suggested its role in female sexual development, although further research was needed to generalize the function of *ILPs* in crustaceans. Additionally, *Cq-ILP1* expression was detected in the gill, indicating its potential involvement in glucose metabolism. Although the expression level was examined in several tissues, the *ILP1* expression in the ovary was much higher than in other tissues, suggesting the dominant role of *ILP1* in sexual determination. While *ILP1* was expressed in several tissues, its significantly higher expression in the ovary suggested its dominant role in sexual determination.

Tissue distribution analysis clearly indicated that *Cq-ILP1* might play a key role in regulating ovarian development in crayfish, prompting further investigation into the expression profiles of *Cq-ILPs* during ovarian development. qRT-PCR results showed that *Cq-ILP1* expression was significantly higher at the ripe stage compared to other ovarian developmental stages, exhibiting a consistent expression pattern with other sex-related genes, and was closely associated with ovarian development in *C. quadricarinatus*.^{14,28} Additionally, the expression of *Cq-ILPs* during early embryo development was also examined. qRT-PCR results revealed that *Cq-ILP1* was first detected in female crayfish at a body length of 2 cm, with expression levels increasing as body size grew. Interestingly, genital pores were also observed on the third cheliped when the body

length approached 2 cm. These findings suggested that sexual differentiation in *C. quadricarinatus* might begin at a body length of 2 cm, and the expression of *Cq-ILP1* in early developmental stages can be marked as the initiating point of sexual differentiation.

The explicit mechanisms underlying sex differentiation and determination of non-IAG *ILPs* remain unclear in crustaceans. It has been established that vitellogenin (*vg*) synthesis is a key process in ovarian development in oviparous animals, with *vg* abundance serving as an important biomarker in studies of ovarian development in crustaceans.²⁹⁻³¹ Endogenous *ILPs* and their associated signaling pathways play critical roles in regulating *vg* synthesis in decapod crustaceans.³² RNAi is a widely used tool for exploring gene function in crustaceans,^{33,34} and in this study, RNAi was employed to investigate the role of *Cq-ILP1* in ovarian development. As expected, *Cq-ILP1* mRNA expression was significantly inhibited following dsRNA injection, demonstrating the effectiveness of RNAi in gene silencing. Furthermore, RNAi-mediated knockdown of *Cq-ILP1* significantly blocked the stimulation of *vg* expression, suggesting that *Cq-ILP1* acted as an intermediary between insulin superfamily polypeptides and *vg* synthesis. These results provided strong evidence that *Cq-ILP1* played a pivotal role in sex differentiation. However, further studies were required to elucidate the interaction between *Cq-ILP1* and *vg* in the regulation of female reproduction in *C. quadricarinatus*.

In conclusion, two non-IAG *ILP* genes, *Cq-ILP1* and *Cq-ILP2*, were characterized from *C. quadricarinatus*. *Cq-ILP1* was predominantly expressed in the ovary, with expression detectable as early as the 2 cm body length stage, implying its involvement in sexual development. Moreover, RNAi-mediated silencing of *Cq-ILP1* significantly reduced *vg* expression, indicating that *Cq-ILP1* played a role in regulating ovarian development. These findings enhanced our understanding of insulin superfamily polypeptides and provided a valuable resource for future studies on dsRNA-mediated regulation of ovarian development in crustaceans.

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AUTHORS' CONTRIBUTION

Conceptualization: Jianbo Zheng, Haiqi Zhang; Methodology: Yi Xu, Chao Zhu; Formal analysis and investigation: Wenping Jiang, Shun Cheng; Writing - original draft preparation: Yi Xu, Jianbo Zheng; Writing - review and editing: Haiqi Zhang; Funding acquisition: Meili Chi; Resources: Shili Liu; Supervision: Fei Li.

COMPETING OF INTEREST – COPE

No competing interests were disclosed.

ETHICAL CONDUCT APPROVAL – IACUC

All experiments in this study were conducted in accordance with the guidelines for scientific purposes and approved by the Animal Ethics Committee of Zhejiang Institute of Freshwater Fisheries.

INFORMED CONSENT STATEMENT

All authors and institutions have confirmed this manuscript for publication.

DATA AVAILABILITY STATEMENT

All are available upon reasonable request.

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