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Investigating the Expression and Function of *HIF-1α* in *Neocaridina davidi* During Embryo Cleavage Stage

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Abstract

Neocaridina davidi, a member of Decapoda within Crustacea, stands out due to its petite stature, robust reproductive capabilities and short molting cycle. Consequently, it has emerged as a valuable experimental model for investigations spanning ecology, development, physiology, and toxicology. Despite the pivotal role of Hypoxia Inducible Factor-1*α* (*HIF-1α*) in diverse physiological processes like biological hypoxia stress, cellular growth, and stress resistance, its functionality in crustaceans remains underexplored. Moreover, the role and function of *HIF-1α* in crustaceans, especially in embryonic stages, have been insufficiently addressed. This study delves into the function of *HIF-1α* during embryonic development. Initially, the complete sequence of the *HIF-1α* gene was acquired. Notably, the expression of *HIF-1α* during the cleavage stage surpassed that of subsequent phases. Subsequently, dsRNAHIF-1α was introduced into sexually mature shrimp, revealing that the inhibitory impact of dsRNA on gene expression in the parental generation could be inherited by the offspring, exerting a specific gene silencing effect in the embryo. The consequences of silencing *HIF-1α* in embryos manifested as varying degrees of premature division termination, besides, the glycolysis in the embryos was also affected by *HIF-1α* suppression. These results provide data support for understanding the early embryonic development of crustaceans and *HIF-1α* functions within it.

Introduction

Embryonic development stands as a pivotal facet within crustacean science, offering profound insights into the physiology and evolutionary trajectory of these organisms. The exploration of crustacean zoology dates back to the early 19th century, gaining widespread traction in the late 19th century. Anderson's (1982) discourse, coupled with subsequent decades of research, delineates three distinct phases: the classical period of crustacean embryology, the developmental era of crustaceans, and the establishment of modern crustacean embryology. During the classical period, the focus on oviparous species, such as Cladocerans, Copepods, Cirripedes, and Decapods, primarily involved external descriptions of crustacean embryos (Wolff, 2009). During the development of crustaceans, Cannon used tissue sectioning technology to complete the study of gill-foot embryos for the first time. Manton (1928) improved the method in the later study and provided guidance for the later embryo research (Sullivan & Macmillan, 2001). The modern era of crustacean embryology witnesses the integration of advanced techniques, including scanning electron microscopy (SEM), transmission electron microscopy (TEM), and biomolecular methods, providing nuanced insights into embryonic development and internal structures (Chen et al., 2021; Tserevelakis et al., 2023). While morphological studies dominate the embryonic development exploration of shrimps and crabs (Meng et al., 2000; Mu et al., 2007; Castejón et al., 2022; Hamasaki et al., 2023), there is a conspicuous gap in our understanding of the molecular regulatory mechanisms governing crustacean embryogenesis.

Comparatively, in mammals, early embryo development hinges on maternal proteins, messenger ribonucleic acids, and subcellular organelles encoded by maternal effector genes (Gouw et al., 2009; Tadros & Lipshitz, 2009). Mammalian embryogenesis, initiated by the fusion of sperm and egg, progresses through cleavage, blastocyst hatching, uterine colonization, and subsequent stages to form a complete individual. Notable events during preimplantation development include maternal-zygotic transformation, zygotic gene activation, and cell lineage differentiation (Caserta et al., 2011). In insect studies, maternal effector genes play crucial roles in inducing anterior-posterior polarity, regulating egg diapause, enhancing immunity, and influencing sexual differentiation and offspring development (Kaelin & Jr- Ratcliffe, 2008). Maternal effector genes also affect embryonic development after zygotic gene activation. For example, the absence of some maternal factors can lead to embryonic arrest at different stages of development (Ji et al.,2023). However, the investigation of crustacean maternal effect genes remains conspicuously absent from the scientific discourse thus far.

Hypoxia-inducible factor 1 (HIF-1) is ubiquitously present in animal cells, serving as a crucial transcription factor enabling cells to sense and adapt to fluctuations in oxygen levels while playing a pivotal role in regulating oxygen homeostasis. Comprising two distinct subunits, *HIF-1α* and *HIF-1β*, each subunit assumes specific responsibilities. Notably, *HIF-1α* is indispensable for gene expression in hypoxic cells (Rashid et al., 2021). The dynamic activity of HIF-1 predominantly hinges on the regulation of *HIF-1α*, with *HIF-1β* exhibiting relatively stable expression. The *HIF-1α* protein structure encompasses the basic helix-loop-helix (bHLH), Per-Arnt-Sim (PAS) domain, and oxygen-dependent degradation domain (Wang et al., 1995). The BHLH domain heterodimerizes *HIF-1α* and *HIF-1β*, and its N-terminal region contains a DNA binding site that mediates binding to the hypoxia response element (HRE) of the target gene. The PAS domain can sense changes in oxygen partial pressure or light intensity. The oxygen dependent degradation domain (ODDD) contains proline residues that hydroxylate and degrade *HIF-1α* under normoxic conditions. The topologically associating domain (TAD), which recruits coactivators and interacts with them to regulate the transactivation of target genes, is required for the full activity of HIF (Jiang et al., 1996). Under normoxic conditions, Pro402 and Pro564 of *HIF-1α* undergo hydroxylation by prolyl hydroxylases (PDHs), are recognized by von Hippel-Lindau protein (VHL), and subsequently subjected to E3 ligase components. Conversely, under hypoxic conditions, *HIF-1α* translocates to the nucleus, forming a complex with the *HIF-1β* subunit, activating downstream target genes with HRE (Kaelin & Jr-Ratcliffe, 2008; Tian et al., 2011). This stabilization of *HIF-1α* in the nucleus facilitates dimerization with *HIF-1β*, instigating the activation of target genes involved in functions such as glucose metabolism (Semenza, 2011), erythropoiesis (Cong et al., 2024), vascular development, skin growth, and cell proliferation and apoptosis (Zhao et al., 2019).

In the context of tumors, *HIF-1α* plays a pivotal role in the Warburg effect, driving heightened aerobic glycolysis and the conversion of glucose to lactic acid (Vander-Heiden et al., 2009; Gonzalez et al., 2014). This metabolic shift involves the transcriptional activation of glycolysis-related genes, including hexokinase (HK), phosphofructokinase (PFK), Phosphoglycerate kinase (PGK), and pyruvate kinase (PK) (Pavlova et al., 2022), and the glycolysis products pyruvate and lactic acid in turn promote the accumulation and stability of aerobic *HIF-1α* (Lu et al., 2002). While extensively studied in mammals, the role of *HIF-1α* as a transcription factor in regulating aerobic glycolysis remains relatively unexplored in arthropods. In *Drosophila melanogaster*, *HIF-1α* has been implicated as a regulatory switch in glucose metabolism, mirroring similar changes observed in mammalian macrophages during bacterial infection (Krejčová et al., 2019). Additionally, *HIF-1α* in Drosophila can bind to the DNA binding domain of its estrogen-related receptor (ERR), influencing adaptation to hypoxia during larval development (Krejčová et al., 2019). The crustacean *Artemia sinica* has demonstrated elevated expression levels of *HIF-1α* during early embryonic development in hypoxic environments, correlating positively with adaptive responses to adverse conditions (Wang, 2021). Nonetheless, the relationship between *HIF-1α* and embryonic development in crustaceans remains inadequately explored.

In our pursuit of comprehending crustacean embryonic development at the molecular level, we investigated the role of *HIF-1α* in shrimp embryonic development. Intriguingly, *HIF-1α* expression during the cleavage stage significantly surpassed other developmental stages. Our findings suggest that *HIF-1α* regulates glycolytic enzymes, influencing the embryonic development of *Neocaridina davidi* through modulation of the glycolytic process.

Material and Methods

Animals

The embryonic development of *Neocaridina davidi* comprises seven stages: cleavage, blastula, gastrula, egg-nauplius, egg-metanauplius, eye pigment, and prehatching stages, mirroring the developmental stages observed in *Macrobrachium rosenbergii* (Tinikul et al., 2016) and *Macrobrachium nipponense* (Li et al., 2003).

Neocaridina davidi specimens procured for laboratory use were obtained from an aquaculture farm in Changzhi City, Shanxi Province. Well-developed male and female *Neocaridina davidi* with gonads were selected and housed in a laboratory tank at a 1:3 male-to-female ratio, maintaining a water temperature of

27°C. To ensure normal growth and development, compound feed was provided daily, with meticulous removal of food residues and timely water changes. Upon identification of eggs in the female crayfish abdomen, the individual was isolated, and embryos were gently extracted from the abdomen after a holding time exceeding 6 hours. Some embryos underwent water rinsing, followed by freezing in liquid nitrogen and storage at -80°C for tissue expression analysis, while others were designated for dsRNA interference experiments.

Both the experimental and control groups were placed in a 25×15×15 cm mesh isolation box within the same shrimp tank, maintaining a constant temperature of 27°C. Daily feeding was standardized for both groups. When a female shrimp was observed holding eggs, it was transferred to a separate tank. The Nikon microscope was employed at regular intervals to observe and photograph the embryonic development process of *Neocaridina davidi*.

Determination and Phylogenetic Analysis of *HIF-1α*full-Length cDNA

The full-length cDNA of the *HIF-1α* gene was amplified utilizing the RACE method, employing gene-specific primers (Table 1) designed based on the transcriptome database. The first-strand cDNA synthesis was conducted using the RevertAid First Strand cDNA Synthesis Kit (Cat # 1621, Thermo Scientific) following the provided instructions. To acquire the 3' end of *HIF-1α*, PCR was executed using the FirstChoice® RLM-RACE Kit (Cat # AM1700, Invitrogen) 3' end universal primers and *HIF-1α*-Outer, followed by nested PCR using *HIF-1α*-Inner. The final PCR product was ligated into the pMD TM 19-T (Takara) cloning vector, and positive clones underwent Sanger sequencing (BGI). The cDNA sequence of *HIF-1α* was deposited in GenBank with accession no. OR996017.

The HIF-1α protein domain was analyzed using NCBI CDD (https://www.ncbi.nlm.nih.gov/cdd/term), and the *HIF-1α* homologous sequence was retrieved via BLASTP (https://www.uniprot.org/blast/uniprotkb). Multiple amino acid sequence alignment and mapping were executed using the algorithm of MEGA-X software and GeneDoc software. The phylogenetic tree was constructed using the MEGA-X adjacency method with a bootstrap value of 500.

Expression Characteristics of *HIF-1α* in Different Stages of *Neocaridina davidi* Embryonic Development

To compare the expression of *HIF-1α* gene, mRNA in different developmental stages of embryos, gonads with early, middle, and late gonadal development were collected. Simultaneously, embryos at the cleavage stage, blastocyst stage, gastrula stage, pro-nuptial stage, post-nuptial stage, eye pigment stage, and prehatching stage were selected. After three washes with DEPC water, RNA was extracted using TRIZOL reagent (Invitrogen), and total RNA was reverse-transcribed into cDNA using the PrimeScript RT kit (TaKaRa). Following the existing Shrimp transcriptome, the ORF sequence of the *HIF-1α* gene was obtained (GenBank accession no. OR996017). Primer 5.0 was employed to design quantitative primers *HIF-1α*F/R, 18SF/R (Table 1) as the internal reference gene, with a primer concentration diluted to 10 μM. Real-time PCR was conducted on the LightCycler 480 system (Roche). Following standardization of the 18S ribosomal RNA gene, the relative HIF-1α expression levels of different stages were analyzed by the comparative CT method as described by Schmittgen and Livak (34). Each group had three independent repetitions, and the data are expressed as means±S.E. The differences were considered significant for $P<0.01$ by a two-tailed, paired Student's t test.

The embryos of different stages of *Neocaridina davidi* were ground on ice, and PMSF protease inhibitor was added simultaneously. The embryos were placed on ice for 30 minutes, centrifuged at 12,000 rpm for 10 minutes, and the supernatant was transferred to a new tube. Adding the corresponding loading buffer, the mixture was boiled in water for 10 minutes, and the protein of *Neocaridina davidi* embryos was collected.

HIF-1α protein separation occurred via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer to a polyvinylidene fluoride (PVDF) membrane (Merck Milibo Co., Ltd.). TBST containing 5% skim milk (Tris buffer with 0.1% Tween-20) was used to block the membrane for 1 hour at room temperature, followed by overnight incubation at 4 °C with an appropriate primary antibody (1:2000 dilution). After three TBST washes, the membrane underwent incubation with a secondary antibody (diluted 1:10000) at room temperature for 2 hours, followed by three TBST washes. Finally, the ECL luminescence kit (Thermo Fisher Scientific) and a gel imager (Bio-Rad Laboratories) were employed for imaging and PVDF membrane analysis

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Preparation of dsRNA

The nucleotide sequence of *HIF-1α* in the muscle transcriptome guided the design of double-stranded RNA-specific forward and reverse primers, ds*HIF-1α*-F/R, incorporating restriction sites (Table 1) using Primer 5.0 software. The ds*HIF-1α* fragment was cloned, electrophoresed, and purified from crayfish cDNA. Subsequently, the pET-T7 expression vector containing the ds*HIF-1α* target fragment was constructed and introduced into *E. coli* HT115. Following induction with IPTG, total RNA was extracted using Trizol reagent, single-stranded RNA was eliminated, and double-stranded RNA was purified. Detailed experimental steps are available in the referenced article (Li et al., 2023).

Table 1 Primers used in this study

Primer name	Primer sequence (5'-3')	Used for
Nd-β-actin F	TGTGACGATGAAGTAGCAGCA	real-time PCR
Nd-β-actin R	AATCTTTCTGACCCATTCCAAC	real-time PCR
Nd-*PFK*-qF	ACTGCTTATTCTCATCAACGCTGCTT	real-time PCR
Nd-*PFK*-qR	ACCTCTCCGATTCCAACTTCTCACA	real-time PCR
Nd-*HK*-qF	ACTCAACGATACCACAGGCACA	real-time PCR
Nd-*HK*-qR	TCATCCATAGCACCATCCCAC	real-time PCR
Nd-*PGK*-qF	AACAACATCGGGACGGAACG	real-time PCR
Nd-*PGK*-qR	TGATGCGAGCAGAGCAGGAG	real-time PCR
18S-qF	TATACGCTAGTGGAGCTGGAA	real-time PCR
18S-qR	GGGGAGGTAGTGACGAAAAAT	real-time PCR
3' RACE Adapter	GCGAGCACAGAATTAATACGACTCACTATAGGT $_{(12)}$ VN	RACE
HIF-1α-Outer-f	TTTCAGTCATCGCCTGGAAGTAA	RACE
HIF-1α-Inner-f	CAACTCGGGAAAGAACACACCT	RACE
3' Outer Primer-r	GCGAGCACAGAATTAATACGACT	RACE
3' Inner Primer-r	CGCGGATCCGAATTAATACGACTCACTATAGG	RACE
ds*HIF-1α*-F	GCTCTAGAAAGAAGTGCGAAGGGAGTGA	RNAi
ds*HIF-1α*-R	GGAATTCAGTGGAAGTGGGCAAGGATT	RNAi

Shrimp Intramuscular Injection of Double-Stranded RNA

Female and male crayfish with well-developed gonads and robust vitality were randomly assigned to experimental and control groups, each comprising 300 females and 100 males. Shrimps were positioned in a petri dish, with gills kept moist by an infiltrating paper towel for proper breathing. Gentle pressure immobilized the crayfish to expose the abdomen. Using a microinjection system, double-stranded RNA was injected into the shrimp's muscle near the gonad at a volume of 600 nl per shrimp. Following injection, both experimental and control groups were placed in separate 25×15×15 cm mesh isolation boxes within the same shrimp tank for daily feeding.

Detection of RNA Interference Effect by qPCR and Western Blot

Within three days of double-stranded RNA injection, shrimps with eggs were selected. Cleavage stage embryos and corresponding muscle samples were collected and stored at -80°C. Muscle RNA was extracted using the Trizol method, subjected to electrophoresis for quality assessment, and reverse transcribed to obtain cDNA. Real-time fluorescence quantitative PCR was employed to detect the relative expression of the *HIF-1α* gene in both the experimental and control groups, analyzing and confirming the interference effect. Western blot analysis was used to assess the expression of the HIF-1α protein in both groups.

Detection of Relative Expression of Glycolysis Key Enzyme Genes by qPCR

Following double-stranded RNA injection, shrimps with eggs within three days were selected. Cleavage and blastocyst stage embryos were washed three times with DEPC water, and samples from three shrimps were pooled and stored at -80°C. Muscle RNA extraction using the Trizol method was followed by electrophoresis to assess quality. Reverse transcription yielded cDNA, and the relative expression levels of *PFK*, *HK*, and *PGK* genes in both experimental and control groups were quantified by real-time fluorescence quantitative PCR post ds*HIF-1α* injection. *PFK*, *HK* and *PGK* primers such as Table 1. The cDNA sequence of Nd-*PFK* was deposited in GenBank with accession no. OR996020.The cDNA sequence of Nd-*HK* was deposited in GenBank with accession no. OR996018. The cDNA sequence of Nd-*PGK* was deposited in GenBank with accession no. OR996019. Using 18S ribosomal RNA gene as the internal control, the relative expression levels of these genes in control and test groups were analyzed by the comparative CT method. Each group had three independent repetitions, and the data are expressed as means ± S.E. The differences were considered significant for $P<0.01$ by a two-tailed, paired Student's t test.

Results

Sequence Analysis and Phylogenetic Analysis of *HIF-1α*

The full-length cDNA of the *HIF-1α* gene was 4254 bp, comprising a 3162 bp Open Reading Frame (ORF) encoding 1053 amino acids, with a predicted molecular weight of 115 kDa. Conserved domain prediction revealed two Per-Arnt-Sim (PAS) domains in *HIF-1α*, spanning 112-168 aa and 240-339 aa, respectively. The C-terminal featured the *HIF-1α* characteristic transactivation domain (CTAD), positioned at 1020-1053 aa (Figure. 1).

Amino acid multi-sequence alignment of the *HIF-1α* protein demonstrated conservation within its putative domain when compared to other species

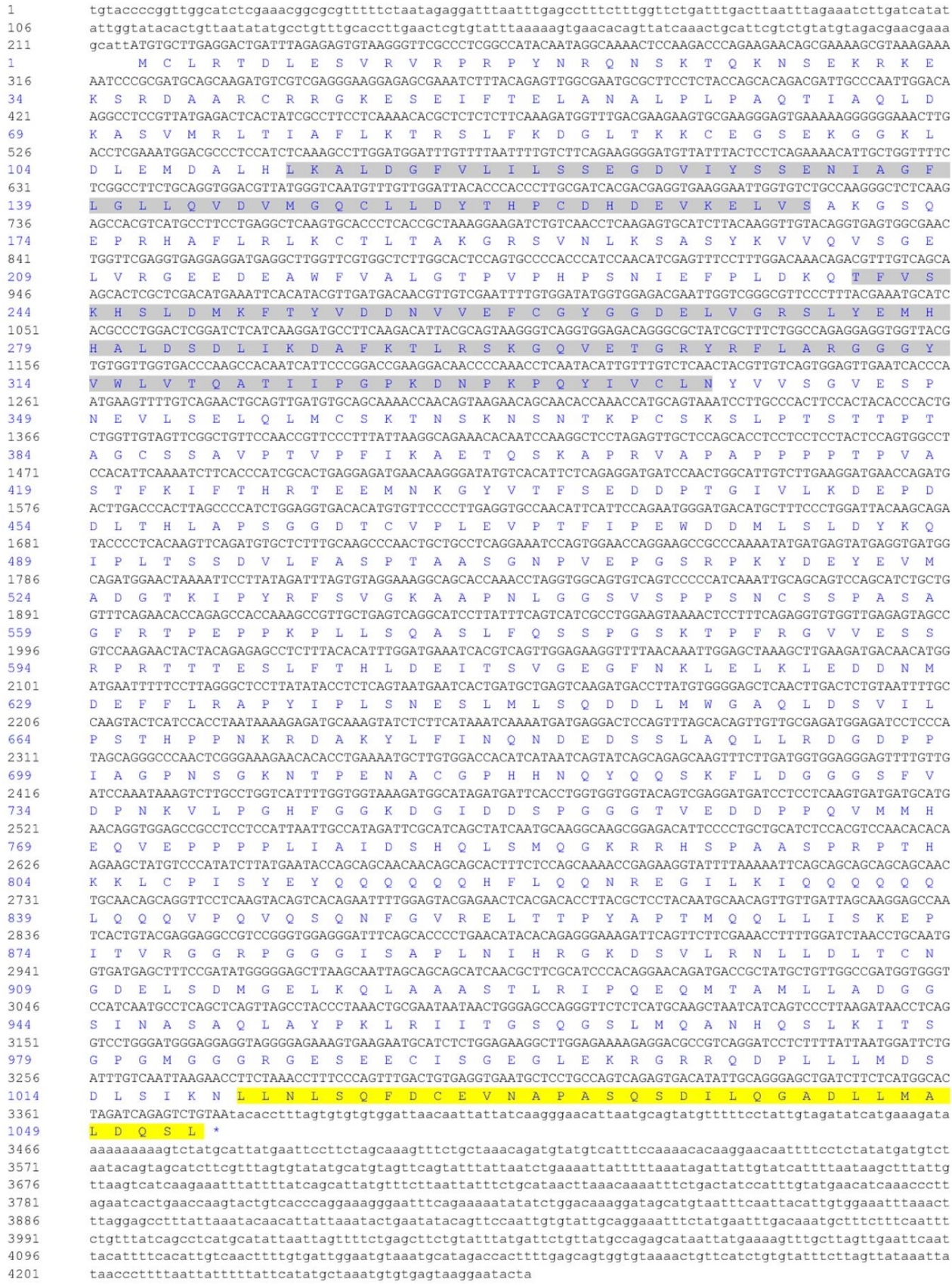

Figure 1. Full-Length *HIF-1α* cDNA and Predicted Amino Acid Sequences.

Numeric annotations for nucleotides and amino acids are provided on the left of the sequences. The amino acid sequence is represented using one-letter codes positioned above the nucleotide sequence. The Open Reading Frame (ORF) is highlighted in uppercase letters, while the 5'and 3'untranslated region (UTR) sequences are presented in lowercase. Two PAS domains, spanning residues 112 to 168 and 240 to 339, are shaded in blue, and the *HIF-1α*-CTAD domain from amino acids 1020 to 1053 is highlighted in yellow.

(Figure. 2). Notably, *HIF-1α* possessed a basic bHLH domain that heterodimerizes with the β subunit, along with two critical PAS domains for sensing changes in oxygen partial pressure and light intensity. Additionally, the conserved TAD domain recruited coactivators, interacting to regulate the transactivation of target genes, crucial for the full activity of HIF.

Clustalx1.83 and MEGA7.0 were utilized to construct the Maximum Likelihood (ML) phylogenetic tree of the *HIF-1α* gene of *Neocaridina davidi* and other species (Figure. 3). This analysis aimed to elucidate the genetic relationships between *Neocaridina davidi*, invertebrates, vertebrates, and 14 other species. The phylogenetic analysis indicated a closer relationship of *Neocaridina davidi* to Arthropoda Crustacea, specifically *Macrobrachium nipponense*, *palaemon carinicauda*, and *palaemon pugio*.

Expression Characteristics of *HIF-1α* Gene in Different Developmental Stages of Gonads and Embryos of Shrimp

To elucidate the functional role of *HIF-1α*, we conducted an investigation into its specific expression during various embryonic developmental stages. Quantitative PCR was employed to detect the mRNA expression of the *HIF-1α* gene in the early, middle, late, and distinct stages of embryonic development. As illustrated in Figure. 4A, the expression of *HIF-1α* was notably higher during gonadal development than embryonic development. Within gonadal development, early and middle gonads exhibited higher expression compared to late gonads. During embryonic development, the cleavage stage demonstrated significantly elevated expression compared to other embryonic stages.

Subsequently, western blot analysis was employed to further assess the expression of the HIF-1α protein throughout early, middle, late, and diverse embryonic developmental stages (Figure. 4B). Utilizing *α*-tubulin as the internal reference protein, the target band of the HIF-1α protein was approximately 115 kD. The western blot results revealed that HIF-1α protein expression was absent during gonadal development, with a gradual down-regulation observed during embryonic development. Specifically, the cleavage stage exhibited the highest expression of HIF-1α protein, while the lowest expression was observed in the prehatching stage.

RNAi

To delve deeper into the functionality of *HIF-1α*, we executed gene knockdown through intramuscular injection of ds*HIF-1α*, investigating alterations in the expression levels within the muscle and embryos of oviparous female shrimp. Quantitative analysis of muscle cDNA samples from both the control and experimental groups, conducted within 3 days post-injection, revealed a significant down-regulation ($P<0.05$) in the expression of *HIF-1α* in both muscle and cleavage stage embryos of the experimental group when compared to the control group (Figure. 5A, 5B).

Subsequently, western blot analysis was employed to further examine the expression of the HIF-1α protein in both muscle tissue and cleavage stage embryos post double-stranded RNA injection. The Western blot bands depicted in Figure. 5C, 5D confirmed a down-regulation of HIF-1α protein levels in the experimental group in comparison to the control group. These results underscore that double-stranded RNA not only impacts injected individuals but can also be transmitted to offspring through oocytes.

Impact of Interference on Embryonic Development

The disruption observed in the normal early embryonic cleavage process upon *HIF-1α* knockdown highlights its pivotal role in this phase. To comprehend the mechanisms underlying *HIF-1α*'s function, we turned to the glycolysis process, crucial for cell growth and proliferation. Quantitative PCR was employed to assess the transcriptional levels of three glycolytic enzyme genes—*PFK*, *HK*, and *PGK*—in the interference group and the control group during both cleavage and blastocyst stages of embryonic development. The results illustrated in Figure. 6 indicated a down-regulation in the expression of these three genes during embryonic development, specifically at the cleavage and blastocyst stages, compared to the control group. This implies that *HIF-1α* plays a role in the early cleavage process of *Neocaridina davidi* embryos by regulating downstream glycolytic key enzymes.

Impact of *HIF-1α* Knockdown on Embryonic Glycolysis

To assess the intergenerational repercussions of *HIF-1α* knockdown, we meticulously examined female shrimp injected with double-stranded RNA and scrutinized the fertilized eggs post-oviposition. The embryonic development of *Neocaridina davidi* was meticulously documented and photographed using an upright stereomicroscope (Nikon).

Upon expulsion from the mother shrimp, the fertilized eggs adhered together and affixed to the abdominal appendages of the female shrimp, exhibiting an oval shape. Throughout in vitro observation, the color of the fertilized eggs underwent dynamic changes. In the early stages, the color deepened from yellow-green, transitioning to a yellow and transparent appearance in the later embryonic phases, concomitant with the emergence of eye spots. The embryonic development in control group, injected with ds*EGFP*, progressed through seven stages: cleavage, blastocyst, gastrula, egg-nauplius, egg-metanauplius, eye pigment, and prehatching stages (Figure. 7A) (Tinikul et al., 2016). This is consistent with the previous observations in *Neocaridina davidi*. Notably, the embryo exhibited

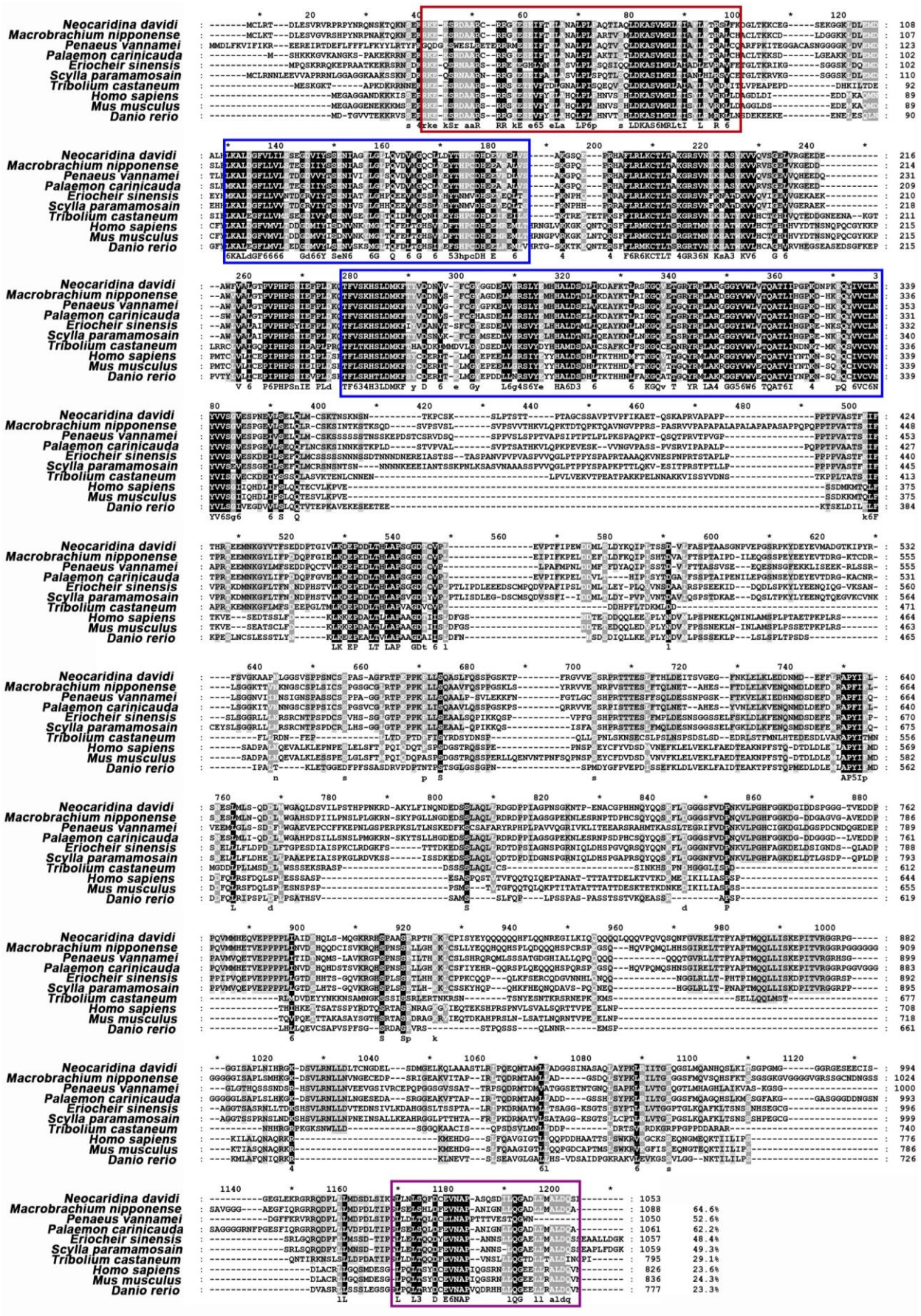

Figure. 2. Multiple Amino Acid Sequence Alignment of *HIF-1α*.

The identical amino acid residues are shaded in black, and similar residues are shaded in gray. The basic Helix-Loop-Helix (bHLH) domain is highlighted with red lines, while the two Per-Arnt-Sim (PAS) domains are enclosed with blue lines, and the *HIF-1α* characteristic transactivation domain (CTAD) is delineated with purple lines. Amino acid identities of *HIF-1α* with other *HIF-1α* proteins are provided on the right. The analyzed *HIF-1α* proteins include: *Macrobrachium nipponense HIF-1α* (AKC54666.1); *Penaeus vannamei HIF-1α* (ACU30154.1); *Palaemon carinicauda HIF-1α* (APU88435.1); *Eriocheir sinensis HIF-1α* (AHH85804.1); *Scylla paramamosain HIF-1α* (ARO76395.1); *Tribolium castaneum HIF-1α* (EFA04586.2); *Homo sapiens HIF-1α* isoform 1 (NP_001521.1); *Mus musculus HIF-1α* (AAC53461.1); *Danio rerio HIF-1α* (AAQ91619.1).

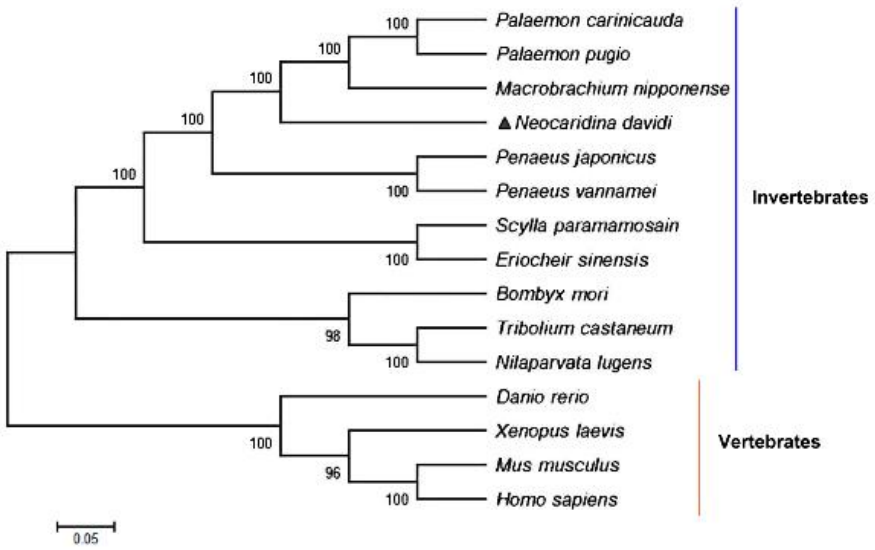

Figure. 3. Phylogenetic Tree Analysis of HIF-1α Proteins in Invertebrates and Vertebrates.
The accession numbers of HIF-1α proteins included in the phylogenetic tree are as follows: *Homo sapiens HIF-1α* isoform 1 (NP_001521.1); *Macrobrachium nipponense HIF-1α* (AKC54666.1); *Palaemon carinicauda HIF-1α* (APU88435.1); *Palaemon pugio HIF-1α* (AAT72404.1); *Penaeus japonicus HIF-1α* (BBC61944.1); *Penaeus vannamei HIF-1α* (ACU30154.1); *Scylla paramamosain HIF-1α* (ARO76395.1); *Eriocheir sinensis HIF-1α* (AHH85804.1); *Tribolium castaneum HIF-1α* (EFA04586.2); *Nilaparvata lugens HIF-1α* (XP_039282885.1); *Bombyx mori HIF-1α* isoform 1 (XP_037876179.1); *Danio rerio HIF-1α* (AAQ91619.1); *Xenopus laevis HIF-1α* (NP_001165655.1); *Mus musculus HIF-1α* (AAC53461.1).

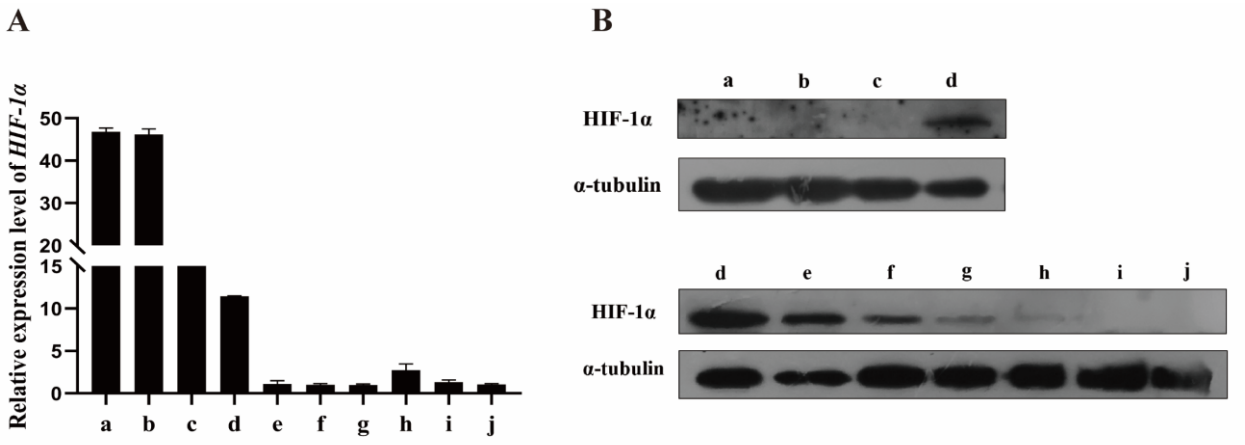

Figure. 4. Protein Expression of HIF-1α in Gonads and Embryos at Different Stages of *Neocaridina davidi*.
mRNA (A) and protein (B) expression of *HIF-1α* in gonads and embryos at different stages: a: Early gonadal b: Mid-gonadal c: Late gonadal d: Cleavage stage e: Blastula stage f: Gastrula stage g: Nauplius h: Metanauplius i: Eye pigment stage j: Prehatching stage.

distinct morphological changes during these stages, from cleavage groove formation to the appearance of protuberances and the eventual differentiation of abdominal limbs and antennae.

Contrasting with the control group, female shrimp injected with ds*HIF-1α* demonstrated the disability to undergo normal embryonic cleavage, presenting three distinct cases: (1) Cleavage groove visibility at 13h, albeit with shrunk or unclear cell edges; abnormal coloration and developmental cessation at 74h (Figure. 7B). (2) Initial normalcy at 12h followed by vesicle-like structures at 14h, progressing to the appearance of white flocculent villi at 40h, leading to hindered development (Figure. 7C). (3) Normal oviposition, yet embryos unable to initiate the cleavage process normally; evident white flocs on the surface at 22h, stalling further development (Figure. 7D).

Discussion

The initiation of early embryonic development in animals is orchestrated by maternal mRNAs and proteins stored within fertilized eggs. Maternal genes are expressed and preserved during oocyte maturation (Pan & Chen, 2004; Rong et al., 2022). Chemical inhibition of transcription, affecting the dizygotic genome allocated to the blastomere through mitosis, does not hinder early cleavage, highlighting the

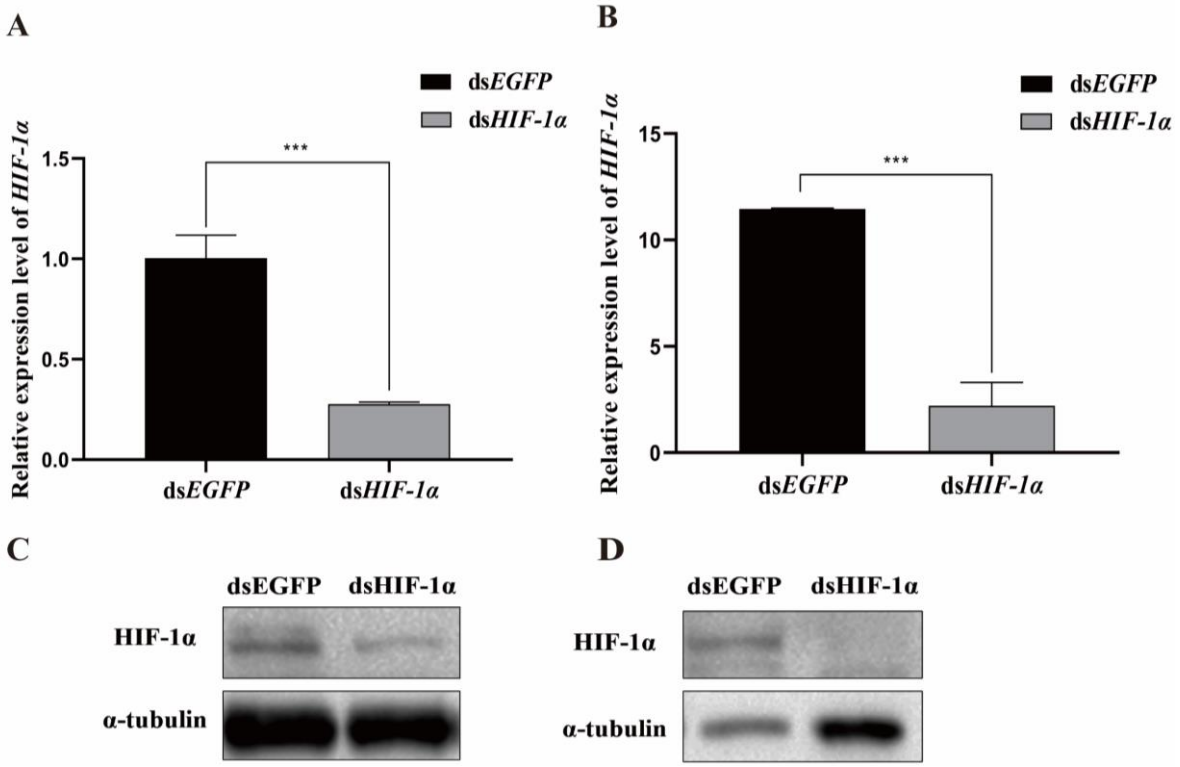

Figure. 5. Relative Expression of Gene *HIF-1α* after RNA Interference.
Relative expression levels of *HIF-1α* in the control (GFP RNAi) and RNAi groups in muscle (A) and embryos (B) determined by QPCR. Relative expression levels of *HIF-1α* in the control (GFP RNAi) and RNAi groups in muscle (C) and embryos (D) determined by immunoblotting. *P<0.05 indicates a significant difference; **P<0.01 indicates an extremely significant difference; ns signifies no significant difference.

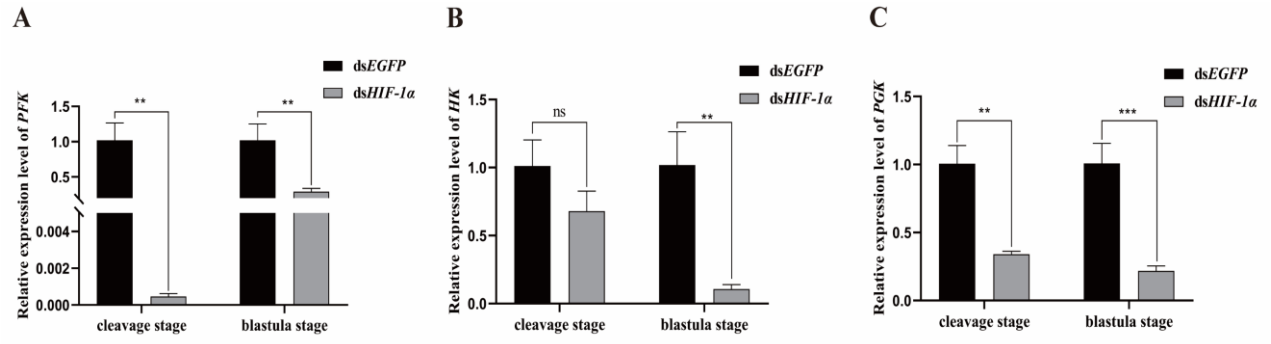

Figure. 6. Alterations in the relative expression of (A) *PFK*, (B) *HK*, and (C) *PGK* genes in muscle following RNA interference.
*P<0.05 indicates a significant difference; **P<0.01 indicates an extremely significant difference; ns denotes no significant difference.

predominant role of cytoplasmic components in guiding the development from cleavage to the blastocyst stage. Until the early gastrula stage with late cleavage, early embryos predominantly transcribe and synthesize mRNAs, marking the transition from maternal to zygotic regulation (Zhang et al., 2022; Brantley & Di-Talia, 2024). By scrutinizing the expression patterns of *HIF-1α* throughout different stages of gonadal and embryonic development, we propose that the gene operates as a maternal effect gene, potentially influencing the embryonic development pattern in *Neocaridina davidi*.

To unravel the interplay between *HIF-1α* and glycolytic key enzymes (*PFK*, *HK*, *PGK*) and their roles in *Neocaridina davidi* embryo development, we utilized RNAi technology to silence *HIF-1α* expression in shrimps with mature gonad. Our findings revealed a down-regulation of *HIF-1α* mRNA and protein levels in the experimental group compared to the control, highlighting the intergenerational transmission of double-stranded RNA. Observations in *Caenorhabditis elegans* previously suggested that dsRNA molecules with silent gene function in the parent could be directly transmitted to and maintain gene silencing in the offspring (Burton et al., 2011). Our results establish, for the first time, that crustacean double-stranded RNA can be transmitted intergenerationally.

Quantitative PCR analysis of key glycolytic enzymes in cleavage and blastocyst stages post ds*HIF-1α* injection exhibited inhibition, suggesting that the *HIF-1α* gene may directly or indirectly regulate glycolytic enzymes, influencing the glycolytic metabolism of *Neocaridina davidi*. The observed developmental impacts in shrimp revealed two scenarios: delayed normal cleavage with subsequent embryo death and a complete lack of cleavage leading to death around 33 hours. Knocking down the *HIF-1α* gene hinders or delays shrimp embryonic development. In summary, we hypothesize that *HIF-1α* regulates glycolytic enzyme expression, mediating the glycolytic process in *Neocaridina davidi* embryos. As a transcription factor, there is already a lot of data supporting the regulatory mechanism of HIF on downstream glycolytic key enzyme expression (Kierans & Taylor, 2021; Wang et al., 2021; Pavlova et al., 2022). Our results further confirm that this effect also exists in early embryonic development. Further exploration is needed to elucidate the specific regulatory mechanisms.

The termination of embryonic development in female shrimp injected with ds*HIF-1α* occurs at varying time points. Differing amounts of ingested double strands during the intergenerational transmission process may lead to distinct interference doses of gene expression. This reaffirms that HIF, functioning as a maternal effector protein, is pre-stored in oocytes and transmitted to offspring. Reduction in HIF content due to interference directly influences its role in early embryonic cleavage, providing a novel perspective on crustacean early cleavage and the role of HIF in embryonic development.

Ethical Statement

All process and experimental protocols have been approved by Ethics approval and consent to participate.

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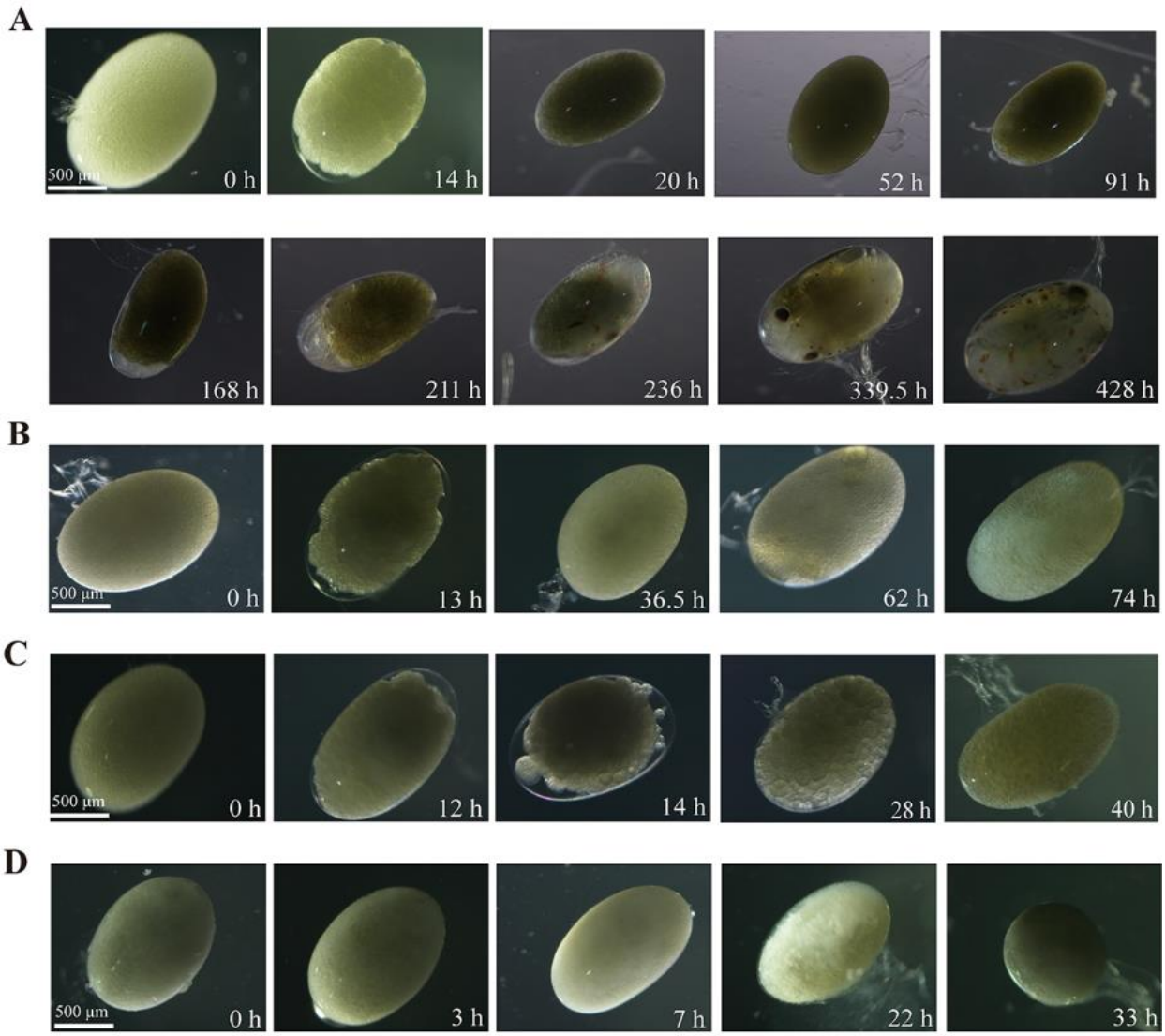

Figure 7. Embryonic development of *Neocaridina davidi* after the injection of ds*EGFP* (A) and ds*HIF-1α* (B, C, D).

Author Contribution

First Author: Conceptualization, Writing -review and editing; Second Author: Data Curation, Formal Analysis, Investigation, Methodology, Visualization and Writing -original draft; Third Author: Funding Acquisition, Project Administration, Resources, Writing - review and editing; and Fourth Author: Supervision, Writing - review and editing.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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