Expression patterns of genes related to growth and immunity in different life stages of spiny lobster, *Panulirus homarus*

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Abstract. Spiny lobster, *Panulirus homarus*, is a species of significant economic value in aquaculture. This study investigates the expression patterns of growth-related (growth hormone/GH and crustacean hyperglycaemic hormone/CHH) and immune system-related (lectin and phenol oxidase as an inactive pro-enzyme/proPo) genes across various life stages of the spiny lobster, *P. homarus*. Understanding these patterns is essential for enhancing productivity in aquaculture. Gene expression was found to be lowest during the nauplisoma stage (0.003±0.0002 CHH, 0.0084±0.0002 GH, 0.003±0.001 lectin, and 0.0033±0.0009 proPo) and highest in 5 cm seed lobsters (1.25±0.11 CHH, 2.14±0.53 GH, 0.78±0.19 lectin, and 1.62±0.24 proPo). These findings suggest potential applications in timing immunostimulant administration to enhance immunity, as well as in developing strategies related to feed supplements, disease management, and environmental factors that influence CHH and GH expression. This research provides crucial insights into the growth and immune development of *P. homarus*, paving the way for improved aquaculture practices.
Key Words: productivity, aquaculture, nauplisoma, environmental, development.

Introduction. The spiny lobster, *Panulirus homarus*, is widely distributed throughout the Indo-Pacific region, with dense populations in East Africa and Indonesia (Berry 1974; Pollock 1993). It is a species of significant economic value, particularly in Vietnam and Indonesia, where it is cultured extensively (Jones 2010). The aquaculture of *P. homarus* relies on the collection of naturally occurring post-larval pueruli, which are then raised to marketable size in marine cages (Do Huu & Jones 2014). However, current culture practices are suboptimal, with issues such as poor nutrition and overcrowding leading to health problems and severe mortality in farmed populations (Behringer et al 2012). Crustacean hyperglycaemic hormone (CHH) plays a crucial role in the life cycle of crustaceans, significantly influencing their growth. It is involved in carbohydrates metabolism and inhibits molting, reproductive activities, and osmoregulatory processes (Fanjul-Moles 2006; Lacombe et al 1999). CHH induces hyperglycemia and hyperlipidemia in the hemolymph, providing necessary glucose and lipids to meet the energy demands of lobster organs and tissues (Kummer & Keller 1993).

The insulin and insulin-like signaling pathway, known to regulate metabolism and reproduction in fish and invertebrates, also appears to play a similar role in crustaceans (Das et al 2013; Das & Arur 2017; Lopez et al 2013). Insulin-like peptides in crustaceans are associated with metabolic control, particularly glucoregulation (Xu et al 2023). Exogenous insulin has been shown to regulate carbohydrate metabolism in crustaceans, similar to vertebrates (Gutiérrez et al 2007). The role of insulin-like growth factors (IGF) in anabolic processes is well-documented in vertebrates, but less understood in crustaceans. However, evidence suggests a similar functional role, with insulin-like

peptides influencing growth and metabolism (Debnath 2010; Moriyama et al 2000; Wong et al 2006). Crustaceans lack adaptive immunity, relying instead on innate immune mechanisms. Lectins in the hemolymph are potential molecules for immunological detection and microbial phagocytosis by opsonization (Wang & Wang 2013). Additionally, the prophenoloxidase (proPO) system in hemolymph, which includes the enzyme phenol oxidase (PO), serves as a marker of crustacean health and is responsive to infection stages (Johansson & Soderhall 1989; Sunish et al 2020). Therefore, lectins and proPO are important components of the crustacean immune response.

In this study, the mRNA levels of growth-related genes (GH and CHH) and immune-related genes (lectin and proPO) was measured to better understand their expression patterns at different life stages of *P. homarus*. These genes were selected because GH and CHH are crucial for regulating growth and metabolic processes, while lectin and proPO are key components of the innate immune system. By elucidating the expression patterns of these genes, the study aimed to provide insights that can enhance our understanding of growth and immune system development in spiny lobsters, which is essential for improving aquaculture practices and productivity.

Material and Method

Lobsters, tissue sample, RNA isolation, and cDNA synthesis. Identification and observation of gene expression related to growth and immunity were carried out on six life stages of *P. homarus*: nauplisoma stage (newly hatched larvae), larvae aged 3 and 6 days after hatching (DAH), 5 cm seeds, 50 g seeds, and adult lobsters. The developmental stages of *P. homarus* are illustrated in Figure 1. Samples of larvae at the intermoult stage were obtained from hatcheries, while seeds and adult lobsters were collected from wild populations in the coastal waters of Situbondo, Indonesia. Tissue samples from pleopods (n=5 individuals) were analyzed for 5 cm, 50 g, and adult lobsters (approximately 200 g) (Thanh et al 2010; Sopian et al 2017; Phan et al 2023). Whole bodies of nauplisoma and larvae (n=10 individuals) aged 3 and 6 days were used for gene analysis. Total RNA was isolated from the tissue using the IQ 2000 RNA extraction kit. Complementary DNA (cDNA) was synthesized using the Ready-To-Go You-Prime First Strand Beads kit (GE Healthcare, USA). A total of 20 mg of tissue sample was homogenized using a pellet pestle. Total RNA isolation and cDNA synthesis were performed according to the manufacturer's instructions. Total RNA concentration and quality were assessed using a NanoDrop spectrophotometer (Thermo Scientific), measuring the ratio of absorbance at 260 nm and 280 nm to ensure RNA purity. DNAse I treatment was applied to remove genomic DNA contamination from the RNA samples.

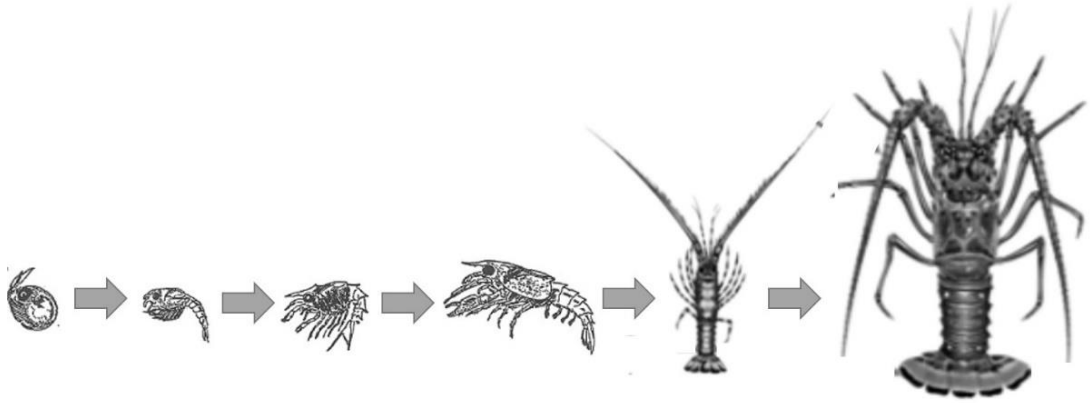

Figure 1. The developmental stadia points of *Panulirus homarus*. The picture from left to right indicated (newly hatched larvae), larvae aged 3 and 6 days after hatching (DAH), seeds size 5 cm and 50 g, and adult lobsters.

Target gene isolation and expression analysis. A total 3 µg RNA in 30 µL DEPC (diethyl pyrocarbonate)-treated water was converted to cDNA. Isolation of target genes was carried out using cDNA as a DNA template. Each gene was amplified by PCR using a

specific primer that had been designed. The identified growth-related genes were GH, MIH and CHH, and the immunity-related genes were lectins and proPO. β-actin gene was used as endogenous control. Gene expression analysis was performed using real-time PCR (qPCR). The qPCR premix for each sample consisted of 4 µL Evagreen (Solis BioDyne) containing PCR reaction materials and fluorescent dye, 1 µL forward primer, 1 µL reverse primer, 12 µL sterile distilled water (SDW), and 2 µL template cDNA. The primers used for gene-specific real-time PCR are detailed in Table 1. The qPCR was conducted using a Rotor-Gene system (Corbett Research) with the following cycling conditions: initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 62.9°C for 30 seconds, and extension at 72°C for 30 seconds. A melting curve analysis was performed from 62.9 to 99°C with temperature increments every 5 seconds. Amplification data were processed using the Schmittgen & Livak (2008) method to calculate mRNA expression levels, normalized to β-actin as an internal control. Data analysis results are presented descriptively.

Table 1

Gene-specific primers are used for amplification by real-time PCR analysis of differentially expressed genes in different life stages of *Panulirus homarus*

Genes	*Primer sequence (5'-3')*	*Reference*
CHH	F: 5'-ATCTGCTGCTGTCCGAAGAGATAGA-3' R: 5'-ACATAAGCTCGTTACCCAGACTTGC-3'	*Panulirus homarus* GeneBank: FJ946880.1
GH	F: 5'-AGAGTCAGCTGTTAAGGGCAAAATT-3' R: 5'-TGGCCCATTGGTGATTTAAATACTG-3'	*Penaeus monodon* GeneBank: GO075401.1
Lectin	F: 5'-AAAGGAGAACTGGTTGCCATCACCA-3' R: 5'-GGATAAACAGTGTTCATTCCCGAGG-3'	*Penaeus monodon* GeneBank: DQ871244.1
proPO	F: 5'-TTGGTGAATGAGAGCCTCTTCGTGT-3' R: 5'-GCAAGCATTTGCTGGTGCATGTAGA-3'	*Panulirus longipes* GeneBank: GQ240941.1
β-actin	F: 5'-GACTCAGATCATGTTCGAGTCCTTC-3' R: 5'-GTGGTGGTGAAAGAATAGCCACGTT-3'	*Penaeus monodon* GeneBank: JQ241179.1

Statistical analysis. Tissue samples were tested in triplicate, and the arithmetic means of all data across all parameters were used for statistical evaluation. Analysis of Variance (one-way ANOVA) was conducted to assess the parameters with a 95% confidence interval. Duncan's test was performed to determine the significance of gene expression levels using SPSS software, version 7.0. Results are presented as means±standard error of the mean (SEM).

Results. The current study investigated gene expression related to growth and immunity in *P. homarus* across different life stages, revealing distinct trends. During the larval phase, the expression patterns of the CHH, GH, lectin, and proPo genes displayed a significant upward trend ($P<0.05$) as the larvae aged. Figure 2 illustrates these trends in gene expression. The lowest expression levels were observed in the nauplii stage, while the highest were in larvae aged 6 days after hatching (DAH). Specifically, the nauplii stage had expression values of 0.003±0.0002 for CHH, 0.0084±0.0002 for GH, 0.003±0.001 for lectin, and 0.0033±0.0009 for proPo. In contrast, the 6 DAH larvae exhibited significantly higher expression levels of 1.25±0.11 for CHH, 2.14±0.53 for GH, 0.78±0.19 for lectin, and 1.62±0.24 for proPo. In contrast to the larval stage, gene expression in the seed-adult stages demonstrated a noticeable downward trend, as depicted in Figure 3. The expression levels of CHH, GH, lectin, and proPo genes uniformly decreased as the lobsters grew larger, with statistically significant reductions ($P<0.05$) observed. The highest expression levels were found in 5 cm seed lobsters, with values of 1.98±0.27 for CHH, 4.01±0.62 for GH, 1.30±0.34 for lectin, and 2.46±0.34 for proPo. Conversely, the adult stage showed the lowest expression levels: 0.40±0.16 for CHH, 1.70±0.18 for GH, 0.38±0.13 for lectin, and 0.33±0.14 for proPo.

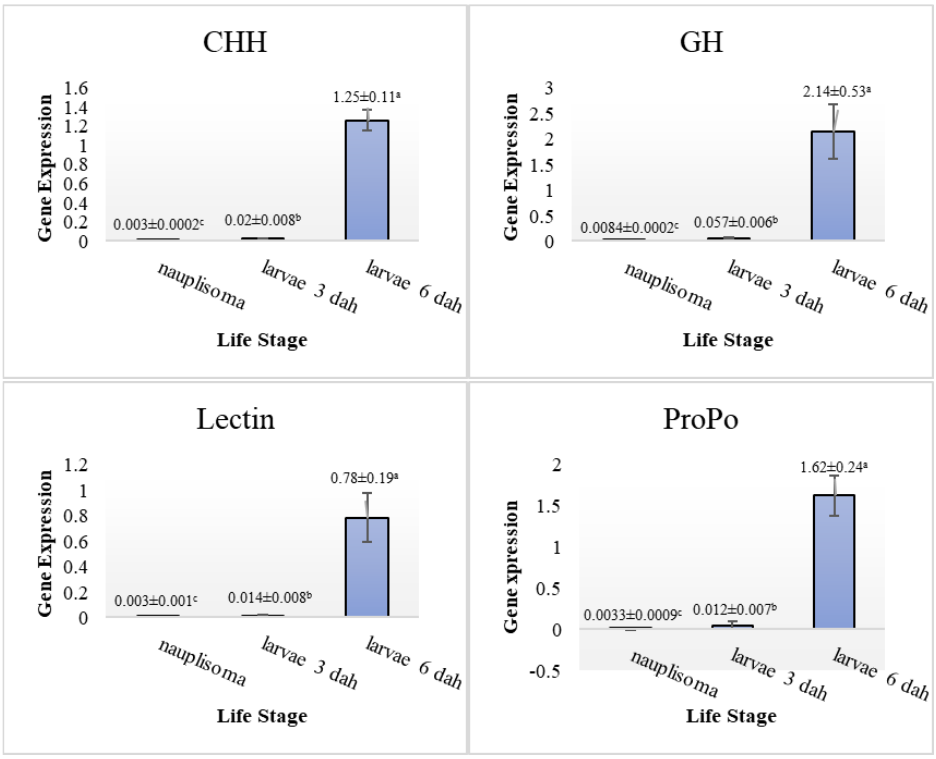

Figure 2. Crustacean hyperglycemic hormone (CHH), growth hormone (GH), lectin, and inactive pro-enzyme (proPO) gene expression in larval stage of *Panulirus homarus*. The columns show average values (from three replication) while the bars illustrate the standard error of the mean (±SEM). Distinct letters above the bars denote a statistically significant difference (P<0.05).

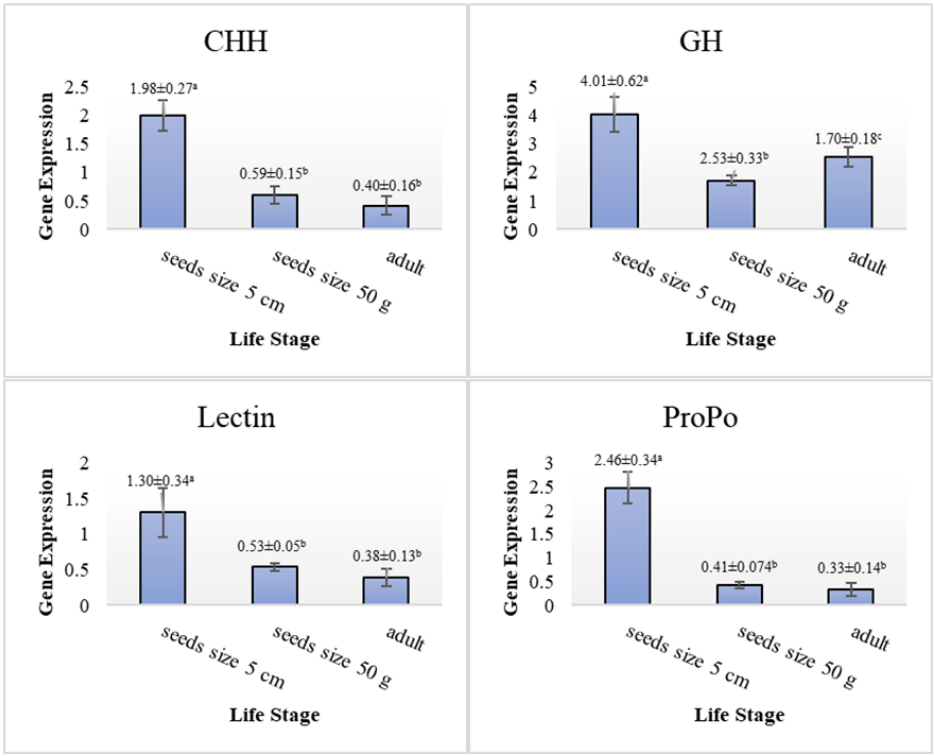

Figure 3. Crustacean hyperglycemic hormone (CHH), growth hormone (GH), lectin, and inactive pro-enzyme (proPO) gene expression in seed-adult stage of *Panulirus homarus*. The columns show average values (from three replication) while the bars illustrate the standard error of the mean (±SEM). Distinct letters above the bars.

Direct comparison of gene expression between the larval and seed-adult stages is challenging due to differences in the samples analyzed. For seed-adult lobsters, pleopod tissue was used for gene analysis, while for the larvae, whole bodies were utilized due to their small size (approx. 2 mm), making it impractical to isolate specific tissues like pleopods. Despite these differences, the overall trend indicates minimal gene expression during the nauplii phase, with peak expression observed in 5 cm seed lobsters.

Discussion. This study provides comprehensive insights into the ontogenetic expression of growth and immune-related genes in the spiny lobster (*P. homarus*). The findings highlight that the gene expressions of crustacean hyperglycaemic hormones (CHH), growth hormone (GH), lectin, and phenoloxidase precursor (proPO) are significantly regulated by developmental stages. CHH and GH are pivotal for crustacean growth and metabolic regulation. CHH is known to play multiple roles, including the regulation of carbohydrate metabolism, suppression of molting, reproductive activities, and osmoregulation (Fanjul-Moles 2006; Lacombe et al 1999). Our study demonstrates that CHH expression increases significantly during the larval stages, peaking in 6-day-old larvae. This peak corresponds to a critical period for growth and energy requirement, as CHH induces hyperglycemia and hyperlipidemia, providing necessary energy substrates (Kummer & Keller 1993). In the seed-adult stages, CHH expression shows a marked decrease, particularly from the 5 cm seed stage to adulthood. This decline reflects the changing energy demands as the lobsters mature, with the highest CHH expression in 5 cm seeds indicating their heightened energy needs for rapid growth. The observed trend in GH expression parallels that of CHH, further corroborating the role of these hormones in promoting growth during early developmental stages and their subsequent decrease as the lobsters reach maturity. GH in crustaceans, similar to human growth hormone (hGH), influences growth and development. The highest GH expression in the 5 cm seeds aligns with previous studies indicating that growth hormone levels peak at specific intermolt stages (Chang et al 2001; Fingerman 1997). The relationship between GH and insulin-like growth factors (IGFs) underscores the complex interplay of these hormones in growth regulation (Chen et al 1994; Christie 2011). Our results provide a deeper understanding of GH gene expression patterns, essential for optimizing growth conditions in aquaculture. The expression of immune-related genes, lectin, and proPO, follows a similar developmental regulation pattern. Lectins are crucial for immunological detection and microbial phagocytosis, acting as primary defense molecules in crustaceans lacking adaptive immunity (Wang & Wang 2013). ProPO plays a significant role in microbial recognition and defense, being activated in response to infection (Gollas-Galvan et al 2017). Our study shows that the expression of lectin and proPO genes is lowest in the nauplii stage and peaks in 5 cm seeds, indicating a robust immune response mechanism during early growth stages. This pattern suggests that as *P. homarus* grow, their immune system becomes more active, reaching a peak when the lobsters are at a critical size for growth and survival. The observed gene expression trends have practical implications for *P. homarus* aquaculture. Understanding the peaks in CHH and GH expression can guide the timing and dosage of nutritional and hormonal supplements to enhance growth rates. Similarly, knowledge of the immune gene expression patterns can inform the administration of immunostimulants to boost *P. homarus*' defense mechanisms during vulnerable stages. Future research should focus on functional assays, such as RNA interference (RNAi) experiments, to further elucidate the interconnections between growth and immune processes. Silencing CHH or GH and observing subsequent effects on lectin and proPO expression would provide definitive evidence of these interactions. Additionally, exploring the environmental and dietary factors that influence gene expression could lead to optimized rearing protocols, reducing health problems and improving productivity.

Conclusions. This study elucidates the ontogenetic expression patterns of growth-related (CHH and GH) and immune-related (lectin and proPO) genes in *P. homarus*, revealing significant regulatory changes across different life stages. The findings indicate that the expression of these genes is lowest during the nauplisoma stage and peaks in

the 5 cm seed stage, reflecting critical periods for growth and immune development. These insights underscore the importance of these hormones in crustacean metabolism and immunity, providing a deeper understanding of their role in the lifecycle of *P. homarus*. The practical implications of these findings for aquaculture are substantial. Knowledge of the gene expression peaks can inform the strategic timing of nutritional and hormonal supplements to optimize growth rates, as well as the administration of immunostimulants to enhance immune responses during critical growth stages. Future research should delve into functional assays, such as RNAi experiments, to explore the interconnectedness of growth and immunity processes. This could lead to the development of optimized rearing protocols, ultimately improving the health and productivity of spiny lobster aquaculture.

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Conflict of interest. The authors declare no conflict of interest.

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