

Expression analysis of the *PEPCK* gene in the shrimp *Metapenaeus affinis* under two different salinity conditions in Southern Iraq

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Abstract

Phosphoenolpyruvate carboxykinase (*PEPCK*) is a key enzyme in gluconeogenesis, playing a vital role in metabolic adjustments during environmental stress. The results indicate that the *PEPCK* gene is responsive to salinity changes in *Metapenaeus affinis*, as the qPCR assays demonstrated successful and reliable amplification, confirming the quality of the generated data. Shrimp collected from the low-salinity environment exhibited the highest gene expression levels, particularly in the hepatopancreas, which showed the lowest ΔC_t value 4.41 and the highest relative expression level (fold change = 8.53). In contrast, increasing salinity resulted in higher ΔC_t values and a marked reduction in gene expression, with this decrease being statistically significant in the hepatopancreas, whereas the reduction observed in the gills was not statistically significant. Furthermore, gene expression in the hepatopancreas was significantly higher than that in the gills under low-salinity conditions, while no significant differences between the two tissues were detected under high-salinity conditions. Overall, given that osmoregulation is a complex physiological process involving multiple regulatory pathways and genes, these findings demonstrate that the *PEPCK* gene is affected by salinity changes. Consequently, these expression patterns suggest that the *PEPCK* gene can be considered a reliable biomarker for monitoring physiological and ecological conditions in *M. affinis*, particularly in the hepatopancreas.

Keywords: Gene expression, *PEPCK*, *Metapenaeus affinis*, salinity, Iraq.

Introduction

Crustaceans are considered important biodiversity resources globally because they possess high nutritional and economic value and contribute to supporting food security (FAO, 2022). One such crustacean is the shrimp, which plays an ecological role in food webs and nutrient recycling in estuarine and marine environments (Gutiérrez *et al.*,



2016). Crustaceans exhibit a high degree of adaptability to environmental changes thanks to specialized physiological systems that maintain internal ion and fluid balance (Thabet *et al.*, 2017). The shrimp *Metapenaeus affinis* is an important species belonging to the family Penaeidae and is widely distributed in the waters of the Arabian Gulf, the Indian Ocean, and adjacent coastal areas. It spends most of its life cycle in shallow coastal environments and estuaries characterized by fluctuating environmental conditions (Dall *et al.*, 1990; Li *et al.*, 2022). This species is also the main component of commercial shrimp fisheries in Iraq and the northwestern Arabian Gulf, constituting a significant proportion of the economically exploited marine shrimp production (Salman *et al.*, 1990 and Mohamed *et al.*, 2025). This species is constantly exposed to changes in environmental factors, particularly salinity, which necessitates physiological and metabolic adaptations to maintain homeostasis and sustain vital functions (Rahi *et al.*, 2021).

Studying the metabolic responses of aquatic organisms to environmental changes is crucial for understanding the mechanisms of adaptation and survival in fluctuating environments. These responses rely on regulating energy production pathways and providing the necessary metabolic compounds for various vital activities (Nelson and Cox, 2021). Osmotic regulation is a fundamental process that allows shrimp to maintain internal ionic balance despite variations in environmental salinity (Liu *et al.*, 2025). Recent studies have shown that gills are the primary organ responsible for ion transport in shrimp under salt stress conditions (Li *et al.*, 2022).

The *PEPCK* gene is one of the key genes involved in gluconeogenesis, where it catalyzes the conversion of oxaloacetate to phosphoenolpyruvate, a crucial regulatory step in the metabolic pathway (Nelson and Cox, 2021). Studies have demonstrated that *PEPCK* plays a role in regulating carbohydrate metabolism and responding to environmental stresses that require increased energy production (Rosas *et al.*, 2002). A study by Schein *et al.* (2004) showed that exposure to salt stress led to an increase in *PEPCK* gene mRNA levels and gluconeogenesis activity in the muscle of the marine crab *Chasmagnathus granulata*.

The *18SrRNA* gene plays a crucial role in shrimp molecular studies, as it has been used in molecular diagnosis and gene expression analysis. It is also a key component of RT-qPCR due to its stability across various tissues and environmental conditions. Chen *et al.*, (2025) highlighted the importance of selecting appropriate reference genes for shrimp gene expression studies using RT-qPCR. Their study aimed to evaluate the stability of several reference genes and found that the *18SrRNA* gene exhibited a high level of stability compared to some other genes. This was confirmed by Hu *et al.* (2018) when evaluating the stability of reference genes used in gene expression analysis of freshwater shrimp (*M. nipponense*) using RT-qPCR. Their study involved testing the *18SrRNA* gene alongside other reference genes under different conditions and tissue types. They observed that the *18SrRNA* gene possessed high stability in its expression levels, making it suitable for use as a reference gene in crustacean molecular studies.

Measuring gene expression levels is a crucial step in understanding gene regulation in organisms. Quantitative polymerase chain reaction (qPCR) is one of the most widely used

and accurate methods for quantifying gene transcripts (mRNA) and analyzing gene expression variations between different samples (Chen *et al.*, 2025). Despite the importance of the shrimp *M. affinis* in the coastal fisheries of the Arabian Gulf region, molecular studies on its salinity-regulating genes are scarce (FAO, 2022).

The significance of this study lies in the fact that, although progress has been made in investigating the molecular mechanisms of osmoregulation in some commercial shrimp species, relevant data remain limited (Schein *et al.*, 2004). There are no published data documenting the gene expression of the *PEPCK* gene under salinity gradients in this specific species. Therefore, studying the expression of this gene under different salinity conditions fills a significant knowledge gap in understanding the mechanisms of osmotic adaptation in this species. Consequently, the primary objective of this study is to evaluate the expression levels of the *PEPCK* gene in the hepatopancreas and gills of *M. affinis* under varying salinity conditions. By using qPCR and employing the *18SrRNA* gene as an internal reference, this research aims to elucidate the role of *PEPCK* in the physiological and metabolic adaptation of this species to environmental salinity fluctuations. Despite the ecological and economic importance of *M. affinis* in Iraq, the molecular mechanisms underlying its osmotic adaptation remain largely unexplored, with no published data documenting the expression of the *PEPCK* gene under varying salinity gradients. Therefore, the primary aim of this study is to evaluate and quantify the expression levels of the *PEPCK* gene in the hepatopancreas and gills of *M. affinis* under different environmental salinity conditions. By using qPCR with *18SrRNA* as a housekeeping gene, and elucidate the role of *PEPCK* in the osmotic adaptation of this species to environmental salinity fluctuations.

Materials And Methods

Sample Collection

Shrimp were collected from two natural water environments in Basrah Governorate, Southern Iraq at mid-November 2025, and identified to the species level as *M. affinis*. The low salinity environment was the Al-Musahab Marsh (30°39'22.0"N, 47°39'39.8"E) and the hypersaline marine environment with high salinity was the Khor Al-Zubair Channel (47°51'49.6"E, 30°13'30.1"N). Six shrimp samples were collected from each environment, for a total of twelve specimens. These sites were selected primarily to contrast the influence of environmental differences between low-salinity and hypersaline marine waters on shrimp traits. This was achieved using common fishing nets for collections during the fishing season. Shrimp in the same size were targeted to minimize individual variances in gene expression. Environmental parameters including salinity, temperature, electrical conductivity (EC), total dissolved solids (TDS) and pH were measured by using a multimeter (HORIPA- Japan) which was calibrated on the morning of sampling day.

Dissection and Tissue Preservation

For this, dissection was carried out (on live specimens) immediately after sample collection so that RNA addition or degradation is limited. RNase enzymes free non-

destroyed sterile surgical instruments were used. Gills and hepatopancreas tissues were removed owing to their functions in osmotic ion regulation and metabolism. The tissues were then placed into 1.5 ml microcentrifuge tubes containing 1 ml of GENEzol directly after excise. The samples were then rapidly frozen by using liquid nitrogen to preserve the intracellular gene expression instantaneously. Samples were subsequently transported in ice box to the Biotechnology laboratory at Department of Natural Marine Science, College of Marine Sciences, University of Basrah. The tissue samples were stored at approximately -196°C in a liquid nitrogen container until further molecular analysis.

RNA Extraction

Gill and hepatopancreas tissues were used for total RNA extraction, using the GENEzol TriRNA Pure Kit (Geneaid/Taiwan) following the provided protocol with minor modification for cortical tissues. 50-100 mg of the tissue was ground using a ceramic mortar with GENEzol solution until homogenized. The mixture was added in 1.5 ml RNase-free microcentrifuge tubes and incubated at room temperature for 5 minutes to ensure complete cell lysis and the disruption of protein complexes. Supernatants were obtained by centrifugation of the cell lysate at $16,000\times g$ for 1 min and then mixed with an equal volume of 96% ethanol and added onto the RNA binding (RB) column. Following the centrifugation, a total of 400 μl of wash buffer was used to wash the column. The DNase I solution (5 μl DNase I and 45 μl reaction buffer) was directly applied to the center of the column for a 15-min incubation at 25°C to remove genomic DNA contamination in an on-column DNase I digestion. Then underwent slush wash steps with 400 μl of pre-wash buffer followed by two washing steps, each involving the centrifugation at $16,000\times g$ for a total of 600 μl of wash buffer, each step was performed for ensure abounding column drying (3-min in the end). Finally, 50 μl of RNase-free water was added in the center of the column and incubated at room temperature for 5 min before centrifugation at $16,000\times g$ for 1 min to elute the purified RNA. The concentration, quality and purity of RNA were determined using a Nanodrop Spectrophotometer (IMPLEN/Germany) by measuring absorbance at 260 and 280 nm. The extracted RNA was normalized using a A_{260}/A_{280} ratio (used as an indicator of purity and protein contamination): if the ratio is within 1.8–2.1, then it is considered to have sufficient purity and other possible contaminating substances and therefore stored in liquid nitrogen until being analyzed later.

Complementary DNA (cDNA) Synthesis

Synthesis of cDNA was performed according to manufacturer instructions using the AccuPower® RT PreMix kit (Bioneer/South Korea). In order to be consistent and eliminate fluctuations in the technique, the volume containing exactly 5 μg of extracted RNA was added to 200 μl PCR tube. Then, these PCR tubes which were containing the RT PreMix pellet were added with 1 μl of oligo dT (15) primer (5'-TTT TTT TTT TTT TTT-3') (Livak, and Schmittgen, 2001), and 1 μl of random hexamer primer. Then, the total volume of reaction was adjusted 20 μl with diethyl pyrocarbonate (DEPC)-treated water, as an

RNase and DNase inhibitor that protects the template RNA and the cDNA from degradation. The reaction components were mixed with a micropipette and centrifuged for 30 seconds to allow the reagents to mix well with the RT PreMix pellet. The samples were then placed into a thermocycler and the reaction was performed as described in the cycling program as follows: Oligo dT and Random hexamer primer was allowed to anneal at 42°C for 60 minutes, cDNA synthesis at 95°C for 5 minutes, finally cold inactive at 4°C for 5 minutes.

Primer Design

Due to no specific primers were available for *M. affinis*, so specific forward and reverse primers for both target gene *PEPCK* and the housekeeping reference gene *18SrRNA* were designed. Primers came from the reference genomic sequences acquired from NCBI, GenBank accession No. FJ441195, and accession numbers MK801122 for *PEPCK* and *18SrRNA*, respectively. Usually, the design process was conducted using the IDT (Integrated DNA Technologies) software package to limit secondary structure (i.e., hairpins and primer-dimer formation) and ensure high amplification fidelity; the standardized settings used in this report were as follows: 18–22 base pairs for primer length, 40%–60% for GC content and lastly ~55°C–60°C for melting temperature (T_m). In addition, qPCR was performed with a target amplicon size of <200 bp. The specificity of the designed primers was finally confirmed in silico via the use of NCBI BLAST algorithm to ensure accurate target and non-target amplification.

Optimization of Annealing Temperature (T_a)

Before gene expression analysis, a gradient PCR was performed with selected cDNA samples to optimize the T_a for primer pair designed in this study for *PEPCK* and *18SrRNA* genes. PCR conditions included initial denaturation (95°C, 15 sec) and extension steps (72°C, 1 min), while, T_a varied from 52–63°C. The optimal T_a for target gene was subsequently identified by assessing the clarity and intensity of the resulting bands following gel electrophoresis on a 2% agarose gel.

Gene Expression Analysis using qPCR

All of the *PEPCK* gene expression were analyzed among groups using quantitative qPCR kit (GO TaqR qPCR master mix kit, Promega/USA). For a reaction volume of 20 μ L, the final composition (in each reaction) was: 10 μ L SYBR Green Master Mix, 0.5 μ L of forward primer and reverse primer for each reaction, 2 μ L cDNA as template and Nuclease-free water to complete the curling to 20 μ L. These reactions were performed in a qPCR-thermocycler (Agilent/USA), using a thermal programs comprised by one cycle at initial denaturation at 95°C for ten minutes, followed by 40 cycles consisting of denaturation phase at 95°C for 30 seconds, annealing temperature at optimized T_a for 30 seconds, and extension stage at 72°C for 30 seconds, then one cycle for final extension cycle (72°C for 5 minutes). The specificity of the amplification products was verified by melting curve analysis. *18SrRNA* reference gene (Housekeeping), was utilized for the

normalization of target gene, *PEPCK* gene expression. Gene expression levels were determined according to the cycle threshold (C_t) values. Relative expression was determined based on ΔC_t , where the target gene C_t value minus reference gene C_t value of the same sample. And then the $\Delta\Delta C_t$ value was calculated as the differences between the ΔC_t from one group vs. that of other group in expression level. Finally, the relative fold change in gene expression for target gene was then determined from two-fold dilution series using the $2^{(-\Delta\Delta C_t)}$ equation (Livak and Schmittgen, 2001).

Statistical Analysis:

Statistical analysis was performed using GraphPad Prism software (version 9.1). A multi-way ANOVA was applied to evaluate the differences in gene expression levels of salt tolerance-related genes in shrimp collected from the two study sites (Al-Musahab Marsh and Khor Al-Zubair Channel). Following the ANOVA, a Least Significant Difference (LSD) test was used as a post-hoc test for pairwise comparisons. Differences were considered statistically significant at a probability level of $P \leq 0.05$.

Results

Environmental Factors

Selected environmental parameters measured at the two study sites, Al-Musahab Marsh and Khor Al-Zubair Channel, are presented in Table 1. Salinity and TDS levels were significantly lower at Al-Musahab Marsh compared to Khor Al-Zubair Channel. Conversely, both sites exhibited moderate and comparable values for temperature and pH.

Table 1: The values of environmental factors that measured at Al-Musahab Marsh and Khor Al-Zubair Channel, Southern Iraq

No	Factors	Khor Al-Zubair Channel	Al-Musahab Marsh
1	Temperature (°C)	27.60±1.65	30.70±2.03
2	Salinity (mg/L)	50.40 ^a ±3.24	9.80 ^b ±1.10
3	pH	7.50±0.24	7.90±0.20
4	TDS (g/L)	40.00 ^a ±2.37	9.40 ^b ±0.81
5	DO (mg/L)	9.70±1.76	7.20±1.00

Morphological Diagnosis

All adult *M. affinis* specimens were confirmed to species-level and identified based on morphological taxonomy. Individuals were then identified based on typical morphological and reproductive characteristics (rostrum structure, arrangement of carapace spines, morphology of pereopods and pleopods). Male species identity was confirmed uniquely by a chitinous spiral tubercle on the merus of the fifth pereopod and a crescent-shaped median process on the petasma, while female species identity was confirmed by the

presence of a longitudinal thelycum plate with a deep median groove and parallel lateral ridges. Morphometric analysis indicated differences in growth parameters, with the highest recorded maximum total lengths being 8.4 cm for males and 10.6 cm for females at low-saline (the Al-Musahab Marsh) and hypersaline (the Khor Al-Zubair Channel) sites, respectively (Josileen and Pillai, 2013).

Total RNA Extraction, Purification, and Reverse Transcription

Total RNA extraction from *M. affinis* gill and hepatopancreas tissues obtained from Al-Musahab Marsh, Khor Al-Zubair Channel at November 2025. The RNA was extracted successfully with high yield and quality 547 to 5500 ng/ μ L (Table 2) by spectrophotometric analysis, confirmed purity of samples between A_{260}/A_{280} ratio, ratios range from 1.8 to 2.1 (Table 2), indicating the RNA obtained is free from proteins and phenols are critical for downstream analyses as this contamination can inhibit reverse-transcription PCR reactions so quantifying them was necessary. Effective diverse synthesis reaction kit was used to reverse-transcribe the purified RNA structure into cDNA prior to the quality assessment. The absolute validity of the conversion was established through analysis of cDNA integrity, which were subsequently utilized for relative *PEPCK* gene expression quantification.

Table 2: Concentration and purity of total RNA extracted from *M. affinis* samples collected from the two study sites.

No.*	Khor Al-Zubair Channel		Al-Masahab Marsh	
	Purity (A_{260}/A_{280})	Concentration (ng/ μ L)	Purity (A_{260}/A_{280})	Concentration (ng/ μ L)
1	1.732	784	2.189	1400
2	2.160	1827	1.912	547
3	2.141	2200	2.0	1726
4	1.991	5500	1.973	1204
5	2.158	4000	1.951	1800
6	1.968	3400	1.941	3115

***Note:** RNA samples 1-3 were obtained from gill tissue, and samples 4-6 were obtained from hepatopancreas tissue of *M. affinis*.

Primer Design and Optimization of T_a for qPCR Analysis

Specific primers for the target gene (*PEPCK*) and the housekeeping reference gene (*18S rRNA*) were successfully designed and validated for qPCR analysis (Table 3), following the methodology described in Method section. To ensure maximum amplification efficiency and specificity, a gradient PCR was performed to determine the optimal T_a , followed by 2% agarose gel electrophoresis. The analysis identified 58°C as the optimal T_a for both genes, yielding clear, specific amplicons (approximately 160 bp for *PEPCK* and 100 bp for *18S rRNA*) without evidence of non-specific amplification or primer-dimer formation (Fig.

1). These optimized conditions confirmed the high precision and suitability of the designed primers. Consequently, these specific temperatures were adopted as the standard for subsequent qPCR experiments, ensuring accurate and reproducible quantification of gene expression levels in *M. affinis*.

Table 3: Sequences and physicochemical characteristics of the primers designed for qPCR analysis

No	Gene name	Oligo sequence (5' 3').	length (bp)	T _m (°C)	G/C %	Amplicon size (bp)
1	<i>PEPCK</i> FW	AACCCCCAACAATGTGAGCA	20	60.0	50	160
	<i>PEPCK</i> RV	AGAGGATGGCAGAGATGGGT	20	60.0	55	
2	<i>18SrRNA</i> FW	CCGCCGAGGTAATGATTAACAG	22	60.1	50	100
	<i>18SrRNA</i> RV	CATGCGACGGTCTCAGAATTT	21	59.5	48	

GEL electrophoresis												
Gene	18SrRNA						PEPCK					
wall	1	2	3	4	5	6	7	8	9	10	11	12
Temp.	58.0	59.0	60.0	61.0	62.0	63.0	58.0	59.0	60.0	61.0	62.0	63.0

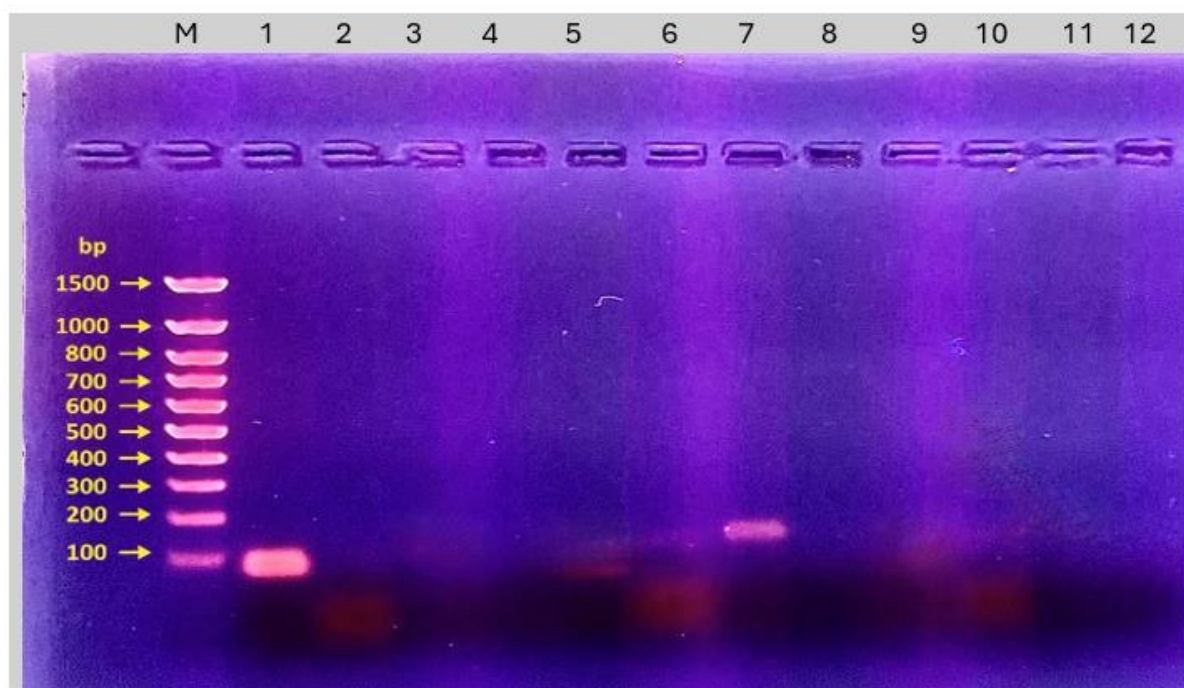


Figure 1: Determination of optimal T_a using gradient PCR for *PEPCK* and *18S rRNA* genes.

Gene Expression Analysis of *PEPCK* via qPCR

To study the molecular responses of *M. affinis* to salinity changes by determining expression levels of the target gene (*PEPCK*) in gill and hepatopancreas tissues. Cycle threshold (C_t) values were used for quantitative analysis using qPCR. Relative fold change in gene expression was determined using the $2^{(-\Delta\Delta C_t)}$ method according to the C_t value of the *18S rRNA* gene, which acted as an internal reference for normalization. The relative expression levels of *PEPCK* gene between samples collected from a low-salinity environment (Al-Musahab Marsh) and a high-salinity marine environment (Khor Al-Zubair Channel) were compared, and statistical significance was assessed to infer differential gene expression patterns in response to salinity gradients.

Amplification and analysis of ΔC_t for the *PEPCK* gene:

Amplification plots for the *PEPCK* gene obtained from qPCR have been represented in (Fig. 2). The plots exhibit a typical sigmoidal amplification profile with high reproducibility between biological replicates, indicating that the genetic template quality and reaction efficiency were high. The plateau phase was achieved around 28 cycles for all samples, thus confirming the reliability of the calculated C_t values when performing gene expression analysis (Fig. 2). The *PEPCK* expression across the environments, indicated by their ΔC_t values and normalized against the *18S rRNA* reference gene, are shown in Fig. 3. The lowest ΔC_t value (4.41) was observed in the hepatopancreas of specimens from the low-salinity environment. Conversely, the highest ΔC_t value (8.18) was recorded in the gills of specimens inhabiting the high-salinity environment. More importantly, the ΔC_t value of the hepatopancreas increased from 4.41 to 7.39 ($P = 0.0386$) as salinity increased, reflecting down-regulation of *PEPCK* expression.

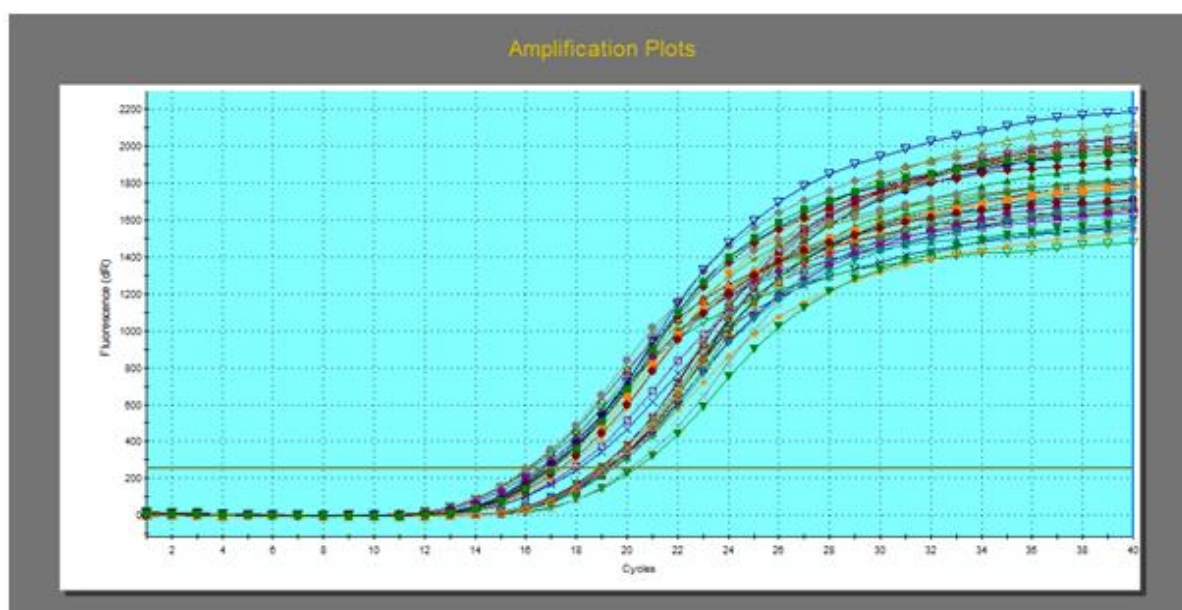


Figure 2: *PEPCK* gene amplification curve in *M. affinis* shrimp samples using qPCR, showing the increasing amplification curves versus the number of reaction cycles.

Conversely, there was a trend in increasing ΔC_t from 6.95 to 8.18 (not significant) in gill tissues. Across both (not significant when comparing like tissues in the same environment, suggesting a similar baseline for expression).

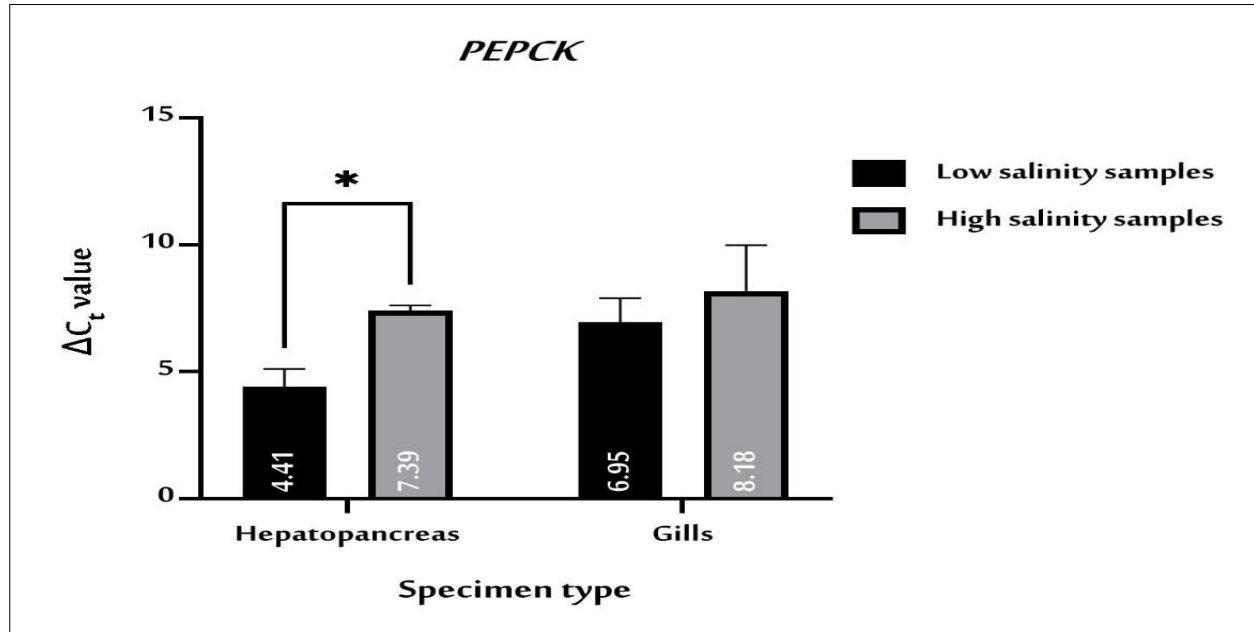


Figure 3: Change in the ΔC_t for the *PEPCK* gene compared to the conservative *18SrRNA* gene in *M. affinis* shrimp samples collected from the low-salinity Al-Musahab Marsh (Riverain) and the high-salinity Khor Al Zubair Channel (Marine).

Relative Expression Analysis of the *PEPCK* Gene:

Analysis of the $2^{(-\Delta\Delta C_t)}$ data revealed significant variations in the relative expression of the *PEPCK* gene between tissues and across salinity gradients (Fig. 4). The hepatopancreas of specimens from the low-salinity environment exhibited the highest fold change (8.53), which was significantly higher ($P = 0.0073$) than that observed in the high-salinity environment (1.01). Similarly, *PEPCK* expression in gill tissues decreased from 3.44 in the low-salinity environment to 2.27 in the high-salinity environment; however, this reduction did not reach statistical significance.

Inter-tissue comparisons within the same environment revealed that, under low-salinity conditions, expression levels in the hepatopancreas were significantly higher than those in the gills (8.53 vs. 3.44, $P = 0.0487$). In contrast, no significant inter-tissue differences were detected under high-salinity conditions. Overall, these findings indicate a decrease in *PEPCK* expression in both tissues as salinity increases, suggesting that high-salinity stress may exert a suppressive effect on *PEPCK* expression in *M. affinis*.

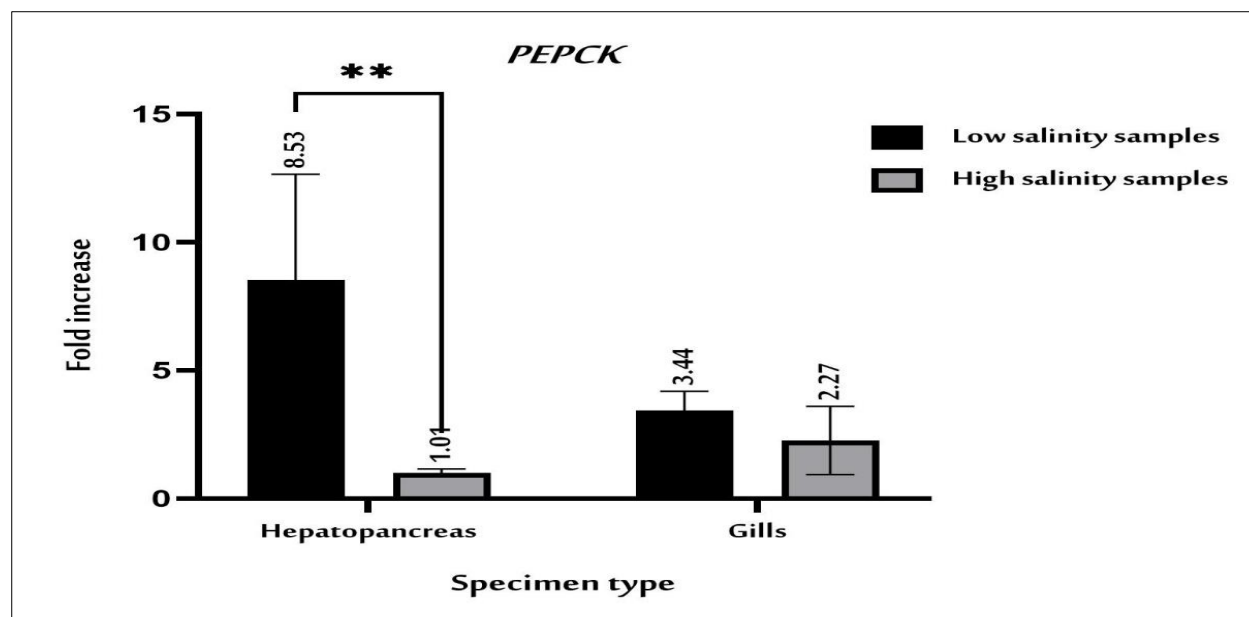


Figure 4: Evaluate the relative gene expression (fold increase coefficient) of the *PEPCK* gene in *M. affinis* shrimp samples collected from the low-salinity Al-Musahab Marsh (Riverain) and the high-salinity Khor Al Zubair Channel (Marine).

Discussion

The results showed that the shrimp *M. affinis* possesses a high capacity for adapting to the prevailing environmental changes in Iraqi aquatic systems. Its presence was recorded in low-salinity environment, specifically the Al-Masahab Marsh, and a marine environment in the Khor Al-Zubair Channel. These two environments differ in the physical and chemical properties of the water, confirming that this shrimp is a widespread species in estuaries and transitional environments that are subject to continuous changes in salinity and other environmental factors (Salman *et al.*, 1990). The study relied primarily on morphological characteristics and morphometric measurements to identify the species, with the morphological results confirming the identity of the studied species. RNA extraction is a crucial step in molecular studies, as the quality of subsequent results, such as reverse transcription, and quantitative polymerase chain reaction (qPCR), depends on obtaining high-quality and pure RNA. The successful design and validation of species-specific primers for *M. affinis* played a fundamental role in ensuring the accuracy and reliability of the gene expression analysis. The optimal annealing temperatures determined via gradient PCR, coupled with the absence of non-specific amplification or primer-dimer formation, confirmed the high specificity of the designed primers. Furthermore, the high quality and purity of the extracted RNA and synthesized cDNA provided a robust template for the qPCR assays. These optimized methodological conditions allowed for precise quantification of *PEPCK* relative expression, ensuring that the observed variations across the salinity gradients reflect true biological responses rather than technical artifacts.

The results indicate that there is a difference in the gene expression level of the *PEPCK* gene between *M. affinis* individuals collected from low- and high salinity environments, suggesting that this gene responds to environmental changes, particularly variations in salinity, these findings indicate that the *PEPCK* gene plays an important role in maintaining homeostasis by enhancing gluconeogenesis pathways and providing the energy required for osmoregulation and ion transport processes (Li *et al.*, 2022).

The successful optimization of T_a via gradient PCR was a critical step in establishing reliable amplification conditions for the *PEPCK* gene. As highlighted by Forootan *et al.* (2017), testing a range of temperatures in a single experiment is essential for achieving optimal amplification efficiency in molecular assays. In the present study, this approach enabled the identification of 58°C as the optimal temperature, yielding distinct single bands of the expected size without non-specific products or primer-dimers. This observation aligns with the principles noted by Dieffenbach *et al.* (1993), who emphasized that single, sharp electrophoretic bands serve as a primary indicator of specific primer binding and robust reaction conditions, thereby validating the precision of the subsequent qPCR quantification.

The significant decrease in ΔC_t values observed in the hepatopancreas under low-salinity conditions compared to the high-salinity environment indicates a relative up-regulation of *PEPCK* expression, as lower ΔC_t values directly reflect a higher abundance of target gene transcripts (Wang *et al.*, 2024). This physiological response is consistent with osmoregulatory mechanisms in crustaceans, where reduced environmental salinity increases the energetic demands required to maintain osmotic balance. Consequently, this metabolic stress stimulates gluconeogenesis pathways to supply the ATP necessary for powering active ion-transporting pumps.

The hepatopancreas serves as the primary metabolic center in crustaceans, rendering it highly responsive to environmental shifts through the modulation of metabolic enzyme gene expression (Henry, 2001; Freire *et al.*, 2008). Conversely, the relative increase in ΔC_t values observed in gill tissues under high-salinity conditions suggests that *PEPCK* plays a less prominent or secondary role in the osmotic response of gills compared to the hepatopancreas. This difference reflects tissue-specific functional specialization; while gills are primarily dedicated to direct ion transport and regulation via membrane channels and pumps, metabolic pathways such as glucose generation play a secondary, supportive role in this tissue (Towle and Weihrauch, 2001).

The relative expression of the *PEPCK* gene was notably higher in the hepatopancreas of *M. affinis* under low-salinity conditions compared to the high-salinity environment. This up-regulation reflects a metabolic reorganization in response to osmotic stress, where increased energy production is essential for ion transport and the maintenance of homeostasis, a process centralized in the hepatopancreas (Li *et al.*, 2014; Chen *et al.*, (2015). In contrast, while the gill tissues also exhibited variations between the two environments, the response was less pronounced than that of the hepatopancreas. This suggests that *PEPCK* plays a more peripheral role in the gills' osmotic response, as this

tissue is primarily specialized for direct ionic transport via ionocytes, rather than serving as a major site for systemic metabolic regulation (Rahi *et al.*, 2021; Liu *et al.*, 2025).

Conclusion

Based on the findings of this study, significant environmental variations in salinity and TDS were established between Al-Musahab Marsh and Khor Al-Zubair Channel. High-quality total RNA and cDNA were successfully obtained, providing a robust template for quantitative assays, while gradient PCR confirmed the high specificity and optimal annealing temperature of 58°C for the designed *PEPCK* and *18SrRNA* primers. The hepatopancreas exhibited the highest *PEPCK* relative expression under low-salinity conditions, which significantly decreased with rising salinity. In contrast, gill tissues showed a less pronounced and non-significant change in expression across the salinity gradient, highlighting tissue-specific functional specialization. Overall, the marked down-regulation of *PEPCK* under high-salinity stress indicates that reduced environmental salinity stimulates gluconeogenesis in the hepatopancreas to meet the increased energetic demands of osmoregulation in *M. affinis*.

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تحليل تعبير جين *PEPCK* في روبيان *Metapenaeus affinis* تحت ظروف ملحية مختلفة في جنوب العراق

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المستخلص

يُعد إنزيم فوسفوينول بيروفات كاربوكسي كيناز (*PEPCK*) من الإنزيمات الرئيسية في عملية استحداث السكر، إذ يؤدي دوراً حيوياً في التغيرات الأيضية استجابةً للإجهاد البيئي. تشير النتائج إلى أن جين *PEPCK* يستجيب للتغيرات في الملوحة لدى الروبيان *Metapenaeus affinis*، إذ أظهرت اختبارات تفاعل البوليميراز المتسلسل الكمي (qPCR) تضخيمًا ناجحًا وموثوقًا، مما يؤكد جودة البيانات الناتجة. أظهرت الروبيانات التي جُمعت من البيئة منخفضة الملوحة أعلى مستويات التعبير الجيني، ولا سيما في الكبد البنكرياسي، إذ سجل أقل قيمة لـ ΔCt بلغت 4.41 وأعلى مستوى للتعبير النسبي، بواقع 8.53 ضعفًا ($\text{fold change} = 8.53$) في المقابل، أدى ارتفاع الملوحة إلى زيادة قيم ΔCt وانخفاض ملحوظ في مستوى التعبير الجيني، وكان هذا الانخفاض ذا دلالة إحصائية في الكبد البنكرياسي، في حين لم يكن الانخفاض المسجل في الخياشيم ذا دلالة إحصائية. علاوةً على ذلك، كان مستوى التعبير الجيني في الكبد البنكرياسي أعلى بصورة معنوية من مستواه في الخياشيم تحت ظروف الملوحة المنخفضة، في حين لم تُسجل فروق معنوية بين النسيجين تحت ظروف الملوحة المرتفعة. وبشكل عام، ونظرًا لأن التنظيم الأزموزي هو عملية فسيولوجية معقدة تتضمن مسارات تنظيمية وجينات متعددة، فإن هذه النتائج تثبت أن الجين *PEPCK* يتأثر بشكل كبير بتغيرات الملوحة، ونتيجة لذلك، تشير أنماط التعبير الجيني هذه إلى أنه يمكن اعتبار الجين *PEPCK* مؤشراً حيوياً لرصد الظروف الفسيولوجية والبيئية في الروبيان، لا سيما في نسيج الكبد البنكرياسي Hepatopancreas.

الكلمات المفتاحية: التعبير الجيني، *PEPCK*، *Metapenaeus affinis*، الملوحة، العراق.