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Gene Expression Analysis in Freshwater Mussels (*Unio stevenianus*) Collected from Pollutant-Associated Environment

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Abstract

Common pollutants found in rivers include sewage, pesticides, heavy metals, nutrients from agricultural runoff, and pathogens. The resulting pollution can alter the physiological processes of living systems and cause environmental stress in aquatic environments. The need to monitor the effects of pollution in aquatic ecosystems has been driven by environmental and human health concerns. Freshwater mussels, which play an important role in freshwater ecosystems, are constantly exposed to a wide variety of contaminants as filter feeders. The sessile nature of mussels and their relatively long life span make them useful indicator species for monitoring and assessment of water quality. The aim of this study is to determine physiological stress in *Unio stevenianus* living in Karasu River, whose pollution level is increasing daily, by *Cox* gene expression. Real-time PCR analysis using *β-actin* as a reference gene was used for gene expression. The expression level of the *Cox* gene was higher in the Zeve Region than in the Arısu Region. The lower level in Arısu region indicates that water quality criteria are better in this region than in Zeve Region. Expression studies should be carried out in *U. stevenianus* by selecting different genes specific to certain pollutants.

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INTRODUCTION

Mussels are invertebrate animals belonging to the class of molluscs. They are found all over the world in fresh and salt waters. The Unionidae family is rich in species diversity, with all members living in freshwater (Joyon, 2020). Freshwater mussels are an essential component of aquatic ecosystems and provide important ecosystem functions and services (Haukenes, 2013; Klishko et al., 2017). Mussels are sessile animals. As they are organisms that obtain their food by filtering the water, they play a role in the filtration of organic matter, bacteria and plankton from the water body (Çetinkaya, 1996; Acarlı et al., 2023). Mussels face persistent and widespread anthropogenic pressures in their aquatic environment. These include excessive nutrient inputs, exposure to toxic substances, sediment loads from agricultural activities, microplastics, changes in hydrological regime, and global climate change. Mussels have become an important indicator for assessing the impact of pollution on aquatic life due to their habitat, nutritional and physiological characteristics (Franzellitti et al., 2010; Haukenes, 2013; Atıcı et al., 2018; Atıcı, 2020; Atici, 2022). With their physiological adaptations, freshwater mussels are mostly able to reduce environmental stressors (Roznere et al., 2018). Important insights for ecological research are provided by the stress-specific expression of cellular proteins in response to exogenous stress and the resulting physiological change. Recently, ecological studies have begun to focus more on the monitoring of various bioindicators to minimize threats to aquatic ecosystem (Viarengo et al., 2007). In this context, the study of gene expression can be an effective method to understand the response of freshwater mussels to many environmental stressors at the biomolecular level (Joyon, 2020; Mehni, 2021).

There are studies that show that physiologically important genes of mussel species are significantly expressed in response to stress and exposure to pollutants. These studies were carried out on gene expression in *Mytilus edulis* held in a copper-polluted region (Dondero et al., 2006); expression of heavy metal-related genes in *Mytilus galloprovincialis* held in the Tunisia coastal region (Banni et al., 2007); expression of stress-related genes in *M. galloprovincialis* exposed to pollutants found on the northern Adriatic coast (Franzellitti et al., 2010).

The *Cox* gene, which was selected as the target gene in the current study, catalyzes the rate-limiting step in the production of prostaglandins, bioactive compounds that are involved in processes such as fever and pain sensitization. It is a target of aspirin-like drugs (Chandrasekharan and Simmons, 2004). There are studies done by different researchers to express *Cox* gene in mussels. These include freshwater mussel (*Elliptio complanata*) exposed to heavy metal contamination (Gagne et

al., 2007); gonads of *M. edulis* exposed to estrogenic contaminants (Cubero-Leon et al., 2010); *M. galloprovincialis* exposed to hypoxia (Woo et al., 2013); zebra mussel (*Dreissena polymorpha*) associated with metal bioaccumulation (Kerambrun et al., 2016); freshwater mussel (*D. polymorpha*) exposed to samarium and yttrium (Hanana et al., 2018).

This study aimed to determine physiological stress by *Cox* gene expression in freshwater mussels (*Unio stevenianus*, Krynicki 1837) living in the Karasu River, particularly exposed to domestic and agricultural waste load.

MATERIAL and METHOD

Mussel collection

A total of 12 mussel samples were collected from two different locations [Arısu (38°36′20.75″ N, 43°14′48.61″ E) and Zeve (38°35′45.13″ N, 43°13′57.26″ E)] of the Karasu River (Figure 1). Morphometric measurements were made on the live specimens brought to the laboratory (Figure 2). Then, a sample of 0.5 g was taken from the whole tissue of the mussel to be used in the gene expression analysis, and the isolation procedures were started.

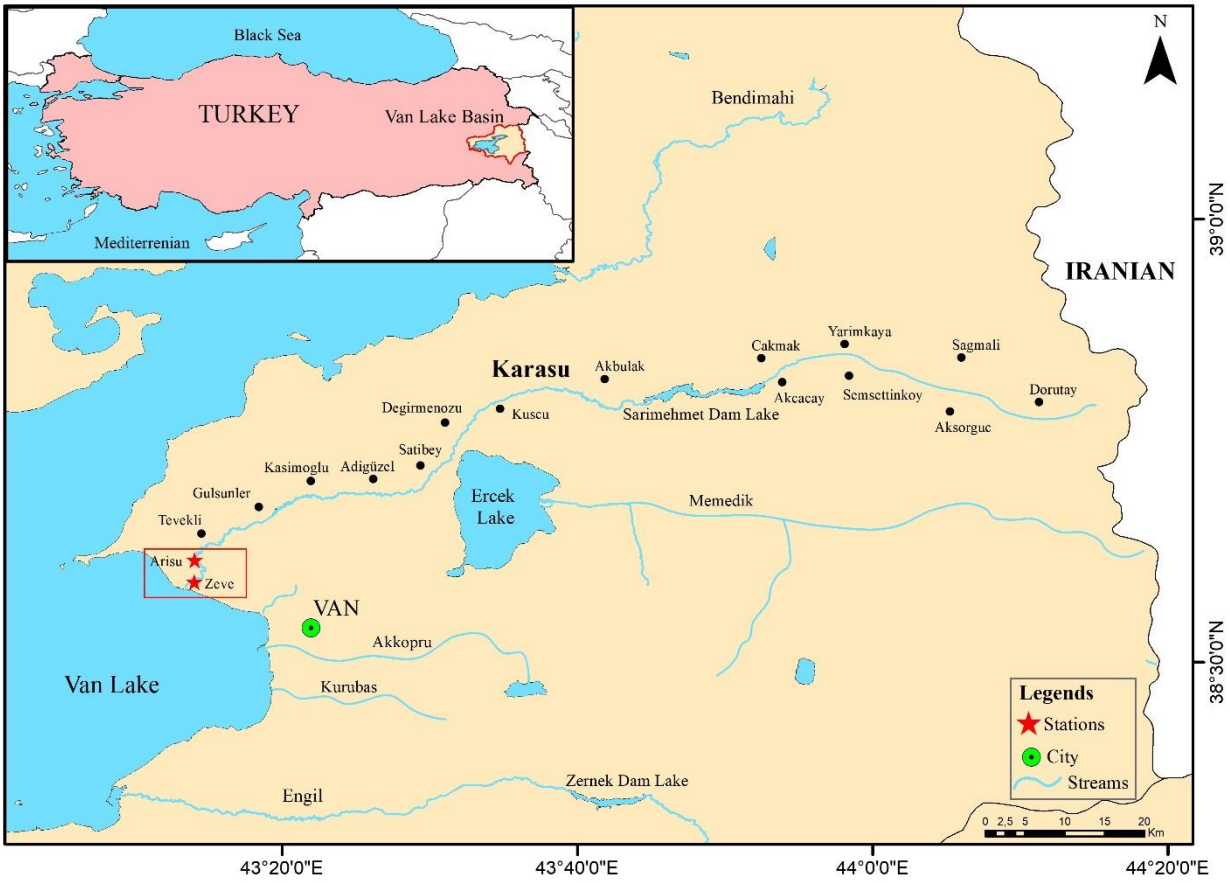

Figure 1. Karasu River and mussel sampling points

Figure 2. Mussel samples collected from Karasu River. (L: Shell length, H: Shell height, W: Shell width)

RNA isolation

A total of 25 mg of tissue was taken from the mussel samples and the isolation was performed using the RNeasy Mini Kit (Qiagen) in the QIAcube instrument. The protocol of the QIAcube instrument was optimized for RNA isolation during the elution stage. The isolation procedures were performed in the following order:

a) Add 350 µL RTL buffer to 25 mg tissue and homogenize in Tissue Lyser LT (Qiagen),

b) Transfer the homogenate to a 2 mL eppendorf tube, add 600 µL 70% ethanol and pipette,

c) Centrifuge at 8000 x *g* for 15 seconds,

d) Transfer the supernatant to spin columns,

e) Add 700 µL buffer RW1 and centrifuge at 8000 x *g* for 15 seconds,

f) Add 500 µL buffer RPE to the spin column and centrifuge at 8000 x *g* for 15 seconds,

g) Add 500 µL buffer to the RPE spin columns and centrifuge at 8000 x *g* for 2 minute,

h) Add 30 µL RNase free water and centrifuge at 8000 x *g* for 1 min,

i) Obtaining RNA elutions (Önalan, 2019).

cDNA synthesis

All steps of cDNA synthesis were performed on a cold plate at +4 °C. RT^2 First Strand cDNA synthesis kit (Qiagen) was used for cDNA synthesis. The following procedures were carried out in the following order:

a) Adjustment of isolated RNAs at 1 ng/µL in a nanospectrophotometer (QIAxpert, Qiagen),

b) Completion of the total volume to 10 µL with ultrapure water,

c) Addition of 2 µL GE Buffer,

d) Incubation of the mixture in a thermal cycler at 42 °C for 5 min,

e) After incubation, 5X Reaction Buffer 4 µL, Primer 2 µL, Reverse Transcriptase Mix 2 µL were added,

f) Keeping the samples at 42 °C for 15 minutes, followed by incubation in a thermal cycler at 95 °C for 5 min (Schwartz et al., 2020).

Gene expression analysis

Real-Time PCR analysis was performed using cDNA products, RotorGene Q 9000 (Qiagen) and RT^2 SYBRGreen qPCR Master Mix (Qiagen). In gene expression analysis, *β-actin* was selected as the reference gene. *Cox* gene was identified as the target gene by sequence data from NCBI gene bank and primers were synthesized. The primers used in the study and their properties are given in Table 1.

Table 1. Primers used in the study and their properties

Gene	Sequence F	tm	bp	Sequence R	bp	tm
β-actin	CTCTTGATTTCGAGCAGGAAA	60	21	AGGATGGTTGGAATAATGATT	21	60
Cox	TTGGGCATCAGTGGTATTGA	60	20	GACCAGGAATGGCATCAACT	20	60

PCR mix was prepared by adding 12.5 µL SYBRGreen qPCR Master Mix (Qiagen), 1µL Forward, 1µL Reverse Primer and 5.5 µL ultrapure water. 5 µL of cDNA was added. Under PCR cycling conditions, the Real-Time PCR protocol was completed with denaturation at 95 °C for 30 seconds and 40 cycles of 5 cycles at 95 °C and 30 seconds at 60 °C.

Statistical analysis

After PCR analysis, gene expression levels were determined with $2^{\Delta\Delta ct}$ -log values using ct values. Normalized values were analyzed by one-way ANOVA, Duncan multiple comparison test to determine the significance of differences between mean values, and paired data differences were analyzed by Student *t*-test for test groups. Statistical difference level was used as $p<0.05$ (Önalan and Çevik, 2020; Xie et al., 2023).

RESULTS AND DISCUSSION

Morphometric measurements

Morphometric values of the mussels collected from the sampling points are given in Table 2.

Table 2. Mean morphometric values±standard deviation of mussels collected from Arısu and Zeve stations (n=12)

	Stations	
	Arısu	**Zeve**
Total weight (g)	43.1±3.8	42.3±5.6
Shell length (cm)	6.4±0.1	6.5±0.3
Shell height (cm)	3.4±0.1	3.3±0.1
Shell width (cm)	2.5±0.1	2.4±0.1
Shell weight (g)	26.7±2.4	26.5±4.6
Soft tissue weight (g)	6.9±0.5	7.9±1.2

Gene expression analysis

In the Real-Time PCR results of the *β-actin* gene, which was used as a housekeeping gene in the study, the automatic threshold value was determined by the device as 0.03. It was observed that all samples were above the threshold value and gave a ct value. *Cox* gene expression values were determined using housekeeping gene ct values. Real-Time PCR result of *β-actin* gene is given in Figure 3.

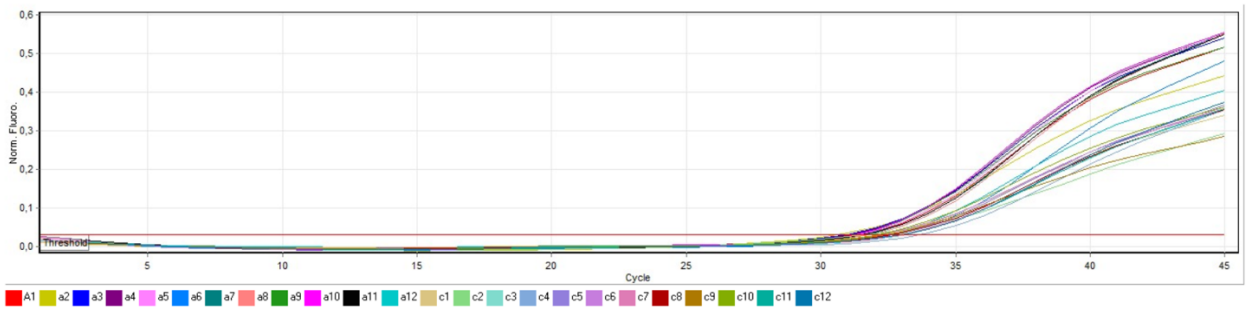

Figure 3. Real-Time PCR results of *β-actin* and *Cox* genes

The expression of *Cox* gene expression levels generated by fold values and $2^{-\Delta\Delta ct}$ -log values after normalization and formulation of ct values obtained from *Cox* gene Real-Time PCR results are given in Figure 4.

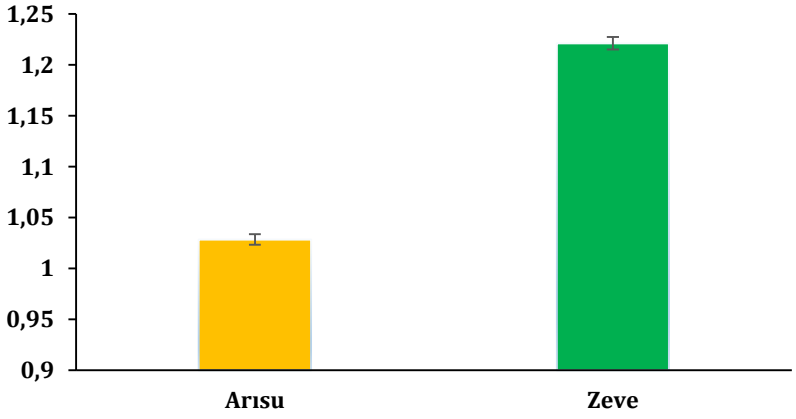

Figure 4. *Cox* gene expression levels observed as a result of gene expression analysis (n=12)

In the results of *Cox* gene expression, an up-regulation of 0.19 fold was observed in the Zeve samples compared to the Arısu group. According to *β-actin* reference gene, it was observed that there was a significant difference between *Cox* gene expression levels in Arısu and Zeve Regions. The expression ratio of 1.02 for Arısu samples was found to be 1.22 for Zeve samples.

The composition and quantity of wastes discharged into the aquatic environment without adequate treatment has increased due to the increasing number of settlements near rivers. These pollutant loads have adversely affected the Karasu River and its inhabitants (Atici, 2022; Kankaya and Atıcı, 2023). This study attempted to determine the effect of physiological stress on *Cox* gene expression in freshwater mussel (*U. stevenianus*) as a result of river pollution.

According to the results obtained, the expression level of *Cox* gene, which is reported to be strongly affected by environmental impacts and water quality criteria, was higher in the Zeve Region. Similar studies on *Cox* gene expression have

also been carried out on mussels found or kept in different geographical areas contaminated by specific pollutants. In these studies, it was reported that the gene activation increased in terms of both gene number and expression change and changed depending on the increase in copper pollution in the study conducted with *M. edulis* kept in the area where copper pollution was found (Dondero et al., 2006). The relationship of bioaccumulation of cadmium, copper, lead, nickel and zinc in *D. polymorpha* with *Cox* gene expression was studied and the gene was significantly expressed with bioaccumulation (Kerambrun et al., 2016). In addition, they reported that *Cox* gene expression was significantly suppressed in *M. galloprovincialis* experimentally maintained in hypoxic environment (Woo et al., 2013). *Cox* gene expression level decreased in *M. edulis* experimentally exposed to estrogenic contaminants (Cubero-Leon et al., 2010). It is observed that *Cox* gene expression tends to decrease or increase in mussels experimentally exposed to contaminants or selected chemicals in the aquatic environment. In clinging mussels, chemical accumulation in their tissues after chemical exposure causes physiological stresses that trigger gene expression (Joyon, 2020). The variability in expression levels is thought to be due to the difference in the target physiological mechanisms of the toxic effects of chemicals and the duration of exposure.

Many freshwater mussel populations are declining worldwide. Unionid species are either threatened or endangered (Klishko et al., 2017). Deteriorating water quality is one of the most important reasons for the decline of freshwater mussels. With growing interest in the effects of pollutant mixtures and emerging contaminants of concern, gene expression-based studies can increase our understanding of the mode of action and interactions of various chemical and biological pollutants (Roznere et al., 2018). The low expression level of *Cox* gene in Arısu Region indicates that water quality criteria in this region are better than in Zeve Region.

CONCLUSION

It is expected that this study on mussels, which are a model for determining both heavy metal levels and bioaccumulation of other pollutants, will be a reference for studies that will be evaluated with more comprehensive results by adding different geographical regions and genes. In addition, it could help future ecological research to identify marker genes for the assessment of water pollution.

COMPLIANCE WITH ETHICAL STANDARDS

Authors' Contributions

1. E. K.: Performed the mussels sample work, designed the study, carried out the laboratory work, interpreted data and prepared the article.

2. A. A. A.: Performed the mussels sample work, prepared the article.

Conflict of Interests

The authors declare that there is no conflict of interest.

Statement on the Welfare of Animals

Not applicable.

Statement of Human Rights

Not applicable.

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