EFFECT OF TEMPERATURE ON *Hsp* 70 AND *Hsp* 83 EXPRESSION IN EARTHWORM *LUMBRICUS TERRESTRIS*

DISSERTATION SUBMITTED TO ST. TERESA'S (AUTONOMOUS) COLLEGE ERNAKULAM IN PARTIAL FULFILLMENT OF THE AWARD OF
DEGREE OF MASTER OF SCIENCE IN ZOOLOGY

SUBMITTED BY,

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2022-2024

CERTIFICATE

This is to certify that the dissertation entitled **"EFFECT OF TEMPERATURE ON HSP 70 AND HSP 83 EXPRESSION IN EARTHWORM"** is an authentic record of original project work carried out by **AISWARYA REMESH (Reg. No:SM22ZOO001)**, during the academic year **2022-2024**, under the external guidance of Dr. BEENA P S, Director, OmicsGen Lifesciences, Kakkanad and the internal guidance of Mrs. INDU VASUDEVAN, Assistant Professor, Department of Zoology, St. Teresa's College, for the partial fulfilment of the requirement of the Degree of Masters of Science in Zoology from St. Teresa's College, Ernakulum.

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This is to certify that the dissertation entitled **" EFFECT OF TEMPERATURE ON *Hsp* 70 AND *Hsp* 83 EXPRESSION IN EARTHWORM *LUMBRICUS TERRESTRIS*"** is a bonafide record of the original research work done by **MS. AISWARYA REMESH**, St. Teresa's College (Autonomous), Ernakulam in partial fulfillment of the requirement for the award of 'Degree of Master of Science in Zoology', during the period – 21st March 2023 to 30th May 2023. The work was done under my direct supervision & guidance; also reported work has not formed the basis for the award of any other Degree/ Diploma/ Associateship/ Fellowship or other similar title to any candidate of any University.

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DECLARATION

I hereby declare that this dissertation entitled **"EFFECT OF *Hsp* 70 AND *Hsp* 83 EXPRESSION IN EARTHWORM"** submitted to Mahatma Gandhi University, Kottayam in the partial fulfilment for the award of **Master of Science in Zoology**, is a record of original project work done by me, and no part thereof has been submitted to any other course. To the best of my knowledge, this project does not include any content that has been previously published or written by someone else, unless proper acknowledgement has been given to the original source.

AISWARYA REMESH

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LIST OF ABBREVIATIONS

ABBREVIATION	EXPANSION
%	Percentage
µg	Microgram
µl	Microlitre
µm	Micrometre
PCR	Polymerase Chain Reaction
Hsp	Heat Shock Protein
ng	Nano gram
TNF	Tumor Necrosis Factor
TCS	Triclosan
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
Cd	Cadmium
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
NFW	Nuclease Free Water
Ct	Cycle Threshold
C	Carbon
N	Nitrogen
&	And
g	Gram
Rps	Ribosomal protein
F&R	Forward and Reverse
ºC	Degree Celsius
E.	Eisenia
ml	Milli liter
rpm	Revolutions per minute

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ABSTRACT

Earthworms are considered to be one of the most economically valuable organisms as they are the natural ploughers as well as one who maintains the fertility and the sustainability of agriculture. They are of greater importance to maintain the eco biodiversity as well as commercial fields. They actually prefer moist conditions in the soil and can be found somewhat deep inside. As the issues of global warming has been of great concern, and the temperature has been globally increasing every year. This study aims to analyse the gene expression of earthworms (*Lumbricus terrestris*) when undergoing a conditioned heat stress in laboratory conditions. In the laboratory the earthworms were given a conditioned heat stress for continuous three days and DNA isolation was done and amplified using qPCR (Real time polymerase chain reaction). This was followed by analysis of the gene expression which reveals a double fold increase in gene expression of the test organism when compared to the control organism. These findings threw light on the importance or paves way for future analysis of earthworm gene expression if the present-day situation of global warming continues which would be a lead towards developing strategies for developing resistance or even assess even their distribution over time.

INTRODUCTION

Earthworms are terrestrial soil-dwelling invertebrates that are classified under the phylum annelida. They exhibit a tubular body plan and shows both internal and external segmentation and also contains setae in every segment. They are commonly found in soil consuming the organic matter present in it. They are of greater economic importance to human beings in many ways (Singh et al., 2018). They are usually known as farmer's friends as it is capable of ploughing the soil naturally. They have the capability to improve the fertility of soil. They can also reduce the acidity or alkalinity of the soil and hence providing better conditions for plant growth and survival.

Different animals in terrestrial ecosystems both above and beneath the surface may be impacted in different ways by climate change. Because of the enormous variety found in soil and the various ecological services that soil organisms regulate, the drivers of soil biodiversity have gained increasing attention. Numerous meteorological factors, such as temperature, precipitation, soil moisture, and extreme weather events like floods and droughts, have been shown to affect the composition and functioning of soil communities (Opute et al., 2022). In the soils of temperate and tropical regions, earthworms are crucial ecosystem engineers for a variety of ecosystem services, including decomposition, nitrogen cycling, and crop output.

Heat shock proteins (Hsps) are related with environmental or abiotic stress or even adverse life conditions. Hsps are proteins or molecular chaperones and they dealt with the maintenance and protection of various cellular activities (Ryan et al.,.2001). They also have an important role in protein translocation or protein repair. Their name comes from the studies which describe their expression by the sudden increase in temperature and now it can be induced as a result of various stress factors. There are various kinds of heat shock proteins; for example, Hsp 70 which is a highly conserved protein and is frequently the main protein expressed during environmental alterations.

Climate change is being a global threat to the present-day situations and emission of greenhouse gases has shown a rapid increase due to growing industrialization and global warming, this can affect the further changes in climatic conditions. The temperature is being increased every year and as a result, distribution of different species or even the chance for

extinctions can occur. The most dramatic effects to be occurred in soil are due to temperature rise (Singh .2019). Soil organisms like earthworms which are abundant and important for soil functioning would be much affected with this kind of altering environmental conditions and hence studies have been done with earthworms (Weber et al., 2007) which being used as a bio indicator of soil contaminants or pollutants. Since Darwin's seminal discovery that earthworms were essential for boosting soil fertility and turnover, earthworm research has become an essential component of biological inquiry. The biological processes that underlie earthworms' beneficial behaviours to preserve the soil ecosystem, boost soil fertility, and manage organic wastes have been studied for many years. It has been determined through physiological and biochemical tests that earthworms are crucial to the first breakdown of plant litter at the soil's surface as well as the release and recycling of the nutrients it contains (Huang et al., 2020). Additionally, it is well known that the faeces of earthworms, known as cast, are much more microbially active than the soil around them and contain plant nutrients in a form that is easily assimilated, increasing the fertility of the soil in which they live.

Thus, the gene expression studies of these species are thus crucial in order to assess their viability or interpreting their survival during heat stressed conditions. Hence the study paves way towards analysing the changes occurring in earthworms at the molecular level which would likely to provide some useful information if certain advancements in the project is to be undergone in future.

AIM AND OBJECTIVES

AIM

The aim of the current investigation is to determine the effect of heat stress in the genes *Hsp* 70 and *Hsp* 83 of the Earthworm *Lumbricus terrestris.*

OBJECTIVE

- To collect earthworm from the soil sample.
- To isolate RNA from collected earthworms.
- To construct cDNA from the extracted RNA.
- To quantify the gene expression using molecular methods

RELEVANCE

Gene expression studies have been important since the changing climatic conditions like extreme temperature conditions even drought, has been arising as a big challenge today. Since the temperature have been rising globally every year, these are causing threat to many of the organisms all around and also it can lead to disturbance in soil ecosystem affecting the soil dwelling invertebrates such as earthworms. Earthworms are economically valuable, their decline in distribution can adversely affect the overall ecosystem. Since this work could be a lead towards developing a strategy in order to mark the changes occurring in earthworm at the molecular level. As these can be helpful to assess the future availability or changes in the distribution of earthworms all around. Further developments in the present study might be useful in developing strategies for improving the stress tolerance of earthworms in future.

REVIEW OF LITERATURE

The literature on heat shock protein and heat adaptation of the organism by Mosley (1997) explains that Acclimatization or thermotolerance are two processes that can lead to heat adaptation, while it's unclear how these two processes are related. The relevance of heat shock proteins (HSPs) in acclimatization is suggested by their significance in thermotolerance and the variations in their accumulation in animals that have adapted to the heat. This overview covers the function of heat shock proteins (HSPs) in the body as a whole as well as the connections between HSPs and endotoxin tolerance, cytokine resistance, and heat adaptation.

Metalliferous soils have been shown to induce HSP-60, -70, and -90 in *L. rubellus* in a dose-response manner (Marino et al., 1999). Moreover transfer from a clean soil to a metalliferous soil quickly caused earthworms to overexpress HSP.

A study conducted by Nadeau et al., (2001) and colleagues highlights the significance of Hsp analysis via western blotting in *L. terrestris*, demonstrating that it is an appropriate and sensitive test for harmful effects in earthworms. An inducible member of the Hsp70 family is shown to be expressed in the midgut/intestinal tissues of *L. terrestris* following heat shock treatment in vitro and exposure to several soil toxins in vivo. Earthworms were similarly impacted by both short- and long-term exposure to chemical standards and heavy metals, and Hsp70 induction was observed in the midgut and intestinal tissues. Hsp analysis in *L. terrestris* midgut/intestine turned out to be a suitable assay for harmful effects in earthworms. They demonstrated a good degree of repeatability with small individual variability. Hsp70 was shown to be temperature-dependently activated in the midgut/intestinal tissues at about 30°C. The study demonstrates that the Hsp 70 induction recognises itself as a biomarker of toxic exposure and that the presence of any hazardous substances or circumstances in soil may be indicated by the detection of Hsp.

HSP70 induction was effective in evaluating environmental pollution/contamination processes due to its many roles in homeostasis of all living organisms and its quick reaction to any agent stressor. HSP70 was evaluated by Nadeau et al., (2001) as a biomarker of numerous soil

contaminants using the *Lumbricus terrestris* earthworm, evaluating if a dose-response connection could be formed and whether the observed reaction to stress was specific.

According to a study by Aamir Nazir et al. (2003) based on heat shock response, Stress gene expression, especially that of Hsp70, has recently been effectively used as an early, first-tier marker for the assessment of environmental toxins. It is believed to be a sensitive indicator of any physical or chemical attack. Because Hsp is largely conserved across species and has a clear correlation with cellular damage and Hsp70 synthesis, Hsp-based tests are applicable to a broad variety of animals.

The subcellular compartments contain the Hsp70 family of proteins, which primarily bind to target proteins to affect protein folding, transport, and repair. Under unfavourable environmental circumstances, the synthesis of Hsp70 increases and it assumes additional functions to protect the cells from proteotoxicity. As a result, these stress proteins are very useful indicators of cellular aggression and have been used to monitor how environmental stressors influence a wide range of animal species. Members of the Hsp70 family are often the primary proteins that are expressed in response to environmental insults. They showed that in these conditions, there was a correlation between the amount of DNA damage and the expression levels of Hsp70. Ordinary soil organisms, like invertebrates, can serve as valuable toxicant Hsp bio monitors (Mukhopadhyay et al., 2003) in areas where heavy metal pollution and pesticide and herbicide buildup have been major problems.

A study based on heat shock response by Nazir et al., (2003) reveals Stress gene expression particularly that of Hsp70, which is thought to be a sensitive sign of any physical or chemical attack and has lately been utilised successfully as an early, first-tier marker for the assessment of environmental toxins. Hsp-based assays can be applied to a wide range of animals because of its highly conserved nature across species and its direct relationship between cellular damage and Hsp70 production, which makes it a very effective marker for environmental monitoring.

A study was conducted by Weber et al., (2007) that aims to study the role of earthworms as biological indicators of soil contamination. As a result of certain exposure to contaminants, proteins are denatured and thus the study of Heat shock protein (Hsp) responses aims to

evaluate such stress protein induction caused by denaturation. Thermal stress has been shown to cause HSP-70 induction in *E. fetida*.

The most frequently used pesticides in the world now are neonicotinoid insecticides. With a thiazolyl ring, the new neonicotinoid pesticide clothianidin has outstanding biological activity against a wide range of pests. The oxidative stress and genotoxicity of clothianidin on earthworms were assessed in the current investigation. Furthermore, for the entire exposure time, the effective clothianidin concentrations in artificial soil were assessed. The findings demonstrated that clothianidin was stable in synthetic soil. These values reflected changes of no more than 10% from the concentrations on the first day. Additionally, clothianidin concentration and exposure duration demonstrated a significant impact on earthworm biomarkers. Reactive oxygen species (ROS) levels were significantly increased, changing antioxidant enzyme activities, harming biological macromolecules, and altering the expression of functional genes. The current findings regarding the Hsp gene expression by Liu et al., (2007) also suggested that heat shock protein 70 (HSP70), DNA damage, and superoxide dismutase (SOD) may be useful environmental risk indicators for clothianidin to earthworms.

As per the study of Kim et al., (2012), the study based on different species earthworms, it has shown both up regulation and down regulation of some of the genes and expressed their products indeed when exposed to low temperatures (0-4 degrees). Ribosomal protein-coding genes and elements of the electron transport chain constituted the majority of the genes in cold-stressed *E. andrei* that were differently expressed. They concluded that the alteration of unsaturated lipid composition in *E.andrei* is important in resisting such cold stress and also many ribosomal proteins have their role in it.

Another study conducted by Tumminello et al., (2013) reveals that the formation of reactive oxygen species (ROS) and it was monitored in order to study the impact of heat stress on celomocytes (leukocytes) from *Eisenia hortensis*. ROS levels were assessed using a flow cytometric technique after celomocytes were cultured for 3–16 hours at temperatures ranging from 4°C (control) to 44°C. At temperatures of 28 °C and above, we consistently saw significant (p 0.05) rises in ROS generation and falls in cell viability. They used antibodies specific for H2AX as a marker of histone alteration to assess the impact of heat stress on histone

phosphorylation. Three different tests were used to compare controls to the heat-stressed samples and they consistently demonstrated the significant H2AX phosphorylation (p 0.05) when comparing both control and heat –stressed.

A study was conducted by Dasong Lin et al., (2014) which proves that the Hsp 70 genes was upregulated under experimental conditions and these genes has shown much sensitivity to Triclosan present in the soil. The study has proved to show high expression of the Hsp 70 gene with the increase of TCS concentration.

The Hsp70 family of proteins is found in the subcellular compartments predominantly binding to the target proteins to influence protein folding, transport and repair. Hsp70 production is increased in adverse environmental conditions (Roy et al., 2017) and it takes on new roles to shield the cells from proteotoxicity. Members of the Hsp70 family are frequently the predominant proteins that are expressed in response to environmental insults; as a result, these stress proteins are highly helpful markers of cellular aggression and have been used to track how environmental stressors affect many animal species. They demonstrated that the levels of Hsp70 expression correlated with the degree of DNA damage under these circumstances. Common soil creatures, such as invertebrates, are helpful toxicant Hsp bio monitors where heavy metal contamination, pesticide and herbicide accumulation have been a serious issue.

Cristina et al., (2018) investigated using stress agent as metals in the earthworm species *L. terrestris*. It revealed that by exposing them with the metal and other chemicals, a decrease in Hsp70 synthesis was detected in the muscle tissues near the middle of the body which was exposed.

To track different pollutants, the ecotoxicologically relevant test species Eisenia fetida earthworm is employed. By subjecting *E. foetida* to soils treated with varying doses of Cd, they demonstrated how high throughput profiling of gene expression may be used to characterise gene expression. In ecotoxicology, earthworms are an essential test animal for monitoring various chemicals. The modification revealed that earthworms in degraded soil ecosystems could reduce the toxicity and bioavailability of Cd through a number of routes. This research, conducted by Chai et al., (2019), has the potential to establish a significant relationship between the transcriptional level of earthworms and the ecological risk of Cd in soil ecosystems.

Singh et al., (2019) examine the research that has been published on the impacts of climate change on earthworm communities and activities. The majority of the time observing the very species- and ecological group-specific reactions to climate change, which are expected to influence the composition of the earthworm community in future ecosystems. While climate extremes like drought have negative consequences, earthworm activity, abundance, and biomass tend to increase with increasing temperature at suitably high soil water content. Higher latitudes and altitudes may see an increase in earthworm invasion, but other parts of the planet may see a decrease in earthworm performance due to drier and warmer conditions. The current assessment of the information available offers an initial baseline for the distribution of earthworms in the future. It also highlights the paucity of research on the interactions between various impacts of climate change on earthworms, such as possible context-dependent impacts of climate change at various levels of soil pollution and in various ecosystem types. For numerous ecosystem services, such as decomposition, nitrogen cycling, and crop yield, earthworms are essential ecosystem engineers in the soils of temperate and tropical climates.

The study conducted by Nerea et al., (2020) intends to evaluate the interactions between cadmium and high temperatures on the various biological complexity levels of earthworms. Earthworms of the species *Eisenia fetida* were kept at temperatures of 19 and 26 °C and subjected to four Cd concentrations at the same time. Endpoints at various biological complexity levels were addressed, including cytotoxicity (coelomocyte viability), buildup of Cd tissue, adult mortality, weight loss, and reproductive impairment (cocoons and juvenile productions). A rise in temperature caused a greater buildup of Cd during the short-term exposure.

Extreme weather events are happening more frequently today as a result of climate change. As a result, extreme ecosystems appear, forcing animals to adapt. For species that live in unstable habitats, such as the riparian earthworm *E. tetraedra*, this problem is particularly important. Its widespread distribution exposes it to a variety of environmental changes, including freezing in subarctic regions and droughts in Mediterranean regions. Therefore, transcriptional alterations in cold and desiccated environments may provide insight into this species' adaptation strategies. For each circumstance, an experiment was conducted by Irene et al., (2022). Temperatures were reduced in the cold experiment from 8 °C to 14 °C ± 2 °C and in the desiccation treatment humidity was lowered from 60% to 15%. A total of 84 genes were found to be differentially

expressed between earthworms that were frozen and control earthworms, while 163 genes were found to be differentially expressed between the desiccation experiment and control. Between the two treatments, there were, no shared responses. The findings imply that activation of genes related to glucose accumulation allows *E. tetraedra* to adapt to low temperatures. The respiratory chain's downregulation, however, may indicate that this earthworm cannot withstand frigid temperatures. Genes involved in DNA repair and cell survival from apoptosis were elevated after desiccation. In contrast, lipid metabolism was downregulated, likely to slow down the rate at which resources are used up.

The focus of research done by Tilikjj et al., (2022) was to assess the impacts of harsh conditions like drought. For that they conducted a global transcriptome study of the endogeic earthworm *Carpetania matritensis*. There were upregulated genes when undergone these effects. The upregulated genes mainly DNA repair genes which was a clear indicator of abiotic stress. This study reveals that the earthworms survive through unfavourable conditions through gene expression changes and many functions like DNA repair and apoptosis is also affected by those unfavourable changes.

MATERIALS AND METHODS

Sample collection and Growth Conditions

A few earthworms were collected from the soil and 4 of them were taken to conduct the experiment trials. Two of them were taken each for control and test sample. The test and control samples were kept for three days treatment. The test sample was treated with 37ºC water twice a day and the control sample treated with normal water.

CONTROL TEST

Fig 1: Control and Test samples

Instruments used

• PCR - BIORAD T100 TM THERMAL CYCLER

• Agarose Gel Electrophoresis- Genei TM MIDI SUBMARINE GEL SYSTEM

• Gel documentation system- BIORAD GelDoc Go Imaging System

RNA Isolation

After the four days of treatment earthworms were separately kept in RNA latter solution and was taken for RNA isolation.

- RNA isolation was done using Takara, RNA iso Plus (Trizol), reagent.

Precautions should be taken in order to avoid RNase contamination. RNA has larger grooves which makes it susceptible to be attacked by enzymes. Intense care should be taken while handling the RNA in plastic or glass wares and it should not be introduced without appropriate care during and after isolation.

Precautions to be taken –

- Wear gloves and change them regularly and make sure that the tubes are kept closed.
- All equipment used should be reserved for the RNA work.
- Non disposable plastics should be treated before usage and the glass wares should be clean.

Procedure

- Wash the mortar and pestle and wipe off the water and wipe them with chloroform using a tissue paper.
- Grind the sample in the mortar and pestle with liquid nitrogen and 1ml Takara, RNA iso Plus (Trizol), reagent.
- Transferring it to a 2 ml tube and adding 200µL chloroform.
- Incubation for 15 minutes at room temperature.
- Centrifuge for 15 minutes at room temperature at 10,000 rpm.
- Collect the aqueous part and add 500µL isopropyl alcohol
- Incubation for 30 minutes at -20°C.
- Pelletization by keeping in centrifuge for 10 minutes at 10,000 rpm.
- Discarding the supernatant and adding 500µL of 75% ethanol.
- Centrifuge for 10 minutes at 10,000 rpm.

- Discarded the liquid and kept for air dry.
- Resuspend the pellet in nuclease free water.

RNA concentration and purity was determined using Thermo scientific Nanodrop 2000 spectrophotometer.

cDNA synthesis

The Prime Script™ RT Master Mix (Perfect Real Time), TAKARA(Japan)kit is designed for effective cDNA synthesis. This reverse transcription reagent kit facilitates cDNA synthesis prior to analysis by real time PCR.

Protocol

1. The reaction mixture for cDNA Synthesis is given in table 1

Reagent	Control	Treatment
5X Primescript buffer	2 µl	2 µl
Primescript RT Enzyme mix	0.5 µl	0.5 µl
Oligo dT	0.5 µl	0.5 µl
RNA	4 µl	2.8 µl
RNase DH2o	3 µl	4.2 µl
Total	10 µl	10 µl

Table 1 showing reaction mixture for cDNA synthesis

2. The reaction mixture was incubated under following conditions in a thermal cycler.

For the conversion of RNA to cDNA, the reaction mixture was incubated for 37°C for 15 minutes which resulted in the reverse transcription. It was followed by incubating the mixture

at 85°C for 5 sec which led to the inactivation of reverse transcriptase with heat treatment. After the inactivation, the mixture was kept at 4°C for infinite hold.

Real time PCR

Real-time PCR uses an increasing variety of chemistries to identify PCR products as they build up inside a closed reaction vessel. Both the particular, fluorophore labelled oligonucleotide probes and non-specific DNA-binding fluorophores fall under this category. prior study aims to inform the scientist about the most recent developments in amplification hardware and fluorogenic detection chemicals.

Real time PCR was carried out using SYBR® Green: TB green premix Ex Taq II Ti RNAse H Plus Takara (Japan).

Reagent	Volume
SYBR Green mix	5 µl
Forward Primer	0.5 µl
Reverse Primer	0.5 µl
Template cDNA	1 µl
ddNFW	3 µl
Total	10 µl

Table 2 showing real time PCR reaction mixture

Primer sequence used for qPCR are given in table 3:

Hsp70	GGGAGAGGGTTGGGCTAGAG	TTGCCTCCTGCCCAATCA
Hsp83	AAGGCCGTTAAGGATCTGGT	CGCTAGTGTGGGGAAGAGA
Rps 57	ATG GTT TTC GGA TCA AAG GT	CGA CCT TGT GTT CAA TGG TG

Table 3 showing the sequence of primers used:

Polymerase activation	95ºC for 10 minutes
Denaturation	95ºC for 15 sec
Annealing/Extension	60º C for 1 minute
Infinite Hold	4ºC

Table 4 showing the Thermal profile

The Ct values were acquired after the real-time qPCR.

The gene expression was analysed by finding the fold induction according to the Pfaffl method using the formula:

$$R = \frac{(E_{\text{target}})^{\Delta C_{\text{Ttarget}}^{(control - sample)}}}{(E_{\text{reference}})^{\Delta C_{\text{Treference}}^{(control - sample)}}}.$$

RESULTS

The RNA isolation of earthworms was carried out using Trizol method and further the cDNA synthesis was carried out

The concentration of the isolated RNA was using Thermo scientific Nanodrop 2000 spectrophotometer.

	CONCENTRATION
CONTROL	544.4ng/ µl
TREATED	788ng/ µl

Table 5 depicts the RNA concentration so obtained

Then real time PCR was carried out using SYBR® Green used: TB green premix Ex Taq II Ti RNAse H Plus Takara (Japan).

Real-time qPCR

Ct values

Well	Fluorescence	Genes	Type	Biological Set Name	Ct
A01	SYBR	Hsp70	C	Gene 1	6.90
A02	SYBR	Hsp70	T	Gene 1	10.43
A03	SYBR	Hsp83	C	Gene 2	8.62
A04	SYBR	Hsp83	T	Gene 2	9.57
A05	SYBR	Rps57	C	HKG	8.22
A06	SYBR	Rps57	T	HKG	11.03

Table 6 showing the Ct values obtained from real time PCR

Analysing steady-state m RNA levels is the method used to evaluate gene expression in response to abiotic stress because it offers a more accurate measurement of gene activation than enzyme activity (Hong et. al., 2009).

The present study investigates and reports the gene expression of earthworm that are subjected to heat stress. The Ct values obtained are used for analysis by Pfaffl method.

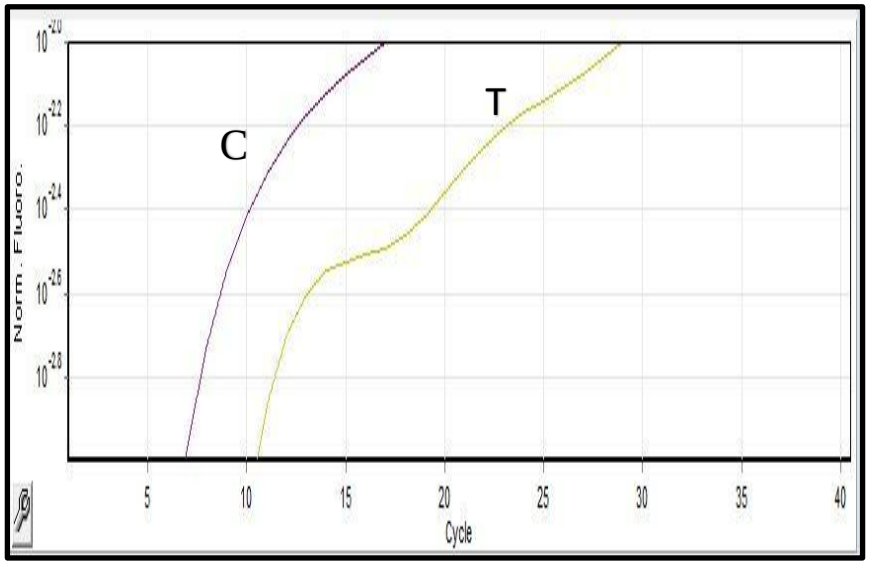


Fig 2 shows the Amplification plot of HSP 70 F&R

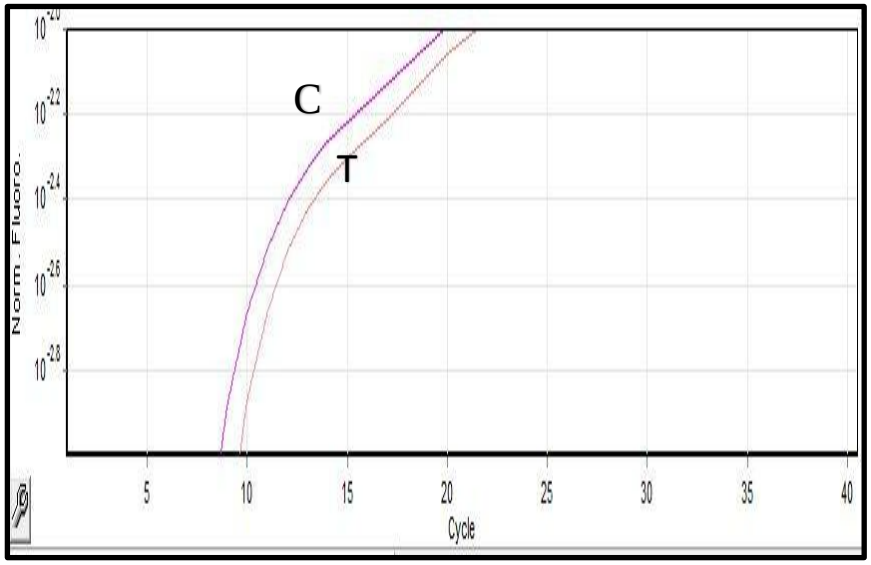


Fig 3 shows the Amplification plot of HSP 83 F&R

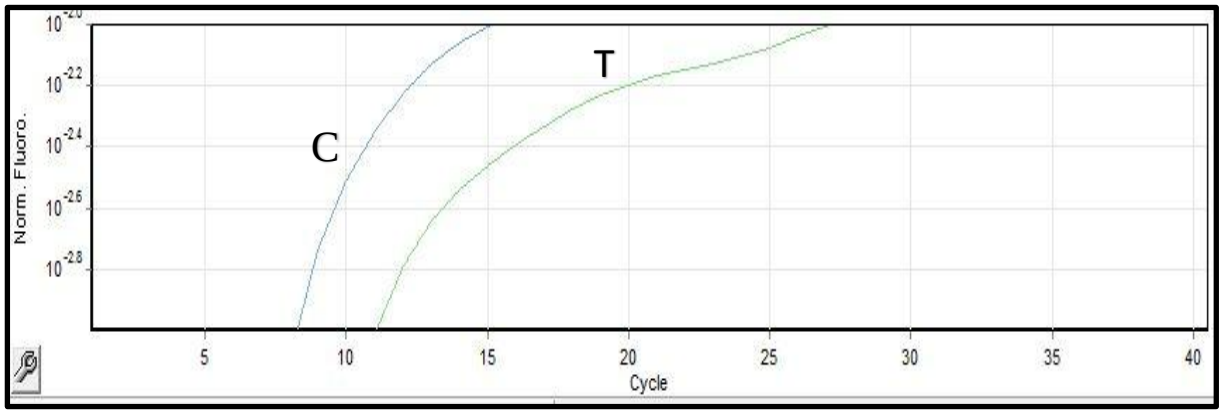
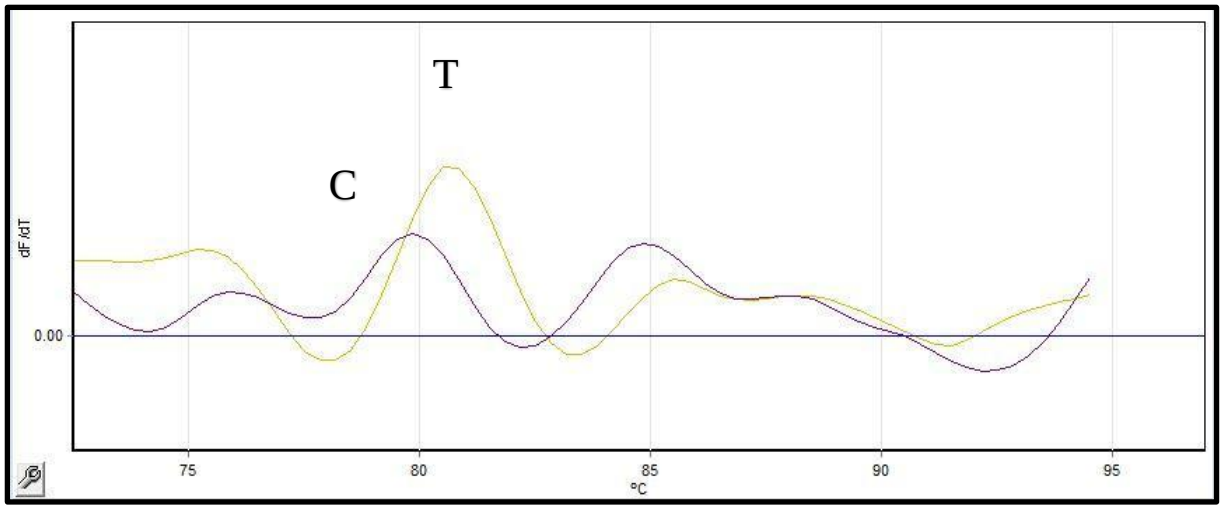


Fig 4 shows the Amplification plot of Rps57

Fig 5 depicts the Melt curves so obtained from the genes

Melt curve of HSP 70 F&R



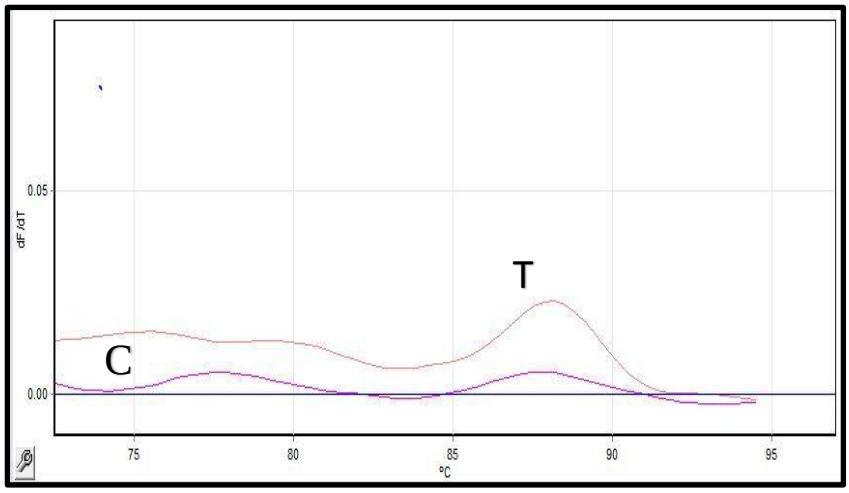


Fig 6 depicts the Melt curve of HSP83 F&R

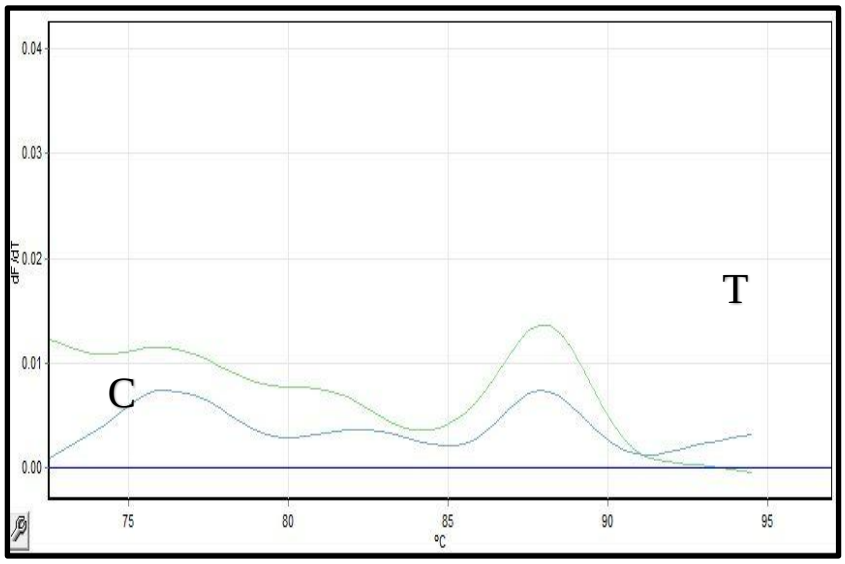


Fig 7: depicts the Melt curve of House keeping gene Rps57

Analysis using Pfaffl method-

- **Hsp70 Gene Expression**

	target gene	reference gene	
PCR efficiency	1.1881	1.8052	
Ct values	Ct target	Ct reference	fold induction
C	6.9	8.22	1
T	10.43	11.03	2.8615747

Table 7 depicts the Gene expression profile of Hsp70 gene

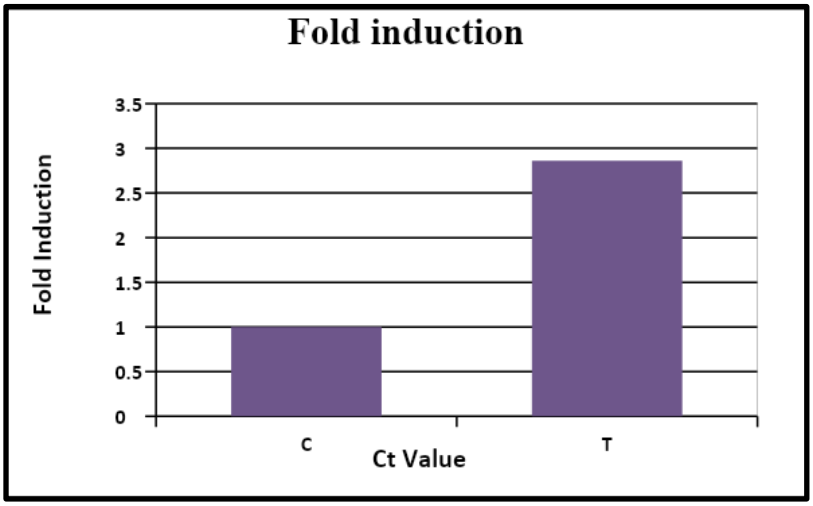


Fig 8 provides the Fold induction of Hsp70 Gene

Hsp 70 showing a 2-fold increase in its expression in the test organism comparing with the control.

- **Hsp83 Gene Expression**

	target gene	reference gene	
PCR efficiency	1.4022	1.8052	
Ct values	Ct target	Ct reference	fold induction
C	8.62	8.22	1
T	9.56	11.03	3.826791475

Table 8 shows the Gene Expression profile of Hsp83 Gene

Hsp 83 is showing a 3-fold increase in its expression in the test organism.

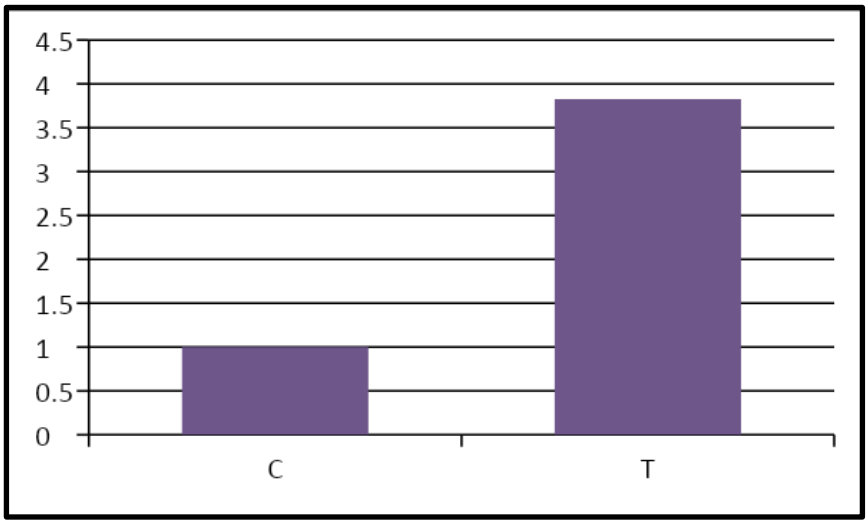


Fig 9 provides the Fold Induction of Hsp83 Gene

DICUSSION

In the current investigation, earthworms were collected from a soil sample which might provide some valuable information about the changes occurring at the molecular level during the present situation of temperature rise as the optimum range is 25ºC. Studying gene expression could help in assessing the changes occurring within these soil dwelling organisms as these would be largely affected by the temperature elevation. In a previous study conducted by (Taey et al., 2021) they proposed that the temperature rise affected the earthworms since the highly fatal temperature falls between 25-48ºC.RNA isolation was carried out and in turn the cDNA was synthesized with the isolated RNA. Similarly, the same procedure was carried out by (Mukopadhyay et al., 1995) in order to assess the gene expression changes in earthworm.

In the present investigation, the quantification of gene expression was done using DNA isolation, PCR amplification followed by analysis using pfaffl method. Using the pfaffl method the gene expression of test organism was compared to the control which shows a twofold increase in its expression when undergone conditioned heat stress. This study confirms that the Hsps has shown to increase in double fold when undergoing a heat stress. In the work of (Will et al., 2016) the expression of Hsp 83 has been strongly induced during the heat stress. Hsps are known to be induced as a result of any physical assault or stress.

The gene expression changes in the situation of elevated temperature were noted in the present investigation. Similar results were found by (Liu et al.,2017) where the Hsp 70 get elevated after the temperature stress has been induced nearly 38ºC. Improvements in biomass as well as plant growth have been largely dependent on both temperature and abundance of these organisms. In a previous study done by (Opute et al., 2021) emphasized that increase in temperature, precipitation can in turn affect the pesticide toxicity. Climate change especially in terms of temperature and soil moisture have a major impact on acidification. An increase in temperature would lead to precipitation which would in turn affect the soil pH. This would create a major impact in reducing the earthworm population as the climate variable have been changing.

In the present investigation the temperature rise has increased the resistance of earthworms by adopting changes or by regulating their expression. As per the study conducted by (Soto et al., 2020) the thermal stress as well as chemical stress have combined effect on the toxicity of the soil. The study proves the combine effect of toxicity with the rise in temperature. The earthworms treated with higher temperature was noticed to be have the larger accumulation of toxins. Earthworms treated with higher temperature shows high mortality rate. Based on our study it confirms that the high temperature has caused variation in the gene expression so for the time being it can be a bio indicator for some kind of stress.

In a study conducted by (Tripathi et al.,2011) focused on the results of temperature variations on the production of various enzymes as well as different mitochondrial proteins were assessed. At low temperatures, high metabolic rate or energy were required. Up to 28ºC the earthworms had shown an increased enzyme specific activity but when the temperature was raised to more than 30ºC the proteins produced was shown to be decreased. One possible explanation for the temperature-related variations in protein concentration is that the ability to synthesise proteins was impaired both above and below the optimal temperature range. This clearly depicts the interconnection between the climate variables with the physiology of earthworms. The current investigation also proves that the beyond their optimum temperature that is 25ºC, they are about to exhibit some changes in their overall viability, different proteins are being expressed or even over expressed.

Earthworms are a major source or an important link for maintaining the soil fertility as well as productivity of plants. Previous study proposed by (Liu et al., 2024) analysed that the nutrient uptake and productivity of plants which have been affected by numerous factors which include climate change and activity of the decomposers. In the experiment, they investigated the effects of future climatic scenarios in terms of warmth. Both the change in climatic condition as well as the earthworm has significantly affected the C:N ratio. The results proved that in the future scenario like extreme warmer conditions especially during summer, the effects of temperature would certainly lower the positives of earthworms on the nutrient uptake of plants due to soil water reduction.

At the present-day situation of global warming, it is of higher importance to conserve these species as these are going to experience much of the adversities of the ever-changing climatic conditions. Marnelis et al., (2024) conducted research on the performance of earthworms while

moving them to another place. The experiment observes various fascinating results regarding the climate variables largely affecting their overall health and viability. The work proved that rather than temperature, there are other factors like pH, humidity which are all interconnected had a major impact on the overall performance of earthworms. It was observed that the changes in these variables had caused weight loss and increased mortality rates of the earthworm. Similarly in the present investigation also, the temperature might have affected the other soil parameters like pH which would adversely affect the viability of these soil dwelling organisms.

The present study identified that the genes expressed during the time of heat stress was over expressed when comparing to the test organisms. This threw light on the changes that have been occurring in earthworms during temperature elevation. This investigation can be provided as a baseline study with regards to the works that might be carried out in the future with advancements in the current study and hence it can be useful to be considered as a biomarker of such stress related events. In a previous study conducted by Ghosh and his colleagues, they successfully proved that the climate changes have been largely affecting the earthworm species since affecting the physiology or even the biochemistry of these organisms. They are not only the source for maintaining the plant productivity but also as an important key player in maintaining the soil structure and also considered as the ecological engineers (Singh et al., 2016).

CONCLUSION

The study examined the effect of heat stress on earthworms by analysing the gene expression of Hsp70 & Hsp83 genes in control and treated conditions. The control and test organisms were obtained in order to analyse the gene expression changes in the test organism in addition to the control. The test organism was subjected to heat stress for continuous three days in the laboratory conditions maintaining the temperature at 37ºC.RNA isolation was carried out using Trizol method followed by cDNA synthesis. Real time PCR was carried out for quantifying the gene expression and final analysis was done using pfaffl method. HSP 70 and HSP 83 were the selected genes for the particular study since it was expressed during stressed conditions in earthworms.

Present study confirms that the gene expression of both HSP 70 and HSP 83 has shown a two-to-three-fold increase in its expression in the test organism after the gene quantification. So during the temperature elevated condition when looking onto the optimum temperature of earthworms that is about 25ºC, these genes are overexpressed and hence can be considered as a variation in its molecular level during heat stress. Further research shall be done on the stress tolerance as well as using this information in order to conserve the species in future with further research developments. The high expression level of these genes indicates their active role in expression during stressed conditions. This in turn proved that these genes are expressed during unfavourable climatic conditions as a result of present global warming issues. These results provide valuable information about the genes expressed under conditioned heat stress, which connects with the present face of changing environmental conditions and provide with valuable insights at the molecular level if further advancements have to be done regarding the current investigation.

REFERENCE

- Hs, K., Ch, A., Park, T., Hd, P., Koh, K. S., Zy, R., Park, T., & S, L. (2012). Gene expression profiling of a cold-shocked earthworm *Eisenia andrei*. *PubMed*, *33*(1), 11. https://pubmed.ncbi.nlm.nih.gov/22434117
- Tumminello, R. A., & Fuller-Espie, S. L. (2013). Heat stress induces ROS production and histone phosphorylation in celomocytes of Eisenia hortensis. *ISJ-Invertebrate Survival Journal*, *10*(1), 50–57. http://www.isj.unimo.it/articoli/ISJ293.pdf
- Nadeau, D., Corneau, S., Plante, I., Morrow, G., & Tanguay, R. M. (2001). Evaluation for Hsp70 as a biomarker of effect of pollutants on the earthworm *Lumbricus terrestris*. *Cell Stress & Chaperones*, *6*(2), 153. https://doi.org/10.1379/1466-1268(2001)006
- Weber, G. B. (2007). The role of earthworms as biological indicators of soil contamination. *Bulletin USAMV-CN*, *63*, 64.
- Mukhopadhyay, I., Nazir, A., Saxena, D. K., & Chowdhuri, D. K. (2003b). Heat shock response: hsp70 in environmental monitoring. *Journal of Biochemical and Molecular Toxicology*, *17*(5), 249–254. https://doi.org/10.1002/jbt.10086
- Hamana, K., Hamana, H., & Shinozawa, T. (1995). Alterations in polyamine levels of nematode, earthworm, leech and planarian during regeneration, temperature and osmotic stresses. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *111*(1), 91-97.
- Moreira-de-Sousa, C., de Souza, R. B., & Fontanetti, C. S. (2018). HSP70 as a biomarker: An excellent tool in environmental contamination analysis—A review. *Water, Air, & Soil Pollution*, *229*, 1-12.
- Moseley, P. L. (1997). Heat shock proteins and heat adaptation of the whole organism. *Journal of applied physiology*, *83*(5), 1413-1417.
- Lin, D., Li, Y., Zhou, Q., Xu, Y., & Wang, D. (2014). Effect of triclosan on reproduction, DNA damage and heat shock protein gene expression of the earthworm *Eisenia fetida*. *Ecotoxicology*, *23*, 1826-1832.
- Urionabarrenetxea, E., Garcia-Velasco, N., Marigómez, I., & Soto, M. (2020). Effects of elevated temperatures and cadmium exposure on stress biomarkers at different

biological complexity levels in Eisenia fetida earthworms. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *231*, 108735.

- Ricketts, H. J., Morgan, A. J., Spurgeon, D. J., & Kille, P. (2004). Measurement of annetocin gene expression: a new reproductive biomarker in earthworm ecotoxicology. *Ecotoxicology and Environmental Safety*, *57*(1), 4-10.
- Liu, T., Wang, X., You, X., Chen, D., Li, Y., & Wang, F. (2017). Oxidative stress and gene expression of earthworm (Eisenia fetida) to clothianidin. *Ecotoxicology and environmental safety*, *142*, 489-496.
- Chai, L., Yang, Y., Yang, H., Zhao, Y., & Wang, H. (2020). Transcriptome analysis of genes expressed in the earthworm Eisenia fetida in response to cadmium exposure. *Chemosphere*, *240*, 124902.
- Singh, J., Schädler, M., Demetrio, W., Brown, G. G., & Eisenhauer, N. (2019). Climate change effects on earthworms-a review. *Soil organisms*, *91*(3), 114.
- De Sosa, I., Verdes, A., Tilikj, N., Marchán, D. F., Planelló, R., Herrero, Ó., .. & Novo, M. (2022). How to thrive in unstable environments: Gene expression profile of a riparian earthworm under abiotic stress. *Science of the Total Environment*, *817*, 152749.
- Tilikj, N., & Novo, M. (2022). How to resist soil desiccation: Transcriptional changes in a Mediterranean earthworm during aestivation. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *264*, 111112.
- Shao, Y., Wang, X., Zhang, J., Li, M., Wu, S., Ma, X., Wang, X., Zhao, H., Li, Y., Zhu, H. H., Irwin, D. M., Wang, D., Zhang, G., Ruan, J., & Wu, D. (2020). Genome and single-cell RNA-sequencing of the earthworm *Eisenia andrei* identifies cellular mechanisms underlying regeneration. *Nature Communications*, *11*(1). https://doi.org/10.1038/s41467-020-16454-8
- Opute, P. A., & S MABOETA, M. (2022). A review of the impact of extreme environmental factors on earthworm activities and the feedback on the climate. *Applied Ecology & Environmental Research*, *20*(4).
- Singh, J. (2018). Role of earthworm in sustainable agriculture. In *Sustainable food systems from agriculture to industry* (pp. 83-122). Academic Press.
- Liu, Q., Eisenhauer, N., Scheu, S., Angst, G., Bücker, M., Huang, Y., ... & Schädler, M. (2024). Climate-dependent plant responses to earthworms in two land-use types. *Oecologia*, *204*(1), 133-146.

- Marnelis, W., Afnan, R., & Mendrofa, V. A. (2024). Performance of Earthworm (*Lumbricus rubellus*) During Different Transportation Durations. *Jurnal Ilmu Produksi dan Teknologi Hasil Peternakan*, *12*(1), 39-45.
- Tripathi, G., Kachhwaha, N., Dabi, I., & Bandooni, N. (2011). Temperature-dependent alterations in metabolic enzymes and proteins of three eco physiological different species of earthworms. *Brazilian Archives of Biology and Technology*, *54*, 769-776.