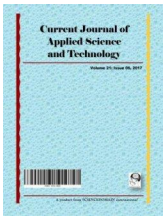

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Thermal Stress Induced Alterations in Tissue Protein, Lipid Peroxidation and Activities of Lactate Dehydrogenase, Acetylcholinesterase and Catalase in the Earthworm *Eudrilus eugeniae* (Kinberg)

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Authors' contributions

This work was carried out in collaboration between all authors. Author CSKM designed and monitored the experiment. Authors SSP and KPM did the sampling and experiments. Author SS did the statistical analysis, interpretation and graphical presentation of data. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To evaluate the effects of different temperatures exposures on tissue protein and certain stress indicating enzymes of the earthworm *Eudrilus eugeniae.*
Methodology: The tissue protein content, lipid peroxidation (LPX) level and activities of lactate dehydrogenase (LDH), acetylcholinesterase (AChE), catalase were studied in the earthworms exposed to 4°C, 30°C, 35°C and 40°C for 15 and 30 minutes durations using a standard protocol.
Results: The highest tissue protein (216.64 mg/g tissue) was recorded at 35°C with 30 min exposure and the lowest (123.19 mg/g tissue) at 4°C with 15 min exposure. LDH activity was the highest (0.14 U/mg protein) at 35°C with 15min exposure and the lowest (0.006 U/mg protein) at 30°C. The maximum AChE activity (0.029 U/mg protein) was observed at 30°C with 15min exposure

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and the minimum (0.005 U/mg protein) at 35°C with 30 min exposure respectively. LPX level was the highest (0.15 nmol/mg protein) at 4°C with 30min exposure and lowest (0.07 nmol/mg protein) at 35°C with 30 min exposure. Catalase activity was the maximum (0.11 U/mg protein) at 30°C with 15 min exposure and minimum (0.02 U/mg protein) at 35°C with 15 min exposure.
Conclusion: The study indicated that tissue protein; LPX level and enzyme activities could be useful biomarkers to study the organismal impact of thermal stress due to climate change.

Keywords: Eudrilus eugeniae; environmental temperature; biomarker.

1. INTRODUCTION

Temperature is one of the most important environmental factors that induce physiological changes in organisms. It is also considered as one of the principal environmental agents determining the activity of biota and rates of decomposition processes in the soil [1]. The potential impacts of climate change on the soil biota have received considerably less attention than other ecosystem components and there are few predictions concerning climate change responses of soil invertebrates [2]. Temperature effects on a number of soil functions have been investigated, including activities of soil invertebrates [3,4]. It has been debated that increasing temperature may lead to the rapid breakdown of organic matter which in turn is likely to affect soil heterotrophic community [5,6]. However, Whitford [7] reported that soil is thermally buffered and soil communities may be less sensitive to changes in atmospheric temperature than above ground fauna. Soil temperature beyond the optimal range of 10°C - 24°C increases the rate of metabolism in soil macro-fauna requiring them to either feed more or burn their own fat stores [8]. Majority of soil macro organisms succumb to high soil temperature which proves unfavourable for their survival. It has been suggested that thermal stress may diminish the antioxidant state and cause oxidative stress which is caused by the imbalance between the production of reactive oxygen species (ROS) and the biological system's ability to detoxify the reactive intermediates [9].

Earthworms have been considered as an important group of soil mesofauna in terms of biomass and activity. These animals have often been designated as ecosystem engineers [10]. Surface dwelling earthworm species are able to indicate minor variations in atmospheric and edaphic environmental factors and therefore considered as important bioindicators for ecosystem monitoring [11]. Soil temperature is likely to influence the life and activities of earthworms. In Indian situations, earthworms operate actively between 0°C - 30°C and their number decrease when soil temperature is below 4°C or above 25°C subject to availability of moisture [12].

Earthworms, in general, are sensitive to alteration in important soil physiochemical properties. Environmental changes are likely to induce physiological stress in soil animals and alter biochemical processes. Therefore, it is hypothesized that temperature variations could influence the synthesis and storage of macromolecules and influence enzyme activities in earthworms.

Antioxidant enzymes and certain biochemical parameters could be used as important tools for diagnosis of oxidative stress. Antioxidant enzyme catalase protects the cell from toxic hydrogen peroxide. Lipid peroxides generated from hydroxyl radical attack on the important polyunsaturated fatty acids could be used as an ideal biomarker of tissue damage [13]. Lactate dehydrogenase (LDH) is an important metabolic enzyme which catalyzes the interconversion of pyruvate to lactate in anaerobic glycolysis. Changes in LDH activity due to environmental impact have been studied in some soil invertebrates [14]. Acetylcholinesterase (AChE) is a key enzyme in the nervous system, terminating nerve impulses by catalyzing the hydrolysis of the neurotransmitter acetylcholine. Combined effects of pesticides on AChE of earthworms have been studied [15].

The present study was therefore designed to evaluate certain stress indicating biochemical parameters such as tissue protein content, LPX level, LDH and AChE and catalase activities in the epigeic worm *Eudrilus eugeniae* in response to variable temperature exposures.

2. MATERIALS AND METHODS

2.1 Experimental Set Up

E. eugeniae was procured from the vermiculture facility of Orissa University of Agriculture and Technology, Bhubaneswar, India. The worms were acclimatised in the organic garden soil in an earthen pot (40cm×40cm) inside the laboratory for seven days. Twelve 250 ml glass beakers with 100ml distilled water were placed at 4 different temperature ranges, 4°C (Refrigerator), 30°C, 35°C, 40°C (BOD Incubator) for 15 minutes. Each set was taken in triplicate. After 15 minutes, 120 clitellated earthworms of identical size and weight were sampled from the culture pot and transferred into the beakers, each beaker containing 10 earthworms. The worms were exposed to different temperatures for 15 minutes. The same procedure was repeated for exposure of worms to various temperature ranges for 30 minutes.

2.2 Sample Preparation

After the exposure period, the worms were washed in distilled water. The gut content was removed by using forceps and needle and then weighed. The tissue was homogenised with phosphate buffer (0.05 M, pH 7.4) and centrifuged at 10,000 rpm for 10 min using a tabletop cooling centrifuge (REMI). The supernatant was collected and stored at -20°C in the deep freezer (Celfrost) for further use.

2.3 Biochemical Analysis

Protein was estimated as per Lowry et al. [16] taking Bovine serum albumin as standard. Reading was taken at 700 nm in UV-VIS Spectrophotometer (Systronics). The OD values thus obtained were converted to concentration of released protein in terms of mg/g tissue. Lipid peroxidation was determined as per Ohkawa et al. [17]. The reaction mixture was prepared by adding 8.1% sodium dodecyl sulphate (SDS), 20% acetic acid, butylated hydroxytoluene (BHT), thiobarbituric acid (TBA) to the sample and boiled for 45 minutes. It was cooled up to normal temperature and then centrifuged at 10,000 rpm for 15 min in a cooling centrifuge. Reading was taken at 532 nm in UV-VIS Spectrophotometer (Systronics). The values were expressed as n mol/mg protein. LDH was determined by Cabaud and Wroblewski [18]. Phosphate buffer (pH-7.4, 0.05 M) was added to the sample followed by addition of NADH and sodium pyruvate. Reading was taken at 340 nm in UV-VIS spectrophotometer (Systronics). The values were expressed as U/mg protein. AChE was determined as per Ellman et al. [19]. The sample was added to the reaction mixture containing phosphate buffer (pH-7.4, 0.05 M) and DTNB solution. Reading was taken at 412 nm in UV-VIS spectrophotometer (Systronics) and values were expressed as U/mg protein. Catalase was assayed as per Cohen et al. [20]. The sample was added to reaction mixture containing phosphate buffer and hydrogen peroxide. Reading was taken at 242 nm in UV-VIS spectrophotometer (Systronics).The values were expressed as U/mg protein.

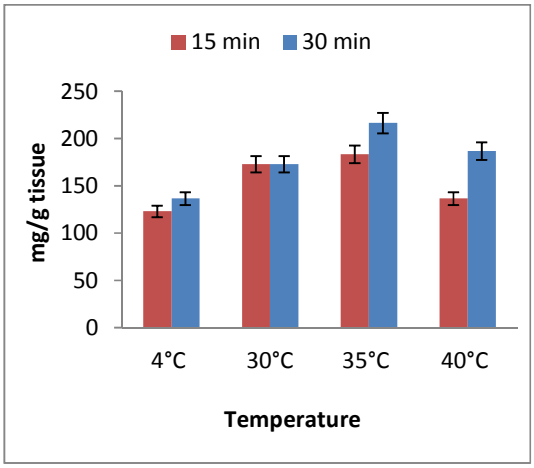
2.4 Statistical Analysis

Statistical analysis of data through one way ANOVA was done using Systat software to determine the level of significance at 0.05 levels between exposure periods.

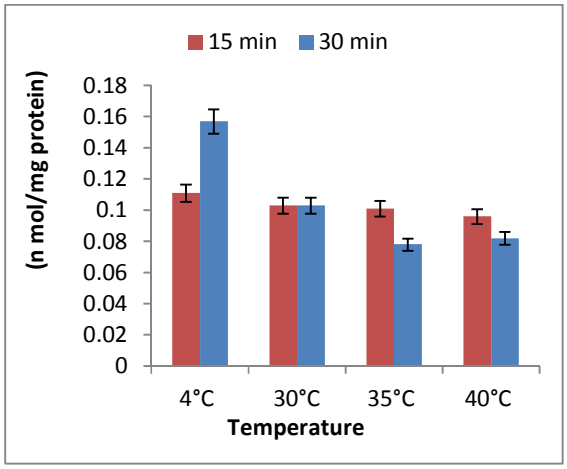
3. RESULTS AND DISCUSSION

Noticeable variation in the tissue protein of the earthworm exposed to different temperatures was observed with both 15 min and 30 min exposure periods (Fig 1a). After 15 min exposure, the highest protein 183.61±3.03 mg/g tissue was observed at 35°C and the lowest of 123.19±0.01 mg/g tissue at 4°C. Interestingly, tissue protein at the highest temperature at 40°C was 136.69±0.02 mg/ g tissue which is higher than that at 4°C. The worms exposed to 30°C indicated 173.12±2.06 mg/g tissue protein. The variation in tissue protein between temperature exposures at 15 min exposure was found to be statistically significant (P=0.05). With 30 min exposure, the highest protein 216.64±4.01 mg/g tissue was observed at 35°C and the lowest, 136.75±0.02 mg/ g tissue at 4°C. The variation in the protein content between temperatures was found to be significant (P=0.05).

LPX indicated minor variation between temperatures and exposure durations (Fig 1b). With 15 min exposure, the minimum LPX level 0.096±0.001 n mol/mg protein was recorded at 40°C and maximum 0.11±0.04 n mol/mg protein at 4°C. The statistical test did not show significant variation in LPX level between temperatures. With 30 min exposure, the highest LPX value 0.15±0.03 n mol/mg protein was recorded at 4°C and the lowest, 0.07±0.02 n mol/mg protein at 35°C. Significant variation (P=0.05) was observed in LPX level between temperature exposures.



(a)



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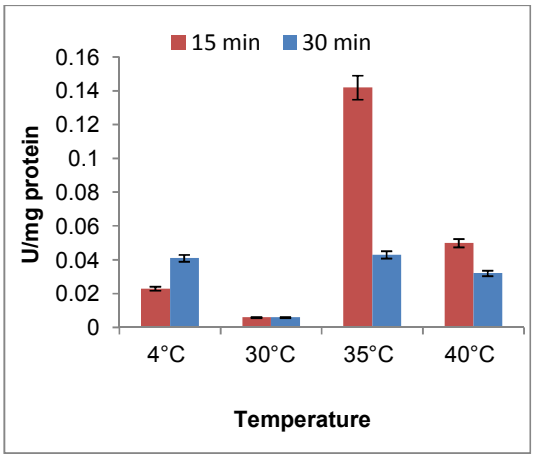

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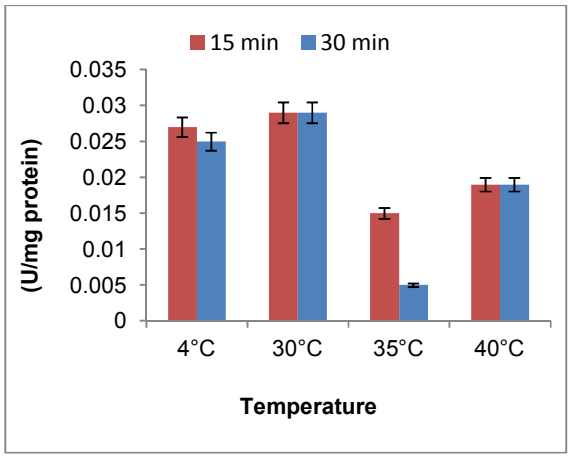

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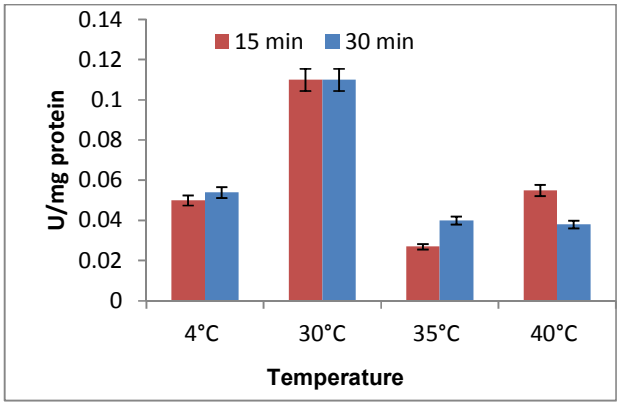

(e)

Fig. 1. Biochemical alterations in *E. eugeniae* exposed to various temperature, a- Protein in mg/g tissue, b- LPX in n mol/mg protein, c- LDH in U/mg protein, d- AChE in U/mg protein, e- Catalase in U/mg protein

With 15 min exposure, the highest LDH activity 0.14±0.012 U/mg protein was observed at 35°C and the lowest 0.06±0.004 U/mg protein at 30°C (Fig 1c). Comparatively higher enzyme activities were observed at 4°C, 35°C, 40°C. Significant variation in LDH activity (*P*=0.05) between different temperature exposures was recorded. With 30 min exposure, the highest LDH activity of 0.043±0.002 U/mg protein was observed at 35°C followed by 4°C,40°C and 30 °C (Fig 1c).

The highest AChE activity 0.027±0.002 U/mg protein was observed at 30°C with 15 min exposure, followed by 4°C, 40°C, 35°C (Fig 1d). However, the variation in this enzyme activity between temperature exposures was not significant. During 30 min time exposure, the highest AChE activity 0.029±0.001 U/mg protein was observed at 30°C followed by 4°C, 40°C and 35°C. Significant variation (*P*=0.05) between temperatures was noticed at this exposure duration.

The catalase activity at 15 min duration indicated the highest value of 0.11±0.02 U/mg protein at 30°C (Fig. 1e) and the minimum 0.02±0.001 U/mg protein was obtained at 35°C followed by 0.05±0.001 U/mg protein at both 40°C and 4°C. Statistical analysis indicated significant variation (*P*=0.05) in this enzyme activity between temperature exposure periods. With 30 min exposure, the maximum catalase activity was observed at 30°C and the minimum 0.03±0.001 U/mg protein at 40°C.

Collet et al. [21] have observed a significant alteration in the population and metabolism of soil invertebrates including earthworms in forest soil due to increased soil temperature after a forest fire and reported that those higher than optimal environmental temperatures negatively influence metabolic rate and survival of the worms. Tripathi et al. [22] have reported a significant variation in the tissue protein of the earthworms *Metaphire posthuma, Perionyx sansbaricus* and *Lampito mauritii* exposed to a temperature range from 12°C to 44°C. They have observed an increase in protein from 12°C to 20°C and subsequent decrease up to 44°C. In the present studies, the highest tissue protein was observed at 35°C exposure which is 5°C higher than the optimal temperature indicating that a marginal increment in temperature could induce higher protein synthesis. It was further observed that worms exposed to extremes of temperatures such as 40°C and 4°C indicated low tissue protein content which implies that very high and low temperature ranges adversely impact tissue protein synthesis and accumulation in this earthworm species. The results are in agreement with the observation reported earlier in other species of earthworms and support the hypothesis that enhanced physiological stress might induce protein synthesis initially but adversely impact the process subsequently.

Geraciano et al. [23] studied antioxidant enzyme activities with lipid peroxidation in the polychaete *Laeonereis acuta* in different seasons and reported higher catalase activity in autumn relative to winter. However, lipid peroxidation did not exhibit seasonal variation. Identical findings were reported by Eseigbe et al. [24] during their study on the effect of hydrocarbon induced stress on the earthworm *E. eugeniae*. Ravi and Aruna [25] observed a wide variation in lipid peroxidation in terms of malondialdehyde (MDA) level with increasing age of earthworm *E. eugeniae*. They have proposed that MDA levels increase the free radicals which might be influenced by age and stress. Parida and Mohanta [26] observed high lipid peroxidation in *E. eugeniae* exposed to the pesticide furadon and malathion. High lipid peroxidation in the tropical earthworms *L. mauritii* and *Drawida willsi* with exposure to elevated concentrations of urea, phosphogypsum and paper mill sludge has also been reported [27]. They have proposed that exposure to stress inducing agents enhance the MDA level in the earthworm with higher lipid peroxidation.

In our study, the highest lipid peroxidation has been observed at 4°C and low lipid peroxidation in the range of 35°C to 40°C. It is therefore apparent that the earthworm suffers from the maximum oxidative damage due to free radical at a very low temperature of 4°C. However, with rising in temperature up to certain threshold, the lipid peroxidation may be neutralised by enhanced activity of antioxidant enzymes in the worm which results in lower lipid peroxidation.

Tripathi et al. [22] observed significant variation in the LDH activity of three earthworm species *M. posthuma*, *P. sansibaricus* and *L. mauritii* exposed to a temperature range of 12°C-44°C. Their results indicated low LDH activity at a high temperature of 44°C and high activity at 12°C. A consistent decrease in the enzyme activity with rise in temperature was observed in all the species of earthworms tested. However, our results on *E. eugeniae* are inconsistent with those reported by previous authors although the

variation in the enzyme activity was significant between temperature exposures in this earthworm. The maximum elevation in the LDH activity indicated that the earthworm surviving in different temperatures exhibited different degrees of metabolic activities. Therefore, to maintain high metabolic activity at high or low temperatures, there was a need for more energy requirement, which was facilitated by higher LDH activity.

Relatively lower enzyme activity was observed at 4°C, 35°C and 40°C. Although information on the effects of temperature variations on the AChE activity of earthworms are not available, earlier studies indicate the effects of other stress inducing agents such as pesticides on this enzyme activity. Pradhan and Mishra [28] reported an initial spike in AChE activity in the earthworms *D. calebi* and *Octochaetona surensis* followed by subsequent inhibition after 24 hours with exposure to the insecticide carbaryl. Earlier reports indicate decreased AChE activity in earthworms due to the toxicity of pesticides [28, 29-31]. Reduced AChE activity results in accumulation of acetylcholine at the synaptic junctions which is likely to disrupt nerve conduction in animals. Our results corroborate these earlier reports and it appears that in *E. eugeniae* higher or lower than the optimal temperature exposure might cause inhibition in the AChE activity, thus affecting nerve conduction.

Catalase is a useful enzyme released in animals under physiological stress to scavenge reactive oxygen species. The catalase activity in the earthworm was found to be the maximum at the optimal temperature of 30°C and lower catalase activity than the optimal was recorded at higher and lower temperatures indicating that the enzyme activity is sensitive to environmental temperature variations. Saint – Denis et al. [32] observed an increased catalase activity with hydrogen peroxide concentration which acts as physiological stress. They have also reported that the enzyme activity increased with temperature from 10°C onwards and a maximum value was obtained at 30°C which subsequently declined with a further rise in temperature. The present findings corroborate those of earlier authors and indicate that the activity of catalase in *E. eugeniae* increases consistently from low to an optimal temperature of 30°C. A further rise in temperature possibly inhibits this enzyme activity increasing the probability of oxidative damage due to free radicals.

4. CONCLUSION

Earthworms have been considered as potential bioindicators of climate change and soil contamination. Biomarker molecules including proteins with certain stress indicating enzymes could be useful to evaluate the sensitivity of surface dwelling (epigeic) earthworms to fluctuations in the environmental factors such as temperature which is likely to affect the metabolism in these animals. A long term study on various earthworm species is desirable to identify sensitive biomarkers for temperature alterations due to climate change.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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