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Impact of Acute Thermal Stress in Male *Drosophila melanogaster* on Climbing Performance, Oxidative stress and Brain Histology

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ABSTRACT

Climate change-induced heat waves pose significant physiological challenges to organisms, yet the sublethal effects of acute heat stress on living organism's health remain poorly understood. This study investigated the effects of acute heat stress (5 and 10 minutes at an elevated temperature of 40ºC) on oxidative stress markers, locomotor function and brain sections in male *Drosophila melanogaster* within a duration of 2 days. Oxidative stress was assessed by measuring malondialdehyde (MDA), superoxide dismutase (SOD), and reduced glutathione (GSH) levels, while climbing performance was evaluated using the Rapid Iterative Negative Geotaxis (RING) assay. Brain morphology was also examined histologically. Results showed decreases in MDA and SOD levels across both heat-exposed groups that was not significant ($p > 0.05$), while GSH levels decreased after 5 minutes but increased after 10 minutes, these changes were also not significant. However, the RING assay revealed a significant decline in climbing performance following 10 minutes of heat exposure ($p < 0.05$ for the control; $p < 0.01$ for the 5-minute group), despite the absence of significant changes in biochemical markers. Brain micrographs from the 10-minute group showed pronounced vacuolation and neuropil disruption in the optic lobes and central brain, correlating with the observed motor deficits. The findings of this study demonstrate that acute heat exposure causes a decline in motor performance and alters brain histological architecture in Drosophila melanogaster. However, no significant changes were detected in the oxidative stress biomarkers.

Keywords: *Drosophila melanogaster*, Heat stress, RING assay, Oxidative stress, Brain histology

INTRODUCTION

Anthropogenic activities such as industrial operations and land modifications (deforestation and urbanization) have significantly contributed to climate change and subsequently elevated environmental temperatures leading to severe heat waves. These human activities have led to a marked increase in the frequency, intensity, and duration of extreme heat events[1,2,3]. While mean global temperature rise exerts chronic selective pressure on biological systems, it is the acute nature of heatwaves which are brief with high-intensity thermal spikes that often poses the most immediate threat to individual survival and population perseverance[4]. Ectotherms specifically insects, whose body temperature and physiological performance are linked to ambient environmental conditions,[5,6] thus, the vulnerability of these organisms to acute thermal stress carries profound implications for biodiversity[7]. Consequently, there is a pressing need to move beyond studies of chronic temperature shifts and static thermal limits to understand the specific biological consequences of realistic heatwaves and implications on living organisms[8,9]. As a suitable thermal stress research model organism, *Drosophila melanogaster*, allows investigating the mechanistic basis of thermal tolerance and the impacts of climate warming[10,11]. Studies have elucidated the critical roles of heat shock proteins, metabolic adjustment, and cellular membrane integrity in surviving sub-lethal temperature increases in this organism[12,13]. However, the vast majority of this research has employed experimental designs involving developmental acclimation, laboratory natural selection, or continuous thermal ramping to determine critical thermal maxima (CTmax)[14,15,16]. These models do not replicate the disrupted and unpredictable nature of acute heatwave exposure in the normal environment. An organism may experience a single, acute afternoon of extreme heat that does not immediately kill it but

nonetheless inflicts latent, sub-lethal damage. The specific, immediate, and delayed consequences of a discrete, acute heat exposure on male *D. melanogaster* remain inadequately characterized within a climate change basis. Therefore, the aim of this study was to evaluate the impact of acute thermal stress in male *Drosophila melanogaster* on climbing performance, oxidative stress and histology of the brain.

MATERIALS AND METHODS

Experimental animals

Harwich wild type strain of *Drosophila melanogaster* was obtained from the Drosophila and Neurogenetics Laboratory, Department of Zoology, Faculty of Life Sciences, Ahmadu Bello University, Zaria, Nigeria.

Experimental design

The flies were reared on standard Corn meal diet (850 ml of distilled water, 8 g of agar, 50g of cornmeal, 10 g of Baker's Yeast and 1 g of Nipagin)[17]. The flies were housed in cylindrical vials at a temperature of 25°C with 60% humidity. Flies were mated and allowed to populate; a total of 270 flies were collected 3 days after eclosion. The flies were aneasthetised using ice, according to the method described by Li *et al*. 2023[18]. Thirty flies were counted into respective plastic vials each in triplicates of each group. The flies were divided into three experimental groups (Groups I – III). Group I served as control and was maintained at 25°C on standard corn meal diet. Group II – III were exposed to heat stress (40°C) for 5 mins and 10 mins respectively for 2 days.

Rapid iterative negative geotaxis (RING) assay

Climbing performance was assessed following heat shock stress exposure using the Rapid Iterative Negative Geotaxis (RING) assay. Nine graduated plastic vials were labeled according to their respective experimental groups, and 20 flies were placed into each vial of the RING apparatus. A camera was positioned in front of the apparatus, and a timer was set for 3 seconds. The RING apparatus was tapped three times, and a video recording captured the flies' climbing activity within the 3-seconds interval. This entire procedure was repeated five times[19].

Biochemical assay

The whole bodies of flies from each plastic vial across the three replicates of both the control and experimental groups were anaesthetised and homogenised in 0.1 M potassium phosphate buffer solution (PBS), pH 7.4, at a ratio of 1:10 (flies to volume in µl). The homogenates were then centrifuged at 5000 rpm for 10 minutes using an Eppendorf Legend Micro 17R Centrifuge. The resulting supernatants were separated from the pellet, transferred into Eppendorf tubes, and stored in a freezer at −20°C for subsequent analysis of oxidative stress markers.

Malondialdehyde (MDA) assay

Lipid peroxidation was determined following the method of Ohkawa *et al*.[49] Lipid peroxidation produces peroxide intermediates that, upon cleavage, release MDA, a compound that reacts with thiobarbituric acid (TBA) to form thiobarbituric acid-reactive substances (TBARS). Accordingly, MDA levels were quantified by measuring TBARS as previously described[20]. Absorbance was measured using a spectrophotometer at 532 nm, and the assessment was performed with a semi-automatic chemistry analyser (MLSBA50).

Reduced glutathione assay

Glutathione activity was determined according to the methods of Rajagopalan *et al*.[21] and Ellman[22]. The assay principle is based on the reaction between 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and reduced glutathione (GSH). Assessment was performed using a semi-automatic chemistry analyzer (MLSBA50).

Superoxide dismutase

The method used to quantify superoxide dismutase activity was based on the procedure of Misra and Fridovich[23]. The assay was performed using a biochemical kit according to the manufacturer's instructions.

Brain histology

Ten whole bodies of flies from each group were fixed in 4% paraformaldehyde for 72 hours, subsequently washed in phosphate buffer saline (PBS). The flies were then dehydrated using ascending graded ethanol concentration (30 %, 50%, 70%, 90% and 100%) for 10 mins respectively. The flies were subsequently transferred in to a clearing agent (xylene) I and equal parts of xylene II and paraffin wax for 30 mins and 90 mins respectively. Infiltration of the whole bodies using paraffin was in 120 mins at 60°C. The flies were then placed into melted paraffin in a stainless base mould and their heads were aligned centrally in the same orientation with the anterior parts facing upwards, the flies were embedded and then were placed on the cooled cryo-plate until the paraffin completely solidifies, the mould was subsequently removed and the flies were sectioned (cross section) at 10 µm serially at 10 to 12 sections of ribbons. Ribbons were placed into a water bath at 55°C, albumin was spread using a small paint brush on each labelled slides and sections were fished gently. Deparaffinization of tissue was done using xylene for three changes, the slides were then rinsed with absolute ethanol and then followed by 96% and 70% ethanol, the slides were then rinsed with H_2O and transferred into Haematoxylin stain for 2 mins, slides were rinsed twice with water and dipped in 1% acid alcohol for 10seconds and transferred in Eosin stain for 2 mins. The tissues were rinsed twice in water then graded ethanol, xylene was used as a clearing agent for just a minute. Mounting agent DPX (Dibutylphthalate polystyrene xylene) was used and

tissues on slides were covered with cover slips. Slides were viewed using an AmScope microscope and specific sections were taken, visualised on the respective software at magnifications of x400. This was according to the method of Sekiya and lijima[24]with some modifications.

Data analyses

Data from the research study are presented as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was employed to compare mean differences between and within the groups, followed by Tukey's *post-hoc* test where applicable. A p-value of less than 0.05 was regarded as statistically significant, whereas a p-value greater than 0.05 was considered statistically insignificant.

RESULTS

Effect of acute heat exposure on MDA Concentration in *Drosophila melanogaster*

There was a decrease in the MDA level of flies exposed to 5 minutes heat exposure when compared to the control, however the decrease was not significant ($p > 0.05$). There was also a non-significant decrease in the MDA levels of flies exposed to 10 minutes heat shock when compared with the control (Figure 1).

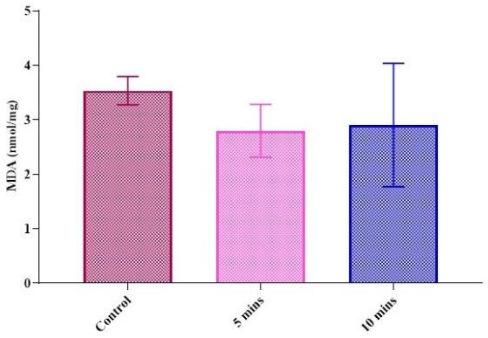

Figure 1: MDA concentration of male *Drosophila melanogaster* exposed to acute heat (40ºC)
mean ± SEM, one way ANOVA, $p>0.05$ when compared across the groups

Effect of acute heat exposure on SOD activity in *Drosophila melanogaster*

There was a decrease in the SOD activity of flies exposed to 5 minutes heat shock when compared with the control. However, the result was not significant. The decrease in the SOD activity of flies exposed to 10 minutes heat exposure when compared with the control group was also not significant (Figure 2).

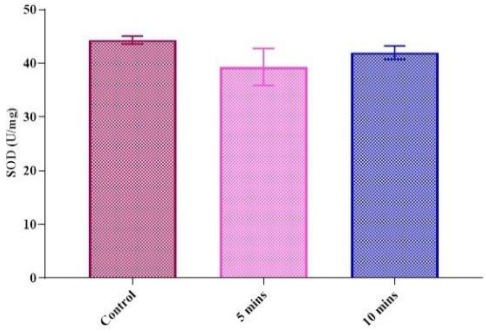

Figure 2: SOD activity of male *Drosophila melanogaster* exposed to acute heat stress (40ºC)
mean ± SEM, one way ANOVA, $p>0.05$ when compared across the groups

Effect of acute heat exposure on GSH Concentration in *Drosophila melanogaster*

There was a non-significant decrease in the GSH levels of the male flies exposed to 5 minutes heat exposure when compared to the control group. However, an increase in the GSH levels of flies induced with 10 minutes heat exposure when compared with the control group were also observed. This difference was also not significant (Figure 3).

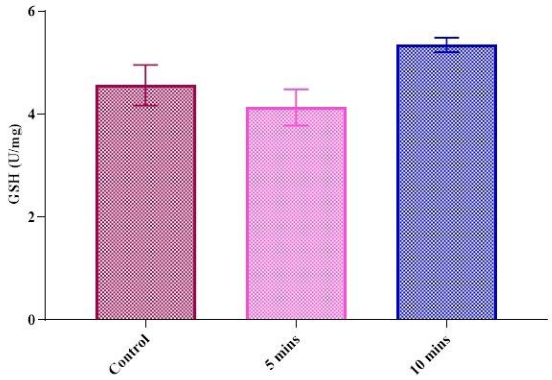

Figure 3: GSH concentration of male *Drosophila melanogaster* exposed to acute heat stress (40ºC). Mean ± SEM, one way ANOVA, $p>0.05$ when compared across the groups

Effect of acute heat exposure on climbing performance in *Drosophila melanogaster* (RING Assay)

There was an increase in the climbing performance of the flies exposed to 5 minutes heat stress when compared to the control group. However, this result was not significant. There was also a significant decrease ($p < 0.05$) in the climbing performance of the flies exposed to 10 minutes heat stress when compared to the control group. There was a significant decrease ($p < 0.05$) in the climbing performance of the flies exposed to 10 minutes heat stress when compared to the climbing performance of the flies exposed to heat stress for 5 minutes (Figure 4).

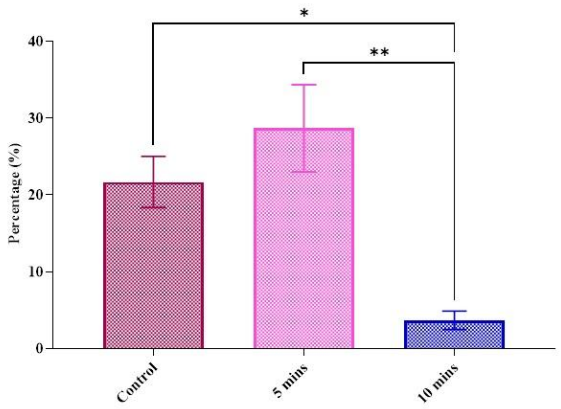

Figure 4: RING assay percentage accuracy in male *Drosophila melanogaster* exposed to acute heat stress (40ºC); mean ± SEM, one way ANOVA, *Tukey post hoc* test, *= p <0.05 when control group was compared to 10 mins group, and **= p <0.01 when 5 mins group was compared to the 10 mins group.

Effect of acute heat exposure on histoarchitecture of brain sections in *Drosophila melanogaster*

Histological sections of *Drosophila melanogaster* brains stained with hematoxylin and eosin stain from the three different experimental groups: Micrograph A (Control group) shows normal, healthy brain structure. The optic lobes (OL) appear well-defined, and the central brain (CB) shows uniform histoarchitecture without vacuolations. Micrograph B (5-minutes heat stress group) also shows normal, healthy brain structure, with obvious uniform histoarchitecture with a very few vacuolations or lesions. Micrograph C (10-minutes heat stress group) shows mild changes, possibly slight vacuolation and subtle disorganization in the neuropil, indicating possibility of early oxidative stress and reduced staining intensity due to prolonged heat stress (Figure 5).

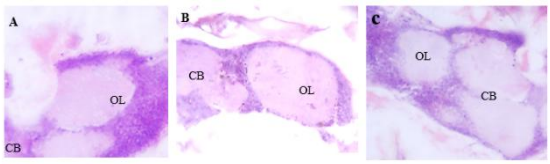

Figure 5: Histological brain sections of male *Drosophila melanogaster* exposed to acute heat stress (40ºC). X400 Magnification. Micrograph A: Control group, Micrograph B: 5-minutes heat shock group and Micrograph C: 10-minutes heat stress group. OL: Optic lobe and CB: Central Brain

DISCUSSION

Heat stress exposed flies (5-10 mins) did not show any significant decrease ($p>0.05$), indicating that the 2 days exposure period and the relatively short heat stress durations (5-10 mins) did not produce a robust increase in lipid peroxidation in male flies A non-significant decline after a mild, acute stressor may reflect upregulation of antioxidant defenses (e.g., superoxide dismutase, catalase, glutathione) within the exposure period, which effectively scavenges reactive oxygen species and reduces lipid peroxidation below baseline in order to neutralize reactive oxygen species (ROS) generated by the stressors[25,26,27,28]. Furthermore, mild stress triggers adaptive pathways that temporarily lower oxidative damage markers[29,30]. While longer heat stress typically increases oxidative stress, the observed decrease within 2 days could be due to the compensatory antioxidant activation that over-compensates, lowering MDA despite greater initial insult[31,32]. Hence, MDA levels can peak within hours and then decline below baseline during recovery due to enhanced clearance or repair mechanisms. The reduction in overall metabolic rate and thus ROS production by the time of sampling could also account for this [33,34].

The non-significant decrease observed in the SOD activity when compared to the control group, after mild heat stress could be due to the fact that acute heat stress may initially disrupt protein homeostasis, including antioxidant enzymes, leading to lower measurable activity within 2 days if recovery is incomplete. However, studies in insects and ectotherms report significant SOD increases during/after heat, not decreases[35,36]. Also, evidence from heat-stressed models indicates that mild stress triggers a coordinated increase in antioxidants, including SOD, CAT, and glutathione-related enzymes (e.g., GR, GPx), to handle ROS flux, rather than causing a compensatory reduction in constitutive SOD[35,37]. Thus, the non-significant SOD drop in this study is likely due to timing (post-peak) or assay limits, not compensation. Furthermore, the decrease in the SOD activity in the 10 minutes exposed flies relative to the control group, is somewhat unexpected because longer heat exposure typically increases ROS production, which might be expected to upregulate SOD. Hence, this could be linked to oxidative inactivation of SOD due to prolonged stress, which can lead to post-translational modifications (e.g., carbonylation, nitration) that reduce enzyme activity without changing protein levels[38]. However, SOD requires metal ions (Cu/Zn or Mn); heat stress might disrupt metal homeostasis, and this could also lower the functional activity of the enzyme. A mild decline in GSH levels after a short heat stress could be from the consumption of GSH[39]. Reports indicates even a brief heat exposure generates reactive oxygen species, and GSH is used up in neutralizing them, lowering its measurable level within the 2-daystime point if resynthesis has not fully compensated[40,41]. Heat exposure may temporarily downregulate enzymes involved in GSH biosynthesis (e.g., glutamate-cysteine ligase) as part of a broader stress response that prioritizes heat shock proteins)[42]. However, the decrease in this study was small and non-significant, suggesting that 5 minutes of heat

shock may not have substantially depleted GSH beyond normal biological variation. Acute heat exposure often depletes GSH levels initially due to heightened ROS production and consumption in antioxidant defenses, rather than increasing the levels. Longer exposures like 10 minutes typically exacerbate this depletion or trigger inconsistent responses[42]. A moderate stress duration can also elevate antioxidant capacity above baseline, a phenomenon often observed with mild-to-moderate oxidative stressors[37,43] 10-minutes heat stress group exhibited a significant decrease in climbing performance compared to the control group. Additionally, the 10-minutes group performed significantly worse than the 5-minutes group ($p < 0.01$). Shorter 5-minutes exposures likely remain sublethal, activating partial heat shock responses (e.g., HSP70 upregulation) that preserve geotaxis without full overload. In contrast, 10 minutes crosses into moderate-severe stress at typical experimental temperatures (37-40°C), inducing protein misfolding, aggregation in neural/muscle tissues, and disrupted Ca^{2+} homeostasis, all impairing coordinated locomotion[44]. Furthermore, Prolonged heat disrupts dopaminergic neurons critical for negative geotaxis motivation, alongside mitochondrial ROS overproduction causing energy deficits (ATP decline) and synaptic vesicle cycling failure in flight/climbing motor circuits[45]. Unlike hormesis in milder protocols similar to this study, single acute 10-minutes sessions could overwhelm refolding capacity, yielding deficits observable immediately post-recovery [45,46].

The histoarchitecture of the brain sections of the flies shows the optic lobes (OL) and central brain (CB), these structures are critical for visual processing (OL) and higher-order integration/motor control (CB). Qualitative differences in tissue integrity, vacuolation, and cellular density are evident across the three groups. Short-duration heat exposure (5 mins) causes minimal, mild structural changes such as slight vacuolation or loosening of the neuropil, but overall architecture remains largely preserved. OL and CB still distinguishable with no gross lesions. This correlates with the RING assay result showing a non-significant increase in climbing performance (mild hormesis). The brain's ability to maintain near-normal morphology after 2 days of heat exposure suggests protective mechanisms (e.g., heat shock protein upregulation). Prolonged heat exposure (10 mins) induces significant brain damage, likely including protein aggregation and membrane disruption. Heat-induced Ca^{2+} influx disrupts mitochondrial integrity, triggering ROS-mediated lipid peroxidation in glial-neuronal interfaces. In Drosophila, this forms electron-lucent vacuoles in neuropils post-37-40°C exposure, paralleling TBI models where 10-30 minutes insults produce similar glial vacuolization without full HSP protection[47,48]. This structural compromise directly explains the significant decrease in climbing performance seen in the RING assay.

CONCLUSION

Ten-minutes heat exposure caused significant locomotor impairment and structural brain damage in male *Drosophila melanogaster* after 2 days, despite no significant changes in oxidative stress markers. These findings suggest that functional and morphological assessments may be more sensitive than biochemical assays alone for detecting heat-induced injury, highlighting the importance of integrating multiple endpoints when evaluating sublethal stress responses and insect vulnerability to heat waves.

Conflict of interest: The authors have no conflict of interests to declare.

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Authors contributions:

ZMB: Conception, interpretation and drafting of manuscript; RNA: Design and Data acquisition

ASI: Interpretation and revising the manuscript
TSA: Data analysis; IM, JO, ZSS, HAU, RKB: Revising the manuscript.

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