Anaerobic respiration and antioxidant responses of *Corythucha ciliata* (Say) adults to heat-induced oxidative stress under laboratory and field conditions

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Abstract High temperature often induces oxidative stress and antioxidant response in insects. This phenomenon has been well documented under controlled laboratory conditions, but whether it happens under fluctuating field conditions is largely unknown. In this study, we used an invasive lace bug (*Corythucha ciliata*) as a model species to compare the effects of controlled thermal treatments (2 h at 33–43 °C with 2 °C intervals in the laboratory) and naturally fluctuating thermal conditions (08:00–14:00 at 2-h intervals (29.7–37.2 °C) on a hot summer day in a field in Shanghai, China) on lipid peroxidation (malondialdehyde (MDA) was the marker) and anaerobic respiration (lactate dehydrogenase (LDH) was the marker), as well as superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and glutathione reductase (GR). The results show that MDA concentration increased significantly in response to heat stresses with similar trend in the laboratory and field. LDH activities did not significantly vary across temperatures in the laboratory-exposed individuals, but they significantly increased by rising temperature in the field. The activities or concentrations of SOD, CAT, GSH, and GR all significantly increased with increasing temperature in the two populations. These findings indicate that high temperature induces oxidative stress, resulting in high anaerobic respiration and antioxidant defenses in *C. ciliata* under both the laboratory and field conditions, which likely provide a defense

mechanism against oxidative damage due to the accumulation of ROS.

Keywords Antioxidative defense · Heat shock · High temperature · Thermal stress · Sycamore lace bug

Introduction

During oxidative metabolism in insects, oxygen is occasionally reduced by single electrons to reactive oxygen species (ROS) (Livingstone 2001), which include the superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($HO^{\bullet}$), and singlet oxygen (1O_2). Although low levels of ROS are beneficial for cell signaling and induction of defense genes in insects (Kamata and Hirata 1999; Wang et al. 2001), high levels of ROS under stressful conditions can lead to oxidative stress (Lopez-Martinez et al. 2008), protein carbonylation, DNA/RNA oxidation and lipid peroxidation (LPO) (Matés 2000; Yang et al. 2010), and then loss of cell function (Stadtman and Levine 2003) or cell death (Imlay 2003; Krishnan and Kodrík 2011). Insects, therefore, should have the ability to eliminate surplus ROS from cells and extracellular fluids (Krishnan et al. 2007).

Previous studies have reported that LPO is mainly caused by the ROS-induced damage, and thus, the increased level of LPO can be directly considered as a marker of oxidative stress (Dubovskiy et al. 2008; Wang et al. 2001). As a major oxidation product of peroxidized polyunsaturated fatty acids in organisms under oxidative stress, MDA is an indicator of LPO and thus, can be an indirect marker to test oxidative stress (Rael et al. 2004; Del Rio et al. 2005). To maintain homeostasis and counteract LPO damage, organisms have an antioxidant defense system including many enzymatic and nonenzymatic scavengers. The enzymatic scavengers include superoxide dismutase (SOD), peroxiredoxin, glutathione reductase (GR), and catalase (CAT), and the nonenzymatic

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scavengers are glutathione (GSH), ascorbic acid, alpha-tocopherol (vitamin E), beta-carotene, and uric acid (Livingstone 2001; Wang et al. 2001; Lopez-Martinez et al. 2008; Yang et al. 2010; Imlay 2003; Rajarapu et al. 2011). When oxidative stress occurs in insects, these ROS scavengers play an important role in protecting cells, decreasing LPO, and repairing protein or DNA and RNA damage (Felton and Summers 1995; Jia et al. 2011). In addition, although there is now no evidence that insects can switch to anaerobic respiration to avoid such damage, previous studies have found that yeast can also employ anaerobic respiration to avoid oxidative damages once oxidative stress happens (Lee and Nam 1998). Insects may switch to anaerobic respiration to avoid such damage because oxygen radical formation is directly dependent on temperature-controlled respiration rates in a mud clam (Abele et al. 2002).

The current study concerns the oxidative damage, anaerobic respiration, and antioxidant responses in the sycamore lace bug, *Corythucha ciliata* (Hemiptera: Tingidae). *C. ciliata* is native to the USA but now recognized as an invasive pest of *Platanus* trees in many countries throughout South and North America (Maceljski 1986), Europe (Mutun 2009), Australia (Dominiak et al. 2008), and Asia (Chung et al. 1996; Ju et al. 2009). In China, *C. ciliata* is invading the subtropical regions where the summer maximum temperatures often reach or even exceed 40 °C, which is far above the optimal temperature range for *C. ciliata* (26–30 °C), and hence causes heat stress to the insect (Ju et al. 2011a). However, we have previously found that high temperatures ranging from 35 to 41 °C with a 2-h exposure did not reduce the survival, longevity, or fecundity of *C. ciliata* adults, indicating that this species has great thermal tolerance (Ju et al. 2011b; 2013) and thus, an effective physiological defense may be induced in the insects at high temperatures. It has been reported that, under thermal stresses, organisms can be affected by oxidative stress which leads to oxidative damage (Abele et al. 2002; Lopez-Martinez et al. 2008). The summer high temperatures in subtropical China, therefore, may cause oxidative stress to *C. ciliata*, in which anaerobic respiration and antioxidant responses may be further induced to counteract oxidative damage in the insects.

Comprehensive laboratory studies at controlled temperatures have shown that antioxidant responses can protect insects against ROS-induced damage caused by thermal stress (e.g., Allen et al. 1983; Paes et al. 2001; Magalhaes et al. 2008; Yang et al. 2010; Jia et al. 2011). In contrast, the implications of antioxidant responses in the field, where temperatures are much more variable than in the laboratory, have received relatively little attention (Diaz-Albiter et al. 2011). We here measured the changes of LPO and the activities or concentrations of antioxidant scavengers, including SOD, GR, CAT, and GSH, as well as the levels of anaerobic respiration in *C. ciliata* adults in both the laboratory and field. The objectives of this study were to identify the oxidative damage

and the antioxidant responses of *C. ciliata* adults to thermal stress under both controlled and natural conditions. Accordingly, we also discussed how antioxidant defenses counteract oxidative damage in insects, and whether this response serves in coordination on thermal tolerance of *C. ciliata* in summer.

Materials and methods

Exposure of *C. ciliata* to controlled thermal treatments (laboratory experiment)

In May 2010, *C. ciliata* adults were collected from London plane trees (*Platanus × acerifolia*) on West Yan'an Road in Shanghai, China (31.2°N, 121.5°E), and the specimens were cultured on leaves of *P. acerifolia* in a mesh cage (50×30×30 cm) in the laboratory. Each cage contained one branch with 5–6 leaves with 100–150 individuals; the branch bottom was inserted in a water-filled bottle. The branches and water were replaced as needed to ensure food availability. The stock culture was maintained at 26±0.5 °C with a relative humidity (RH) of 80±5 % and a 14:10 h (L:D) photoperiod. The *F*₁ generation adults (regardless of sex) were placed individually in a closed Petri dish (diameter 9 cm) and then were exposed to 35, 37, 39, 41, and 43±0.5 °C for 2 h in climatic incubators (MIR 350H, Sanyo Electric Co. Ltd., Osaka, Japan). Adults were then transferred to rearing conditions (26 °C) and allowed to recover for 2 h (according to a pilot test, this was sufficient time for recovery for those that would eventually recover; recovery did not increase with recovery times >2 h). The surviving adults were immediately frozen in a canister containing liquid nitrogen (YDS-10A, Chengdu Jinfeng Liquid Nitrogen Container Limited Corporation, China) and were then conserved in a low-temperature refrigerator (Thermo 702, Thermo Electron Corporation, USA) at –80 °C for further assays. The adults were considered dead if no appendage moved after the appendages were touched with a brush. Each treatment was replicated three times, and each replicate was represented by 0.2 g (fresh weight) adult insects (equivalent to about 200 insects). For the control, adults were maintained at 26 °C throughout the experiment.

Exposure of *C. ciliata* to high temperatures in the field (field study)

The collected *P. acerifolia* trees (height 1.5 m) were planted in the experimental field at the Shanghai Landscape Gardening Research Institute, China (31.2°N, 121.5°E) in December 2009. Original *C. ciliata* adults and nymphs were collected from *P. acerifolia* trees on West Yan'an Road in Shanghai, and they were transferred to each of the experimental trees

in May 2010. The experimental trees and insects were kept pesticide-free in the field. On 29 July 2011, about 600 adults (regardless of sex) for each treatment on the upper leaves in the shadow were collected at 08:00, 10:00, 12:00, and 14:00. At each collection time, the temperatures at a position 20 cm below the leaves that supported the insects were measured with a thermometer (according to the early test, here the temperatures are similar to the fed leaves; the microhabitat difference between the leaf and 20 cm away is significant); the average temperature was 29.7 °C at 08:00, 33.5 °C at 10:00, 35.2 °C at 12:00, and 37.2 °C at 14:00. Adults were recovered, frozen, and stored for the same conditions as described in the laboratory experiment. The specimens collected at each collection time were randomly divided into replicates of 200 individuals (0.2 g, fresh weight) each, such that each determination could be based on three replicates.

Sample preparation

Each 0.2-g sample of *C. ciliata* adults was homogenized in a mortar with 1.8 ml of anhydrous ethanol and then diluted to a 10 % concentration with purified water. All crude homogenates were centrifuged at 10,000×g for 15 min at 4 °C, and the supernatants were used for the following analysis. Each assay required its own specific and unique homogenization buffer in order to maintain the stability and functionality of the target material. Total protein concentrations were quantified based on the method provided by Bradford (1976) with bovine serum albumin as the standard.

Lipid peroxidation assay (TBARS)

MDA (an indicator of LPO) concentrations were measured with an assay kit (Nanjing Jiancheng Bioengineering Inst., Jiangsu, China). The assay was based on a thiobarbituric acid (TBA) method in which MDA reacts with TBA to generate a red species (with a maximum absorbance at 532 nm) that was detected with a DU-800, Beckman spectrophotometer. MDA concentration was expressed as nanomole per milligram protein.

Anaerobic respiration assay

Anaerobic respiration levels were indirectly measured by quantifying LDH activity due to its function on the conversion of pyruvate and lactic acid (Tarmy and Kaplan 1968; Mat-Jan et al. 1989). Under anaerobic conditions, LDH catalyzes lactate acid to generate pyruvate, which then reacts with 2,4-dinitrophenyl hydrazine and forms pyruvate dinitrobenzene hydrazone. The latter compound was quantified with an assay kit (Nanjing Jiancheng Bioengineering Inst., Jiangsu, China) and a spectrophotometer (DU-800, Beckman Coulter Inc., USA) at 440 nm. One unit of LDH activity was defined as

the amount that produces 1 μmol of pyruvate after 15 min at 37 °C. LPH activity was expressed as units per gram protein.

Antioxidant assays

Activities or concentrations of SOD, CAT, GR, and GSH were determined with several commercially available assay kits (Nanjing Jiancheng Bioengineering Inst., Jiangsu, China) following the manufacturer's protocols. As mentioned in above detections, all colorimetric assays used a DU-800, Beckman spectrophotometer.

SOD activity was determined by a colorimetric assay (at 550 nm) based on a xanthine oxidase method. One unit of SOD activity was defined as the amount of enzyme required to cause 50 % inhibition of xanthine and xanthine oxidase system reaction in 1 ml enzyme extraction of 1 mg protein. SOD activity was expressed as units per milligram protein.

CAT activity was detected by an ammonium molybdate method that measured the decrease in absorbance at 405 nm due to H₂O₂ decomposition. One unit of CAT activity was defined as the amount that decomposed H₂O₂ per second per gram protein. CAT activity was expressed as units per gram protein.

GR activity was determined by the decrease in absorbance of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm. One unit of GR activity was defined as the decrease in 1 nmol NADPH per second per gram protein. GR activity was expressed as units per gram protein. GSH concentration was measured by the absorbance of a yellow species formed by the reaction of 2,2'-dithiodipropionic acid and thio compound at 420 nm. GSH concentration was expressed as milligrams per liter.

Data analysis

Data among different treatments were subjected to one-way analyses of variance by using the general linear model procedure of SPSS 15.0 for Windows (SPSS 2006). The results were presented as the mean values ± SE. When treatment effects were significant ($P < 0.05$), means were compared with the Tukey's test.

Results

Lipid peroxidation

MDA concentration (an indicator of LPO level) in *C. ciliata* adults increased significantly in response to thermal stresses in both laboratory ($F_{6, 20} = 12.62$, $P < 0.001$, Fig. 1a) and field ($F_{3, 11} = 40.21$, $P < 0.001$, Fig. 1b). The field experiment used the collecting time of the day from 08:00 to 14:00 to show effects of temperature.

Fig. 1 Maleic dialdehyde (MDA) concentration (an indicator of LPO activity) in *Corythucha ciliata* adults after thermal stress in the laboratory (a) and field experiments (b). In the laboratory experiment, adults were exposed to the indicated high temperatures (33–43 °C) for 2 h; adults that were not exposed to high temperatures but were kept at 26 °C served as the control. In the field study, adults were collected on a hot summer day (29 July 2011) from an experimental field in Xuhui District, Shanghai (31.2°N, 121.5°E). Values are means $\pm$ SE. Values with different letters are significantly different (Tukey's test at $P < 0.05$, ANOVA)

Anaerobic respiration

LDH activity (an indicator of anaerobic respiration) in *C. ciliata* adults was insignificantly affected by laboratory heat treatment ($F_{6, 20} = 0.79$, $P = 0.589$, Fig. 2a). In the field study, however, LDH activity increased significantly with temperature ($F_{3, 11} = 6.69$, $P = 0.014$, Fig. 2b), but with relatively low values compared to those in the laboratory.

Antioxidant response

SOD activity in *C. ciliata* adults significantly increased in response to thermal treatments in the laboratory experiment ($F_{6, 20} = 7.83$, $P < 0.001$) (Fig. 3a), and to temperature or collecting time of the day ($F_{3, 11} = 9.31$, $P = 0.006$) in the field study which is greater at 14:00 (37.2 °C) than at earlier times (29.7–35.2 °C) (Fig. 3b). The results for CAT activity were similar to those for SOD activity, i.e., CAT activity tended to increase with increasing high temperatures in the laboratory experiment ($F_{6, 20} = 12.53$, $P < 0.001$) (Fig. 3c) and with time of day (and thus with temperature) in the field ($F_{3, 11} = 16.06$, $P = 0.001$) (Fig. 3d).

Fig. 2 Lactate dehydrogenase (LDH) activity (an indicator of anaerobic respiration) in *Corythucha ciliata* adults after thermal stress in the laboratory (a) and field experiments (b). In the laboratory experiment, adults were exposed to the indicated high temperatures (33–43 °C) for 2 h; adults that were not exposed to high temperatures but were kept at 26 °C served as the control. In the field study, adults were collected on a hot summer day (29 July 2011) from an experimental field in Xuhui District, Shanghai (31.2°N, 121.5°E). Values are means $\pm$ SE. Values with different letters are significantly different (Tukey's test at $P < 0.05$, ANOVA)

GR activity was greater at 41 and 43 °C than at the lower temperatures ($F_{6, 20} = 11.47$, $P < 0.001$) but did not differ between 26 and 39 °C in the laboratory experiment (Fig. 3e), and it tended to significantly increase with field time of day or temperature with the peak at 14:00 ($F_{3, 11} = 6.69$, $P = 0.014$) (Fig. 3f). GSH concentration tended to significantly increase by laboratory thermal treatment ($F_{6, 20} = 106.36$, $P < 0.001$) and especially by treatments ≥ 37 °C (Fig. 3g). GSH also significantly increased with time of day under the field conditions ($F_{3, 11} = 33.62$, $P < 0.001$) (Fig. 3h).

Discussion

The survival and distribution of ectothermic invertebrates are greatly affected by temperature (Denlinger and Yocum 1998). Generally, insect cells exposed to high- or low-temperature stresses may lead to oxidative damage and generate surplus ROS (Lopez-Martinez et al. 2008; Cui et al. 2011). To avoid this adverse effect, insects have evolved the ability to maintain a balance between ROS and antioxidants (Krishnan and Kodrik 2011). In this study, our results demonstrated that all

Fig. 3 Antioxidant defenses (SOD, CAT, and GR activity and GSH concentration) in *Corythucha ciliata* adults after thermal stress in the laboratory (a, c, e, g) and field experiments (b, d, f, h). In the laboratory experiment, adults were exposed to the indicated high temperatures (33–43 °C) for 2 h; adults that were not exposed to high temperatures but were kept at 26 °C served as the control. In the field study, adults were collected on a hot summer day (29 July 2011) from an experimental field in Xuhui District, Shanghai (31.2°N, 121.5°E). Values are means $\pm$ SE. Values with different letters are significantly different (Tukey's test at $P < 0.05$, ANOVA)

the detected parameters in *C. ciliata* adults changed significantly in response to heat stresses with similar trend in the two populations (except LDH), suggesting that oxidative stress and antioxidant responses can simultaneously occur both under the laboratory controlled conditions and in the naturally fluctuating environments. Comparison of antioxidant metabolisms between the two conditions is important in the near future for the area of comparative biochemistry due to global climate change.

In the present study, increases in high temperature significantly increased MDA concentrations in *C. ciliata* adults under both the laboratory and field conditions, indicating that thermal stress is accompanied by oxidative stress and increased LPO. Although previous studies have shown that relatively low and high temperatures have yielded similar results in other animals, e.g., *Panonychus citri* (Yang et al. 2010), *Scapharca broughtonii* (An and Choi 2010), and *Bactrocera dorsalis* (Jia et al. 2011), these works are mostly

done in the laboratory. Our results suggest that LPO can also be induced by naturally fluctuating high temperatures. But it is worth mentioning that we focus on only LPO of oxidative stress, while injuries of proteins, DNA, and RNA are equally possible targets, and we ignore them in the present study. Therefore, more indicators associated with injuries of proteins, DNA, and RNA also need to be investigated in the further study (Nikolaïdis et al. 2012).

In most cases, when high concentrations of ROS are produced under oxidative stress, many important enzymatic antioxidants immediately act as scavengers to eliminate the surplus ROS generated in the cell (Jovanović-Galović et al. 2004; Meng et al. 2009; Zhou et al. 2010; Wilczek et al. 2013). SOD and CAT are regarded as the two most important antioxidant enzymes that reduce the high levels of superoxide anions and thereby reduce oxidative damage caused by environmental stresses (Park et al. 2009; Bafana et al. 2011; Celino et al. 2011). During ROS scavenging, SOD initially converts $O_2^{\bullet-}$ into O_2 and H_2O_2 , and H_2O_2 is then broken down into O_2 and H_2O by CAT (Jovanović-Galović et al. 2004). In this study, SOD and CAT activities in *C. ciliata* adults significantly increased under heat stress in both the laboratory and field, showing that both SOD and CAT take part in stepwise oxygen reduction, and they act synergistically in antioxidant defenses. Similar results have been reported in some other insects, spiders, and mites, such as *P. citri* (Yang et al. 2010), *Chilo suppressalis* (Cui et al. 2011), and *Xerolycosa nemoralis* (Wilczek et al. 2013).

Besides enzymatic scavengers, many nonenzymatic antioxidants may also help remove the excess ROS associated with oxidative stress. GSH is considered as an important low-weight molecule, nonenzymatic component of the antioxidant defenses (Lalouette et al. 2011). The reduced GSH protects the cell against ROS but also against their toxic products (Jones et al. 1981; Storey 1996; Hayes and McLellan 1999). GSH reduces H_2O_2 and organic peroxides and forms oxidized glutathione, which is then reduced back to GSH by NADPH via a reaction catalyzed by GR (Storey 1996). This cyclic reaction maintains GSH levels and the reducing environment in the cell and therefore enables organisms to periodically reduce oxidative stress (Allen et al. 1983; Paes et al. 2001). In both laboratory and field conditions, GR activities and GSH concentrations increased significantly in *C. ciliata* adults in response to heat stress, indicating that both GSH and GR may partly help protect *C. ciliata* against temperature-induced oxidative stress, although it is difficult to avoid oxidative damage drastically.

A previous study has shown that SOD deficiency in *Saccharomyces cerevisiae* may cause the metabolic flux to shift into anaerobic ethanol fermentation in order to avoid their oxidative damages by Paraquat (a herbicide) stress (Lee and Nam 1998). However, data on the link between anaerobic respiration and oxidative stress in insect species are scarce.

Although physiologies in insects are much different from those in yeasts, it is doubtful that anaerobic respiration is beneficial for some small insects when oxidative stress happens. Nonetheless, other studies have recently found that excessive anaerobic respiration is generally thought to have toxic effects on insect cells, and the energy provided by anaerobic respiration is insufficient to maintain insect development and reproduction (Westneat et al. 2003). Anaerobic respiration, therefore, may only help insects survive brief periods of hypoxia (Lighton and Schilman 2007), and the effects of anaerobic respiration on antioxidant need further study. On the data of LDH in the present study, thermal treatments did not vary among the levels of anaerobic respiration in the laboratory experiment, but the lack of a heat response of LDH may be due to already high LDH activity. In the field study, LDH activity in *C. ciliata* adults increased significantly with temperature as well as the collecting time of the day. It is likely that LDH activity may have been reduced by the lower temperatures of the night and then rapidly increased by sunlight or warming in the field. Under the field condition, there exists the possibility that the effects of temperature and other stresses (such as UV radiation) are mixed with a potential circadian rhythm of anaerobic respiration. Similar features also happen on other scavengers, such as GR, and hence cause their basal level to be quite different between the laboratory and the field. Further experimental evidence, however, is still needed to explain such an inference. In addition, the tested insects were treated in closed Petri dishes for 2 h in the laboratory experiment, whereas they were not similarly enclosed in the field, which might also have affected the oxygen environment for the two experiments, although it is inevitable in this study. This may also account for the difference of LDH (and even more other markers) between laboratory and field, and, thus, the methods for the detection of antioxidants also need to be improved.

Several previous studies have suggested that antioxidants may increase the survival and reproduction of insects. For example, CAT expression increases the fecundity and reduces the mortality of *Lutzomyia longipalpis* (Diaz-Albiter et al. 2011). Inactivation or silencing of CAT in *Musca domestica* (Allen et al. 1983), *Drosophila melanogaster* (Mackay and Bewley 1989), *Rhodnius prolixus* (Paes et al. 2001), and *Anopheles gambiae* (Magalhaes et al. 2008) leads to increased ROS levels, and ultimately increase mortality. However, our understanding of ROS scavenging is still incomplete. The increases in MDA caused by the thermal treatments in the current study suggest the potential for damage caused by ROS accumulation and oxidative stress in *C. ciliata* because MDA is the endpoint of damage. Nonetheless, *C. ciliata* adult can survive, reproduce, and develop to high temperatures from 35 to 41 °C, and it thrive in summer in subtropical China (Ju et al. 2011b, 2013), indicating that oxidative damage is not enough to cause the negative effect on the survival and distribution in

this species. The results suggest that the antioxidant defenses may partly help *C. ciliata* counteract oxidative damage and may reverse appropriate concentrations of intracellular O₂. Although it is unclear as to whether intracellular O₂ contributes to heat tolerance in *C. ciliata*, recent studies have shown that higher levels of environmental O₂ in vitro are predicted to enhance thermal tolerance of insects, and reductions in O₂ are expected to reduce thermal limits (Klok et al. 2004; Stevens et al. 2010; Verberk and Bilton 2011; Verberk and Calosi 2012).

In conclusion, thermal stress results in oxidative damage in *C. ciliata*, as indicated by increased levels of MDA. At the same time, thermal stress also induces enzymes and other factors with antioxidant activity in *C. ciliata* adults, which include SOD, CAT, GR, and GSH. Anaerobic respiration is also induced to help the insects cope with oxidative stress. Although we did not test how these antioxidant responses affect the tolerance of *C. ciliata* to thermal stress in the present study, we could infer from this study together with our previous works (Ju et al. 2011b, 2013) that under antioxidant counteracting, oxidative damage has little influence on population booming in *C. ciliata*. Further studies, however, are needed to examine how these metabolic processes help the insects tolerate thermal stress through more detailed experiments because we did not find antioxidant defenses which immediately lower the oxidative damage in the present study. Furthermore, many follow-up experiments including other important oxidative markers (i.e., hydroxynonenal, prostaglandin, and particularly some recoverable indicators) and antioxidant markers (i.e., alkyl hydroperoxide reductase, peroxidases, glutathione peroxidase, glutathione-S-transferase, etc.) need to be conducted in the animals experiencing even more high temperatures and more durations that if be included would enhance the value of the study on antioxidant responses substantially.

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