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Response of antioxidant enzymes in *Mythimna separata* (Lepidoptera: Noctuidae) exposed to thermal stress

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Abstract

The oriental army worm *Mythimna separata* (Lepidoptera: Noctuidae) is a migratory pest in Eastern Asia and China. Seasonal high temperatures in Southern China and low temperatures in Northern China are pressures favouring the annual migration of this species, while cold tolerance determines the northern limit of its overwintering range. A number of physiological stress responses occur in insects as a result of variations in temperature. One reaction to thermal stress is the generation of reactive oxygen species (ROS), which can be harmful by causing oxidative damage. The time-related effects (durations of 1, 4 and 7 h) of thermal stress treatments of *M. separata* at comparatively low (5, 10, 15 and 20°C) and high (30, 35, 40 and 45°C) temperatures on the activities of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), peroxidase (POX) and glutathione S-transferases (GSTs), and total antioxidant capacity (T-AOC) were determined. Thermal stress resulted in significant elevation of the activities of SOD, CAT and GSTs, indicating that these enzymes contribute to defence mechanisms counteracting oxidative damage caused by an increase in ROS. However, at high-temperatures, POX and T-AOC were also found to contribute to scavenging ROS. Our results also indicate that extreme temperatures lead to elevated ROS production in *M. separata*. The present study confirms that thermal stress can be responsible for oxidative damage. To overcome such stress, antioxidant enzymes play key roles in diminishing oxidative damage in *M. separata*.

Keywords: thermal stress, antioxidant enzymes, oxidative stress, *Mythimna separate*

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Introduction

Temperature is the most critical environmental factor for many organisms; it effects growth, reproduction, distribution and abundance, by inducing numerous physiological responses (Angilletta *et al.*, 2002; Parmesan, 2006; Jia *et al.*, 2011). The thermal stress response, which occurs in all living organisms, is a standard reaction to above normal temperatures (Kotak *et al.*, 2007; Nguyen *et al.*, 2013). Under thermal stress, overproduction of reactive oxygen species (ROS) can cause oxidative damage. In general, the production of ROS and antioxidant processes are synchronized; however, the balance between these activities can be disrupted during periods of environmental stress, leading to synthesis of additional ROS (Joanisse & Storey, 1996; Lopez-Martinez *et al.*, 2008; Lalouette *et al.*, 2011). Overproduction of ROS can disrupt the fluidity of cell membranes, due to lipid peroxidation, and lead to necrobiosis, as well as alterations in cellular DNA (Green & Reed, 1998; Monaghan *et al.*, 2009). A number of factors promote

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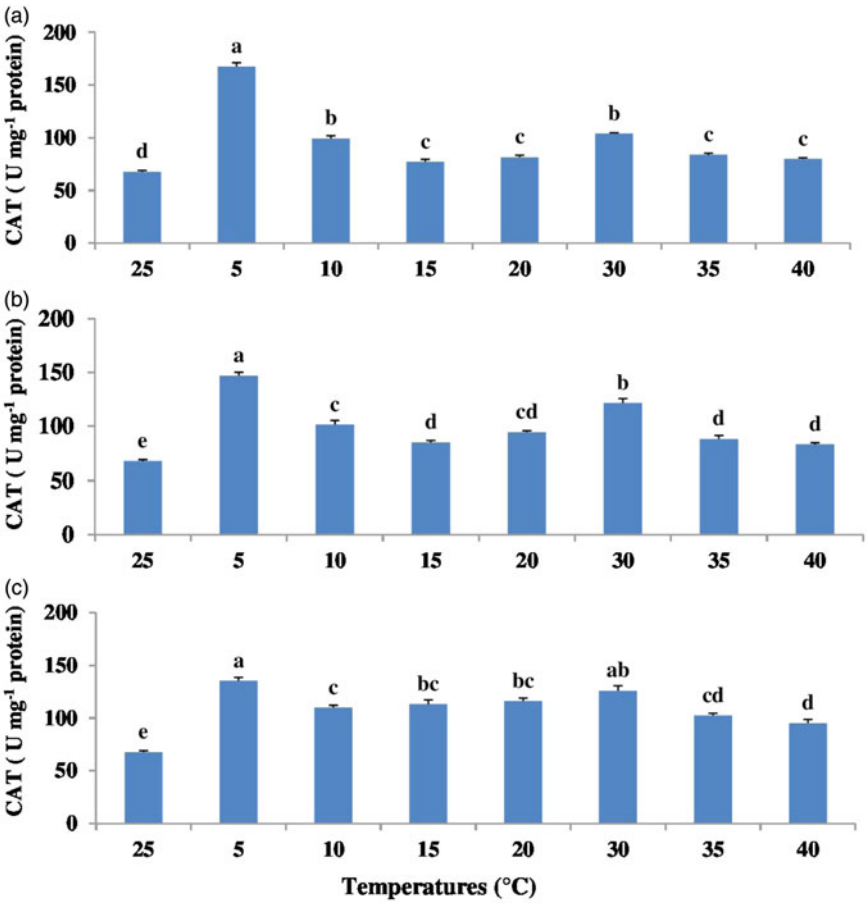

Fig. 1. Effects of treatment of *M. separata* adults with thermal stress for various lengths of time on CAT activity. Data collected after treatment durations of 1, 4 and 7 h are presented in (a), (b) and (c), respectively. Data are presented as means (±SE) of three replicate experiments. Letters above bars indicate significant differences ($P < 0.05$) determined by ANOVA with Tukey's test.

overproduction of ROS in insects, including compensatory growth, ingested plant photo-oxidants and unfavourable environmental conditions (such as the presence of pollutants, adverse temperatures or hypoxic stress) (Aucoin *et al.*, 1995; Zaman *et al.*, 1995; Joanisse & Storey, 1998; Jing *et al.*, 2005; Mangel & Munch, 2005).

To prevent ROS damage, living organisms have developed complex defence mechanisms for handling ROS, which include both enzymes and molecular antioxidants (Howe & Schilmiller, 2002). Anti-oxidative enzymes are the key to removal of ROS from biological systems. The primary anti-oxidative enzymes in insects are superoxide dismutase (SOD), catalase (CAT), peroxidase (POX) and glutathione-S-transferases (GSTs) (Felton & Summers, 1995; Wang *et al.*, 2001; Dubovskiy *et al.*, 2008). SOD catalyses the disputation of superoxide radicals into oxygen and H_2O_2, whereas both CAT and POX catalyse the disputation of H_2O_2 into oxygen and water. Another important enzyme, GST, eliminates lipid peroxidation products (hydroperoxides) from cells (Dubovskiy *et al.*, 2008; Meng *et al.*, 2009). In addition, the ability of all antioxidants in an organism to counter oxidation is described as the total antioxidant capacity (T-AOC) (Ghiselli *et al.*, 2000).

The oriental army worm *Mythimna separata* (Lepidoptera: Noctuidae) is a migratory pest in Eastern Asia and China (Ruilo & Ziangshi, 1987; Rui-Lu *et al.*, 1989; Chen *et al.*, 1995). It has been responsible for damaging millet (*Pennisetum* spp.) and wheat (*Triticum* spp.) crops for thousands of years in China. Recently, it has also been found to damage rice and corn crops (Chen & Hu, 2000; Wang *et al.*, 2006). Seasonal migration of *M. separata* has been observed in China. The organism is mainly present in Southern and Central China and its population is well controlled by reducing the cultivation area of host plants in these regions, although it can survive and reproduce in some southern regions during winter. However, crops in several areas of Northern China, where the insect is unable to survive over winter, are still continuously damaged (Jiang, 2004; Zhang *et al.*, 2006). Seasonal high temperatures in Southern China and low temperatures in Northern China are one of the pressures favouring the annual migration of *M. separata* between these areas as an adaptive life history strategy (Jiang *et al.*, 2000). Conversely, the cold tolerance of this species determines the northern limit of its overwintering range in China. Zhang *et al.* (2008) revealed that cold stress (5°C) experienced during the first 24 h after eclosion can change migrant *M. separata* into resident insects. Jiang *et al.* (2011) reported that, for adults of *M. separata*, flight occurred at temperatures between 11 and 32°C, with an optimum range of 17–22C, and a lower threshold of 8°C. Warmer temperatures generally have a positive effect on developmental

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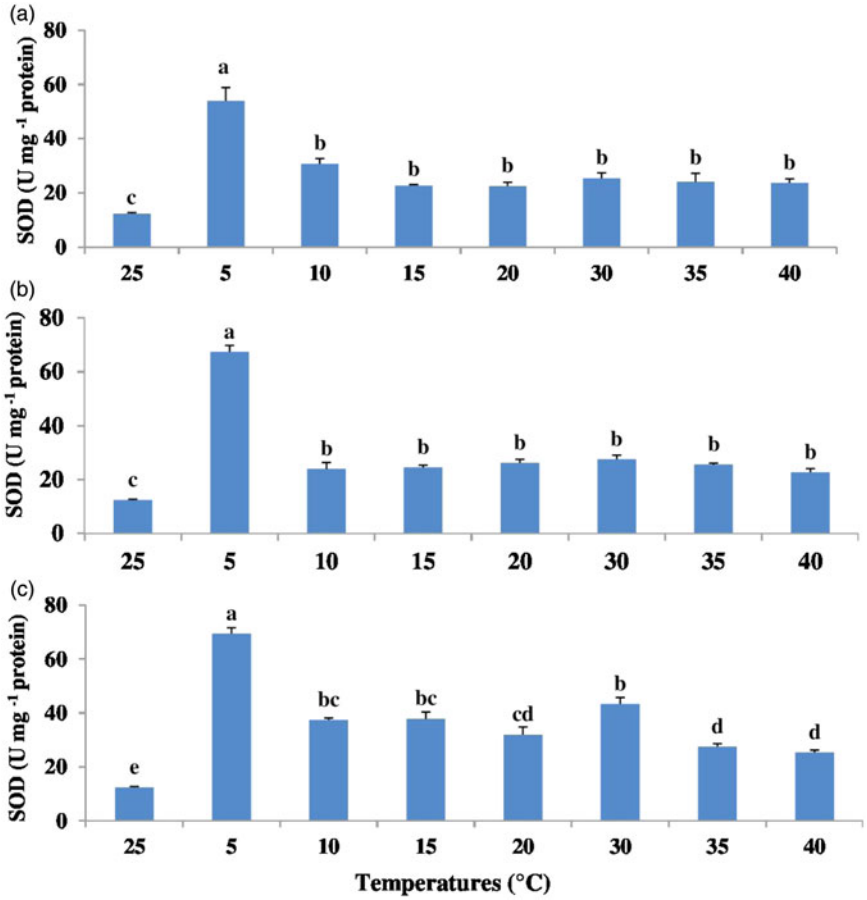

Fig. 2. Effects of treatment of *M. separata* adults with thermal stress for various lengths of time on SOD activity. Data collected after treatment durations of 1, 4 and 7 h are presented in (a), (b) and (c), respectively. Data are presented as means (±SE) of three replicate experiments. Letters above bars indicate significant differences ($P < 0.05$) determined by ANOVA with Tukey's test.

time, lifespan, adult flight activity and reproduction of *M. separata* (Jiang & Luo, 1997; Xinfu *et al.*, 1998); however, very high temperatures can have the opposite effect, and suppress adult reproduction to a greater extent than they promote migratory flight (Jiang *et al.*, 2000). To facilitate growth and reproduction, animals search for balanced sources of nutrition, mates and oviposition sites. This kind of searching behaviour has costs that are offset by the benefits gained from the resource (Crespo *et al.*, 2014). *M. separata* encounters thermal fluctuations during its life cycle. The cost to the adults of extreme temperatures (both low and high) is much higher than that of migratory flight. To date, the effects of thermal stress on *M. separata* have not been reported. The aim of the present study was to determine how variations in temperature affect anti-oxidant enzyme activities in response to oxidative stress as such changes may lead *M. separata* to migrate in order to survive in different seasons.

Materials and methods

Insects

Insects for experimentation were collected from the Key Laboratory of Insect Resources Utilization and Sustainable Pest Management, Huazhong Agricultural University, Wuhan. *M. separata* were reared at room temperature (25 ± 2°C), 60 ± 10% relative humidity, and with 14:10 h light:dark cycles. An artificial diet was used to feed the larvae as described in Chun (1981).

Thermal stress

Three-day-old adults were selected for the experiment. Five adults were transferred into 100 ml plastic containers for each treatment. Insects underwent temperature treatments, at 5, 10, 15, 20, 30, 35, 40 and 45°C, for 1, 4 and 7 h. For all stress treatments, a programmable thermal controller (Ningbo Southeast Instrument, RXZ-260B, China) was used. A temperature of 25°C was set as the control for this experiment. Adult insects were frozen in liquid nitrogen immediately after temperature treatment and stored at −80°C until further analysis. Experiments were performed three times on three different days.

Enzyme extraction

A commercially available assay kit (Nanjing Jiancheng Bioengineering Institute, China) was used for extraction of enzymes, according to the manufacturer's instructions. Samples

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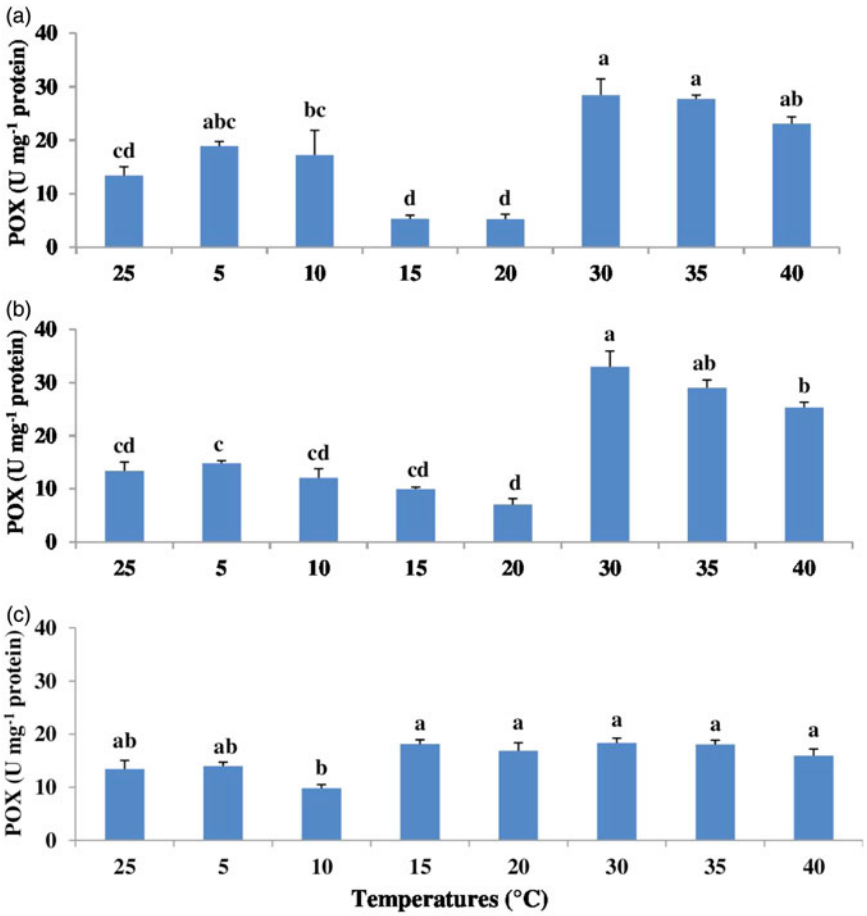

Fig. 3. Effects of treatment of *M. separata* adults with thermal stress for various lengths of time on POX activity. Data collected after treatment durations of 1, 4 and 7 h are presented in (a), (b) and (c), respectively. Data are presented as means (±SE) of three replicate experiments. Letters above bars indicate significant differences ($P < 0.05$) determined by ANOVA with Tukey's test.

were homogenized in 0.9% saline solution at a ratio of 1:9 (W_{flies}:$V_{normal\ saline}$). Homogenates were centrifuged at 10,000 *g* for 15 min at 4°C. After centrifugation, the supernatant was stored at low temperature until tested to determine enzyme activity. The method of Bradford (1976) was used to calculate protein concentrations.

Measurement of T-AOC

T-AOC was measured using an assay kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions. The kit is based on the ability of antioxidant substances present in the supernatant to reduce a pool of ferric iron. This acts as a redox-linked, reductant colorimetric assay, as a relatively stable complex is formed between Fe^{2+} and porphyrin, which absorbs light at 520 nm. The required quantity of protein to elevate the absorbance measurement by 0.01 nm min^{-1} mg^{-1} protein was defined as one unit of T-AOC.

Determination of antioxidant enzyme activities

Spectrophotometry was used to determine the activities of enzymes (SOD, CAT, POX and GST) using assay kits (Nanjing Jiancheng Bioengineering Institute), in accordance with the instructions of the manufacturer.

CAT activity was calculated by gauging the decline in absorbance at 405 nm in response to decomposition of H_2O_2. The amount of enzyme required for decomposition of H_2O_2 per second per mg of protein was defined as one unit of CAT activity. The unit of expression for CAT activity was U mg^{-1} protein.

The xanthine oxidase method was used to determine SOD activity at 450 nm. The quantity of enzyme required for 50% inhibition of the xanthine–xanthine oxidase reaction in a protein concentration of 1 mg ml^{-1} was defined as one unit of SOD activity, expressed as U mg^{-1} protein.

POX activity was measured at 420 nm by the activation of oxidation in the presence of H_2O_2. The quantity of POX enzyme required to catalyse 1 µg substrate min^{-1} mg^{-1} of protein was defined as one unit of POX activity, and expressed as U mg^{-1} protein.

The substrate, 1-chloro-2,4-dinitrobenzene (CDNB) was used to determine the activity of GST. A change in absorbance at 412 nm was observed due to the formation of GSH–CDNB. The amount of GST enzyme required to activate the fusion of 1 µmol l^{-1} GSH with CDNB min^{-1} mg^{-1} protein was

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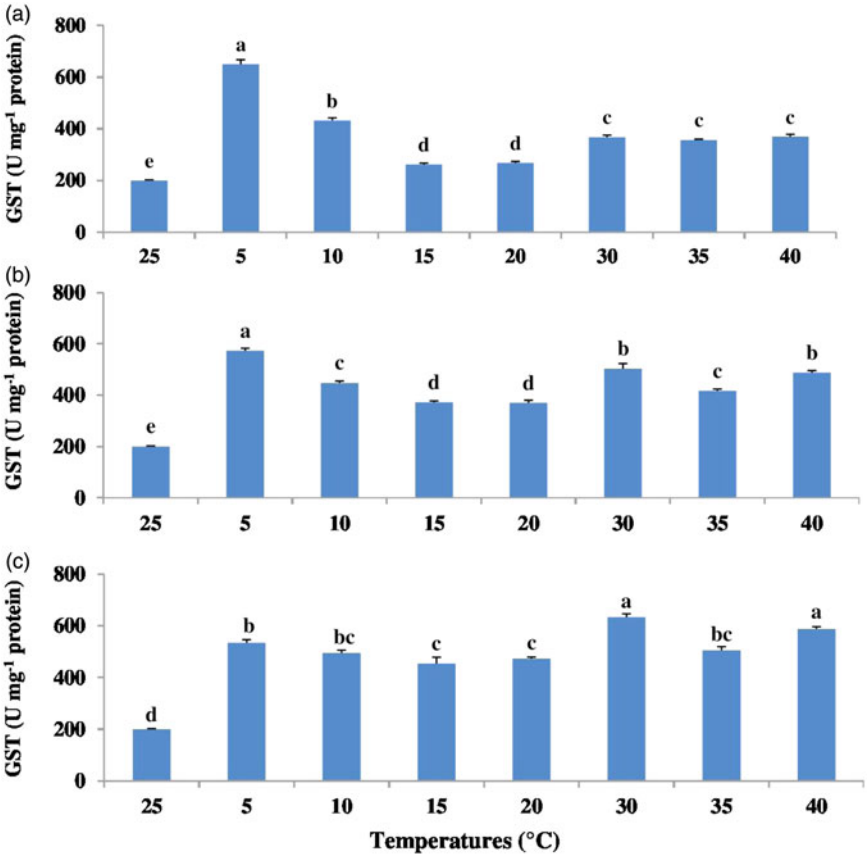

Fig. 4. Effects of treatment of *M. separata* adults with thermal stress for various lengths of time on GST activity. Data collected after treatment durations of 1, 4 and 7 h are presented in (a), (b) and (c), respectively. Data are presented as means (±SE) of three replicate experiments. Letters above bars indicate significant differences ($P < 0.05$) determined by ANOVA with Tukey's test.

defined as one unit of GST activity and expressed as U mg^{-1} protein.

Statistical analysis

Treatment effects (temperature and duration) were subjected to one or two-way analysis of variance (ANOVA) using the general linear model procedure in SPSS 16.0 (SPSS, Chicago, IL, USA); when significant effects were identified, mean differences were separated by Tukey's test, with $P < 0.05$ considered statistically significant.

Results

At 45°C, all adults died, regardless of the duration of the thermal stress treatment.

Antioxidant enzymes

CAT activity in *M. separata* adults was significantly increased at both low and high, compared with the control, temperatures ($P < 0.01$), after treatment for all durations ($P < 0.01$), and the interaction between temperature and duration was significant ($P < 0.01$). Maximum CAT activity values were 167.67, 146.94 and 135.50 U mg^{-1} protein recorded under cold stress (5°C) for 1, 4 and 7 h, respectively (fig 1).

SOD activity was significantly raised at both low and high, compared with the control, temperatures in *M. separata* adults ($P < 0.01$), for all durations of treatment ($P < 0.01$), and there was a significant interaction between temperature and duration ($P < 0.01$). The highest SOD activity levels (53.92, 67.41 and 69.45 U mg^{-1} protein) were observed under cold stress (5°C) for 1, 4 and 7 h, respectively (fig 2).

POX activity in *M. separata* adults was also significantly affected at all temperatures ($P < 0.01$) and for all durations ($P < 0.01$), and temperature and duration interacted significantly ($P < 0.01$). POX activity increased significantly under high-temperature stress (temperatures ranging from 30 to 40°C) at 1 and 4 h, relative to cold stress and control (25°C) conditions; however, after 7 h, while a significant elevation in POX activity was observed at temperatures of 15, 20, 30, 35 and 40°C, relative to that at 10°C, no significant differences were observed at either low or high temperatures compared with the control group (25°C) (fig 3).

Significant increases in GST activity in *M. separata* adults at both low and high temperatures were observed at all temperatures ($P < 0.01$) and durations ($P < 0.01$), compared with controls (25°C), and there was a significant interaction between temperature and durations ($P < 0.01$). The highest values of GST activity recorded were 649.71 and 572.50 U mg^{-1} protein at 5°C for 1 and 4 h, respectively. In addition, after 7 h at 30°C GST activity was 633.15 U mg^{-1} protein (fig 4).

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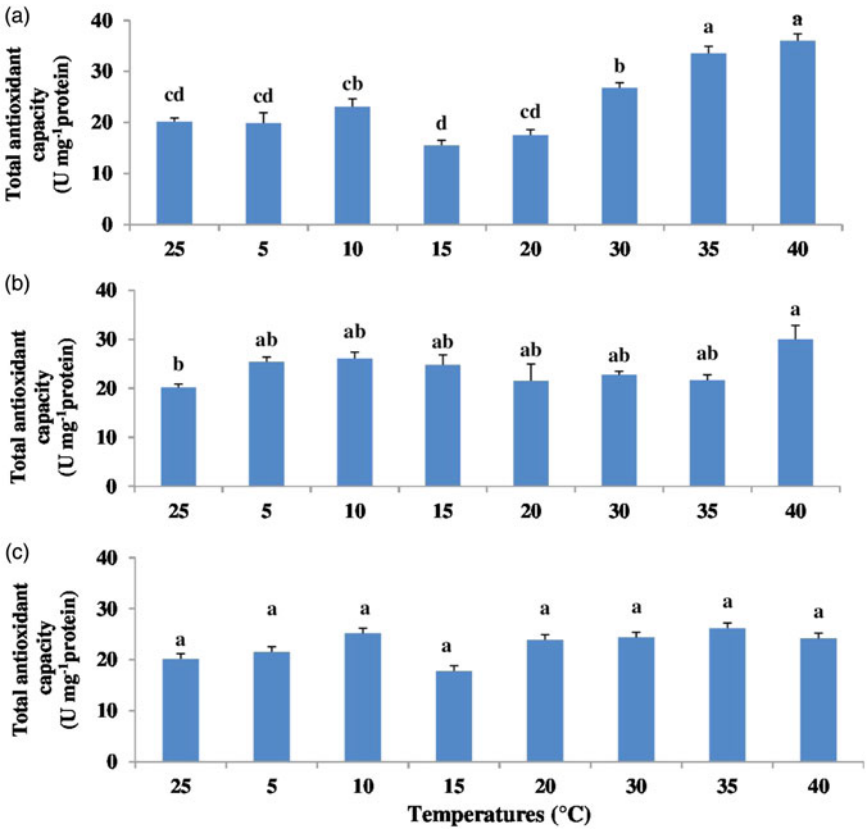

Fig. 5. Effects of treatment of *M. separata* adults with thermal stress for various lengths of time on total antioxidant capacity. Data collected after treatment durations of 1, 4 and 7 h are presented in (a), (b) and (c), respectively. Data are presented as means (±SE) of three replicate experiments. Letters above bars indicate significant differences ($P < 0.05$) determined by ANOVA with Tukey's test.

Total antioxidant capacity (T-AOC)

Significant effects were observed on the T-AOC of *M. separata* adults, relative to the control group, under both low- and high-temperature stresses ($P < 0.01$). The duration of treatment did not result in a significant change in T-AOC ($P < 0.31$); however, a significant interaction between temperature and duration was observed ($P < 0.01$). Heat stress (temperatures ranging from 30 to 40°C) resulted in a significant increase in T-AOC after 1 h, relative to cold stress and control temperature; however, after 4 h of treatment only temperature stress treatment at 40°C resulted in significantly increased T-AOC compared with controls (25°C). No significant changes were observed compared with controls when adults were exposed to low- and high-temperature stresses for 7 h (fig 5).

Discussion

Temperature is a critical environmental variable that engenders physiological changes in organisms (Jia *et al.*, 2011). *M. separata* adults were exposed to different thermal stresses, at both low and high temperatures, and consequent physiological oxidative stress responses explored. The effect of different thermal stress conditions on the activities of the antioxidant enzymes, SOD, CAT, POX, GST and on T-AOC, in *M. separata* adults was examined. CAT, SOD, POX and GST are key antioxidant defence enzymes, which work in a synchronized manner to thwart oxidative stress caused by high concentrations of ROS within cells. Among these antioxidant enzymes, CAT is considered to be the principle H_2O_2 scavenging enzyme in arthropods (Jena *et al.*, 2013), as selenium-dependent glutathione POX (the main catalyser in other organisms) is deficient (Sohal *et al.*, 1990). However, CAT is ineffective for the removal of low concentrations of H_2O_2, as it functions only in the presence of high cellular concentrations (Ahmad *et al.*, 1991). Under thermal stress, CAT activity in citrus red mites is insufficient (Yang *et al.*, 2010); however, in the present study, a significant elevation of CAT activity was observed at both low and high temperatures in *M. separata* adults, compared with controls. These data suggest that overexpression of CAT enhances the removal of H_2O_2 at both low and high temperatures, and prevents oxidative stress damage. Similar results were reported by Jia *et al.* (2011), and Nabizadeh & Kumar (2011), in the oriental fruit fly, *Bactrocera dorsalis* and the silkworm, *Bombyx mori*.

SOD plays a critical role in reducing high levels of superoxide radicals induced by exposure to low and high temperatures (Celino *et al.*, 2011). In the present study, significant enhancement of SOD activity was determined under conditions of thermal stress, compared with controls at 25°C, suggesting that SOD production was induced as a result of temperature fluctuations to protect *M. separata* adults from

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thermal stress. Similar results were reported by McCord & Fridovich (1969) and Jia *et al.* (2011). SOD and CAT can directly remove excess ROS in a coordinated manner. SOD removes O_2^- through the process of dismutation to O_2 and H_2O_2, and H_2O_2 is then sequentially reduced to H_2O and O_2 by CAT (Kashiwagi *et al.*, 1997). The observed higher levels of CAT, relative to those of SOD, in this study indicate that, under thermal stress, H_2O_2 is also synthesized by processes other than SOD activity.

GSTs can metabolize lipid peroxidation products together with POX, which also breaks down H_2O_2 (Jia *et al.*, 2011). In the present study, POX activity increased significantly at high temperatures (ranging from 30 to 40°C) for 1 and 4 h, compared with controls. Similar findings were reported by Zhang *et al.* (2014) in the predatory mite, *Neoseiulus cucumeris*. Our results demonstrate that POX activity was expeditiously induced by thermal stress in *M. separata* adults, which is consistent with the findings of a similar study involving *Helicoverpa armigera* (Meng *et al.*, 2009). However, after the longest duration (7 h) of thermal stress, a significant decrease in POX activity was observed in the oriental fruit fly *B. dorsalis* (Jia *et al.*, 2011) and predatory mite, *N. cucumeris* (Zhang *et al.*, 2014). In contrast, our results indicate no significant changes in POX activity at either low or high temperatures compared with the control temperature after the longest treatment duration (7 h), similar to the results reported by Yang *et al.* (2010). The elevation of POX activity at higher temperatures indicates that it was stimulated by scavenging ROS in *M. separata*.

GSTs are a group of multifunctional dimeric enzymes, which catalyse the conjugation of glutathione to a broad spectrum of endogenous and xenobiotic compounds for detoxification, protection from oxidative damage, isomerization and intercellular transportation (Board & Menon, 2013). These enzymes are involved in the inactivation of toxic lipid peroxidation products created by oxidative stress damage. In the present study, the observation of significantly elevated levels of GST under temperature stress suggests that this enzyme protects *M. separata* adults from oxidative damage under these conditions. Similar antioxidant responses have been reported in *P. japonica* (Zhang *et al.*, 2015), *A. mylitta* (Jena *et al.*, 2013), *B. dorsalis* (Jia *et al.*, 2011) and *P. citri* (Yang *et al.*, 2010).

T-AOC is widely used as a tool to assess redox, and as a representative measure of the total antioxidant capacity existing in an organism (Meng *et al.*, 2009; Yang *et al.*, 2010; Sashidhara *et al.*, 2011). T-AOC was augmented significantly when *M. separata* adults were exposed to high temperatures (ranging from 30 to 40°C) for 1 h and (40°C) for 4 h, compared with controls. These data suggest that T-AOC adapts to deal with oxidative stress and free radical formation and are consistent with the results reported by Zhang *et al.* (2015), Zhang *et al.* (2014) and Jia *et al.* (2011). However, no significant difference was observed compared with controls after treatment for the longest duration (7 h). A similar result was reported by Jia *et al.* (2011) in *B. dorsalis* under thermal stress conditions.

Antioxidant stress is well managed by antioxidant enzymes; however, some non-enzymatic substances, e.g. trehalose (Mahmud *et al.*, 2010) and vitamin E (a-tocopherol) (Kaur *et al.*, 2009) also contribute to this process. A recent study also confirmed the involvement of heat shock proteins, along with antioxidant enzymes, in the response to ROS damage (Rosa *et al.*, 2012). The increase of T-AOC only at high temperatures indicates that *M. separata* uses not only antioxidant enzymes, but also other defence mechanisms, to combat thermal stress and enable survival of the organism (Jia *et al.*, 2011).

Conclusion

Oxidative stress can be generated when environmental factors disturb the balance of redox reactions within an organism. In *M. separata*, thermal stress is the main candidate factor for the induction of oxidative stress. In response to thermal stress, antioxidant enzymes are upregulated as a defence mechanism to mitigate potential cellular damage. The enzymes SOD, CAT and GST undergo significant increases in activity in response to thermal stress in *M. separata*, and may be involved in the management of oxidative damage produced by ROS. Indeed, there was an increased production of ROS at higher temperatures; therefore, these fluctuations may reflect physiological adaptations in *M. separata* related to its migration habits. However, at high temperatures, compared with lower temperatures, POX activity and T-AOC have additional roles in scavenging ROS.

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