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Effects of Low Temperature Stress on Survival, Reproduction and Protective Enzyme Activities of *Ectropis grisescens* Warren 1894 (Lepidoptera: Geometridae)

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Key words

***Ectropis grisescens* Warren, Low temperature stress, Survival, Reproduction, Protective enzyme activities**

ABSTRACT

Ectropis grisescens Warren 1894 is a tea garden pest that poses a significant threat to tea yield and quality. To better understand its ecological resilience and potential population dynamics under changing environmental conditions, this study investigated the tolerance of *E. grisescens* to low temperatures and the consequent effects on its survival, reproduction, and protective enzyme activities. The experimental treatments of a control of 25°C and eight low temperature treatments (-8, -5, 0, 5, 10, 15 and 20°C for 1 h and -10°C for 10 min) were set. Assessment of parameters, including male adult longevity, eggs laying number, egg hatching rate, total protein content, and activities of key antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and total antioxidant capacity (T-AOC) were measured. In the results, as the temperature decreased, the lifespan of both female and male *E. grisescens* adults shortened. At -10°C, the survival periods for females and male adults were only 1.67 and 1.47 days, respectively. Similarly, fecundity was significant impacted, the number of eggs laid and hatching rate gradually decreased with decreasing temperature, eggs laid by per female on average reached only 118.60 with a hatching rate of 0% at -10°C. Moreover, the total protein content reached its highest level in female adults (7435.58 μg mg^{-1}) at 15°C while in males (3790.29 μg mg^{-1}) at -8°C. The SOD activity in female adults peaked at -10°C (18.56 U $mgprot^{-1}$), while that in male adults peaked at 10°C (6.58 U $mgprot^{-1}$), both significantly higher than other treatments. The CAT activity was higher in female adults compared to male adults, with decreasing temperature, the CAT activity decreased in female adults while that increased in male adults. The POD activity reached its peak at -10°C for both female (0.43 U $mgprot^{-1}$) and male (0.84 U $mgprot^{-1}$) adults. The T-AOC content was highest in the control group compared to other treatments. These findings on the changes of protective enzyme activities in *E. grisescens* indicated their important role in adapting to low temperature stress. The study provides a theoretical basis for predicting the development of *E. grisescens* population under low temperature.

INTRODUCTION

Ectropis grisescens Warren, belonging to Lepidoptera, Geometridae, is a major pest of tea plants. It is widely distributed and occurrs in tea-producing regions throughout China (Ma *et al*., 2016; Zhang *et al*., 2016; Qiao *et al*., 2021). Its larvae feed on tea leaves and shoots, leading to substantial damage and affects tea yield and quality (Li *et al*., 2019; Ma *et al*., 2019). Environmental temperature plays a crucial role in the growth, development, reproduction, behavior, and survival of insects (Reinhold *et al*., 2018; McDonald *et al*., 2020). Insects can only grow and live normally within a specific temperature range, and extreme low or high temperatures can have detrimental effects (Reinhold *et al*., 2018).

The development rate of *E. grisescens* in different stages and generations is influenced by temperature, with accelerated growth observed along with increasing temperature. The growth and development of *E. grisescens* in different stages proceed normally within the range of 19-25°C, while the female adults fecundity decreases at 27°C, and the development becomes severely delayed at 19°C (Geng *et al*., 2021). In our previous study, we determined that the optimal temperature for individual and population growth of *E. grisescens* is 25°C (Geng *et al*., 2021). Additionally, we found that the supercooling

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points for female and male adults were -11.07 and -12.73°C, respectively, while the freezing points were -6.51 and -6.39°C, respectively (Geng *et al*., 2023). Low temperatures directly affect proteins, membranes, and nucleic acids in insects, causing metabolic disorders and influencing their reproduction, dispersion, and population dynamics (Bale, 1987; Koštál *et al*., 2007; Rozsypal, 2022). The ability of insects to tolerate low temperatures is crucial for their survival and evolutionary adaptation (Sinclair *et al*., 2013; Teets *et al*., 2020). Therefore, investigating the adaptability of insects to low temperature stress holds significant implications for understanding insect ecology and biological evolution (Bale, 2002).

The adaptation and survival rates of insects under low temperature stress are influenced by various factors, including insect species, gender, developmental stages, and individual differences (Renault *et al*., 2002; Jiang *et al*., 2015). Furthermore, the duration and temperature of exposure to low temperatures also impact the survival rate of insects, with longer exposure times and lower temperatures leading to greater lethal effects (Renault *et al*., 2002). For example, first instar nymphs or newly hatched adults of *Rhopalosiphum padi* experienced a significant decrease in their average total fecundity after being exposed to -7.5°C for 2 h (Hutchinson and Bale, 1994; Sinclair *et al*., 2003; Marshall and Sinclair, 2015).

The balance between the production of reactive oxygen species (ROS) and the antioxidant defense of insects can be disrupted in response to changes in environmental temperature and stress (Cui *et al*., 2011). To mitigate and prevent damage caused by ROS, insects have developed antioxidant defense mechanisms involving enzymes such as catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), and total antioxidant capacity (T-AOC). These enzymes regulate the levels of free radicals and reduce the damage caused by ROS and their byproducts (Dubovskii *et al*., 2010; Xin *et al*., 2017; Kim *et al*., 2018). For example, in *Frankliniella occidentalis*, the SOD activity significantly increased after low temperature treatment, while POD activity was inhibited significantly, and CAT activity initially decreased and then gradually increased (Shi *et al*., 2013). In *Empoasca onukii* Matsuda adults, the activity of protective enzymes showed a sharp decrease at -5°C (Qiao *et al*., 2015). Additionally, the activity of SOD reached the highest at 28°C (Xing *et al*., 2012).

Understanding the changes in population and protective enzyme parameters of *E. grisescens* after low temperature is essential for a better predicting its adaptability. However, more details on the antioxidant enzyme responses of *E. grisescens* under low temperature stress remain unclear. Therefore, the effects of short-term low temperature stress on the longevity, egg production, egg hatching rate, total protein content, POD, SOD, T-AOC, and CAT activities of *E. grisescens* adults were evaluated to reveal the physiological mechanisms of stress resistance, and further provide a theoretical basis for population development and prediction.

MATERIALS AND METHODS

Collection and rearing conditions of E. grisescens *larvae*

E. grisescens larvae were collected from a tea garden in Baimiao village, Shihegang Township, Xinyang City, Henan Province, China (31°59′ N, 113°51′ E, altitude: 235 m). They were fed with fresh tea branches (cultivar Fuding white tea), reared under controlled environment conditions in an artificial climate incubator at a temperature of 25±1°C, relative humidity of 70±5%, a photoperiod of L/D = 12 h/12 h, and a light intensity of 100-150 lx. The experimental insects used were from the fifth generation.

Low temperature stress treatments

To access the tolerance of *E. grisescens* adults to low temperatures, experimental groups were divided and subjected to short-term low-temperature treatments at -10, -8, -5, 0, 5, 10, 15 and 20°C. For each treatment, fifteen male and fifteen female *E. grisescens* adults were transferred into separate 20 mL centrifuge tubes, then sealed with cotton balls. Subseequently, the sealed tubes were placed in low-temperature constant temperature baths (DC-2006, Shanghai Miqingke Industrial, Shanghai, China) set to the respective temperatures. The treatment at -10°C was 10 min, while the treatment time for all other treatments was 1 h. These treatment durations were carefully selected based on preliminary testing to ensuring the survival of test insects for subsequently experiments. The control group was maintained at 25°C for 1 h, each treatment was repeated three times.

Assessment of adult mortality, egg laying, and hatching

Following the above treatments, female and male adults were placed in a 25°C light incubator (PGX-280A 3H Laifa Technology, Ningbo, China), and provided with 10% honey water to supplement nutritional requirements. Daily observations were conducted to record the mortality of adult insects, egg laying, and hatching. The calculations for egg-laying quantity, egg hatching rate, and adult longevity were performed using the established methods described in previous studies (Qiao *et al*., 2015; Han *et al*., 2023).

Sample collection for measuring protective enzyme activities

To evaluate the effects of low-temperature stress

on adult insect's protective enzymes activity, the low temperature treatments were replicated as described above. Following the low temperature treatment, the adult insects were promptly transferred to centrifuge tubes and frozen in liquid nitrogen rapidly. Subsequently, they were stored at -80°C (HD-86L830, Hisense Electric, Quigdas, China) for subsequent analysis.

Enzyme extraction from low-temperature treated adults

For enzyme extraction, three male and three female adults from each low-temperature treatment group were individually placed in separate 1.5 mL centrifuge tubes. Subsequently, 0.7 mL of 50 mmol/L phosphate buffer (pH 7.0) was added to each tube, and the mixture was homogenized evenly in an ice bath (ICE-0.2T, Huaya Brothers Refrigerator Equipment, Zhengzhou, China). The resulting mixture was then centrifuged at 12,000 r/min (TGL-165, Shuke Instruments, Chengdu, China) for 15 min at 4°C, and the supernatant was collected for further analysis.

Determination of protein content and protective enzyme activities

The total protein content was determined using colorimetry at 562 nm. The activity of SOD was measured using the WST-1 method with a colorimetric wavelength of 450 nm. CAT activity was determined by the ammonium molybdate method, and the colorimetric wavelength was 405 nm. POD activity was determined using colorimetry at 420 nm, and T-AOC was determined using the microplate method with a colorimetric wavelength of 405 nm (FG Thermo Fisher Scientific, Shanghai, China). Three replicates were set for each treatment group, and each sample from each treatment was measured three times. The measurement procedures were followed the instructions of the kits (Jiancheng Biotechnology, Nanjing, China).

Data analysis

Data processing was performed using SPSS 16.0. One-way ANOVA and Duncan's new multiple range test were used to assess the significance of differences, with a significance level set at $P < 0.05$. The independent sample *t*-test was used for comparisons between treatments. The percentage data were transformed using the inverse sine square root before statistical analysis.

RESULTS

Effects of low-temperature stress on adult longevity, egg production and egg hatching rate

Across all low-temperature treatment groups, the average fecundity of female adults was markedly lower compared to the control group maintained at 25°C (Fig. 1A). The most pronounced reduction in egg-laying was observed at -10°C, with an average number of 119 eggs, significantly different from the control group (431 eggs). There was no significant difference in the number of eggs

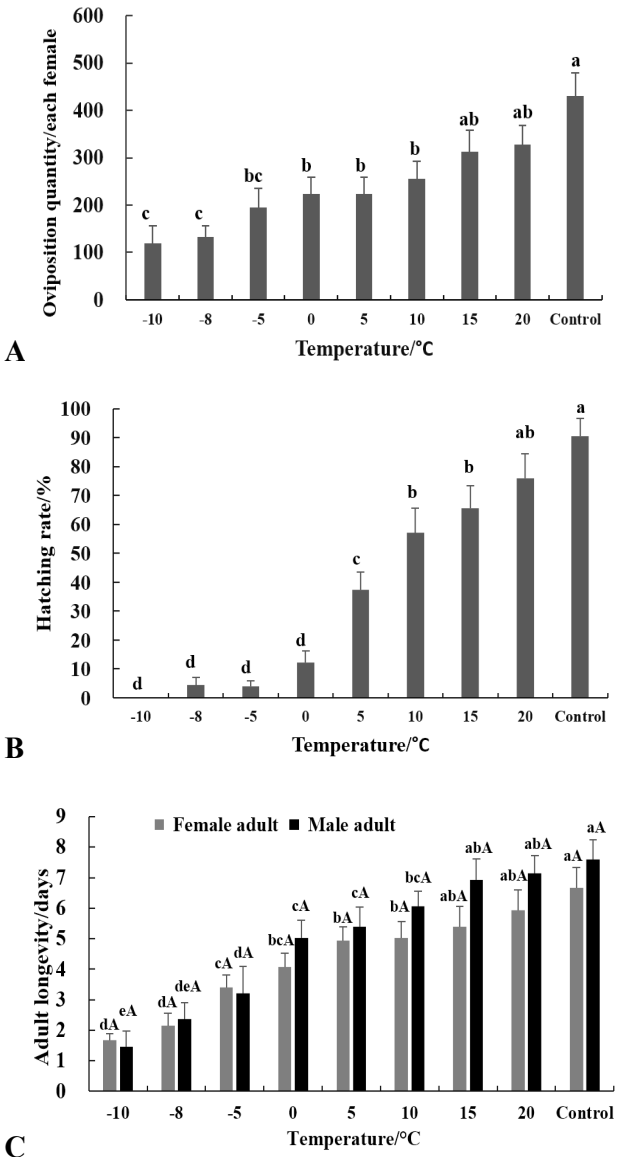

Fig. 1. Effects of low temperature stress on oviposition quantity (A), hatching rate (B), and longevity (C) of *Ectropis grisescens* Warren.
The data are mean±standard error (SE). Different lowercase letters indicate significant differences between male and female adults ($P < 0.05$). Different uppercase letters indicated a significant difference between male and female adults ($P < 0.05$, *t*-test). The control was treated under the conventional feeding condition (25°C).

laid between the 15 and 20°C treatment groups and the control group, as well as the -5, 0, 5, and 10°C groups. However, a significant difference was evident between the 15 and 20°C groups and the -10°C group. Moreover, significant differences were also observed in the number of eggs laid between treatment groups of -8, -5, 0, 5 and 10°C and the control group. These results demonstrated that low-temperature stress reduced the fecundity of *E. grisescens* female adults.

Different low-temperature stress levels exhibited varying effects on the egg hatching rate of *E. grisescens* (Fig. 1B). As the temperature decreased, a downward trend in egg hatching rate was observed. All low-temperature treatment groups, except for 20°C, showed significant differences compared to the control group at 25°C. There was no significant difference was found between the 15 and 10°C treatments. However, under 5°C treatment, the egg hatching rate was significantly lower than that of the control group, and the differences among 0°C, -5°C, -8°C, and -10°C treatment groups were similar, all significantly different from that of the 5°C treatment group. Remarkably, under the -10°C treatment, the hatching rate of eggs reduced to 0%. These findings indicate that short-term low temperature stress affects the egg hatching rate of adults of *E. grisescens*, thus influencing the population size of the next generation.

After exposure to various temperature treatments, the longevity of female and male adults of *E. grisescens* was found to be lower, compared to the control group; and it decreased with decreasing temperature (Fig. 1C). No significant difference was observed in the longevity of female and male adults in different temperature treatments. Notably, after -10°C treatment, the average lifespan of female and male adults was the shortest, which were 1.67 and 1.47 days, respectively. While no significant difference was found between the -8°C treatment and other treatment groups, a significant difference was observed between them. At 15 and 20°C, the average lifespan of female and male adults did not significantly differ from the control group. Male adults at 0°C, 5°C and 10°C exhibited similar levels on lifespan, while there was no significant difference in the average lifespan of female adults at 0°C, 5°C, 10°C, 15°C and 20°C. These results indicate that low temperature stress has the potential to shorten the lifespan of both male and female adults of *E. grisescens*. This could reduce the time available for mating and oviposition, and ultimately led to a decline in the population.

Effect on total protein content

Low temperature stress exerted distinct impact on the total protein content of female and male adults of *E. grisescens*, particularly on male adults (Fig. 2A). In male

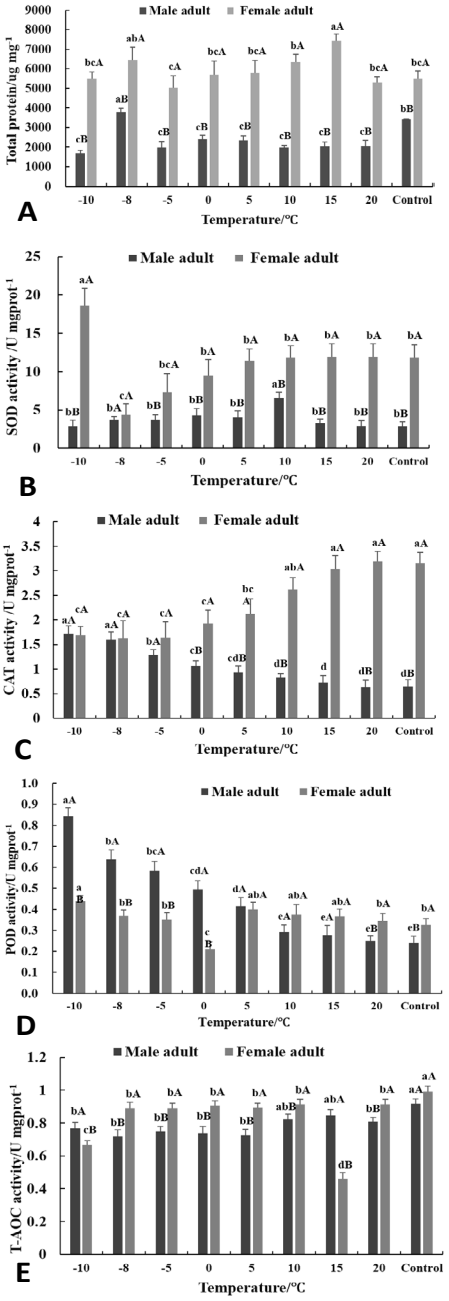

Fig. 2. T-AOC activity effect of low temperature stress on total protein content (A), SOD activity (B), catalase activity (C), POD activity (D), of *E. grisescens* adults. The data are mean ± SE. Different lowercase letters indicate significant differences between male and female adults (P <0.05). Different uppercase letters indicated a significant difference between male and female adults (P <0.05, t-test). The control was treated under the conventional feeding condition (25°C). SOD, superoxide dismutase; CAT, catalase; POD, peroxidase; T-AOC, total antioxidant capacity.

adults, the total protein content reached the highest at -8°C treatment (approximately 3790 μg mg^{-1}), which was significantly different from all other treatments. The second highest value was observed in the control group, which also showed significant differences from all temperature treatment. The total protein content of female adults was significantly higher than that of male adults under all treatments, and showed a fluctuating trend overall. It reached the highest (about 7436 μg mg^{-1}) at 15°C treatment, then gradually decreased, increased at -8°C, and decreased again at -10°C. These results showed that low temperature stress changed the total protein content of *E. grisescens* adults. This may be a mechanism for counteracting the adverse effects of low temperature on growth and survival.

Effects on SOD activity

Different temperature stress levels exerted specific effects on the SOD activity of male and female adults of *E. grisescens* (Fig. 2B). Notably, the SOD activity of male adults was consistently lower than that of female adults, except for the -8°C treatment, where no significant difference between male and female adults.

Specifically, for female adults, SOD activity gradually decreased as the temperature stress decreased. The lowest activity (approximately 4 U $mgprot^{-1}$) was observed at -8°C, significantly different from other treatments, except for a lack of significant at -5°C. However, at -10°C, SOD activity reached its highest level (approximately 19 U $mgprot^{-1}$), significantly different from other treatments.

In male adults, SOD activity reached the highest value (approximately 7 U $mgprot^{-1}$) at 10°C, which was significantly different from all other treatments. At -10°C, SOD activity reached its lowest value (approximately 3U $mgprot^{-1}$), with no significant difference observed compared to other treatments, except for the 10°C treatment. The SOD activity of *E. grisescens* adults increased or decreased under low temperature stress, with the changes in female adults being greater than those in male adults. This difference may be related to the increased low temperature resistance of female adults.

Effects on CAT activity

Low temperature stress affected CAT activity of both male and female adults of *E. grisescens* (Fig. 2C). The lower the temperature, the smaller the difference in CAT activity between male and female adults. There was no significant difference in CAT activity between male and female adults at -5, -8 and -10°C. The CAT activity of female adults showed a downward trend with the decrease of stress temperature, and there was no significant difference between the 10, 15, and 20°C treatments compared to the control (25°C). The significant differences among 0, -5, -8, and -10°C treatments were at the same level.

The CAT activity of male adults gradually increased with the decrease of stress temperature, and reached the maximum value (about 2 U $mgprot^{-1}$) at -10°C. Except for the -8°C treatment, significant differences were observed with other treatments. There was no significant difference in CAT activity between the 10, 15, and 20°C treatments and the control group (about 1 U $mgprot^{-1}$).

Effects on POD activity

Low temperature stress has certain effects on POD activity of male and female adults (Fig. 2D). Except for the 5, 10, and 15°C treatments, significant differences in POD activity were observed between male and female adults in the other groups. The POD activity of female adults showed an overall upward trend with the decrease of stress temperature, reaching the lowest value at 0°C (about 0.2 U $mgprot^{-1}$), gradually increasing at -5°C, and reaching the highest value at -10°C (about 0.4 U $mgprot^{-1}$). The POD activity of female adults of *E. grisescens* at -10°C was significantly different from other temperature treatments, except for no significant difference compared to the 5 and 10°C treatments. There was a significant difference in POD activity between the 0°C and other treatments.

The POD activity of *E. grisescens* male adults increased gradually with the decrease of temperature. The highest POD activity was observed at 10°C (about 0.8 U $mgprot^{-1}$), which was significantly different from other treatments. The activities of POD at 10°C, 15°C, 20°C, and the control were lower, with no significant difference observed. The change in POD activity showed that POD activity in male adults increased significantly with decreasing stress temperature, indicating that the parameters of different protective enzyme activities changed differently in male and female adults under low temperature stress.

Effects on T-AOC

Different temperature stress has a certain impact on the T-AOC of male and female adults of *E. grisescens* (Fig. 2E). Under all stress temperature treatments, the T-AOC of female adults was significantly lower than that of the control, and reached the lowest value at 15°C, which was significantly different from other treatments. The second lowest T-AOC was observed at -10°C, also significantly different from other treatments. The T-AOC of male adults also showed a gradual downward trend with decreasing temperature. Except that there was no significant difference between 10 and 15°C treatments and the control group, the T-AOC of the other treatments was significantly lower

than that of the control group, but there was no significant differences among these treatments.

DISCUSSION

Understanding how insects response and adapt to environmental factors such as light and temperature, is of paramount importance for effective insect prevention and control (Qiao *et al*., 2023). Insects exhibit a wide range of temperature adaptations, varying with species (Sheikh *et al*., 2017). Investigating how insects adapt to low temperature stress can help to predict insect mortality during overwintering, estimate the establishment success of and invasive species in new areas, and the response of different geographical populations of insects to climate change (Hart *et al*., 2002; Hatherly *et al*., 2004; Sinclair *et al*., 2003).

Previous studies have shown that insects lay more eggs in suitable growth of environments. However, when exposed to short-term low temperature stress, their egg hatching rate may decline or even fail (Delisle *et al*., 2013). Short-term low temperature affects all developmental stages of *Bactrocera tau*, and its survival rate and the lifespan of male and female adults gradually decrease with decreasing temperature, which is unfavorable for its growth and development (Huang *et al*., 2021). This study found that the lifespan of both female and male *E. grisescens* adults gradually shortened with decreasing treatment temperature, especially temperatures ranging from -10 to 10°C, where the lifespan of male and female adults was significantly shorter than that of the control group, indicating a significant impact of temperature decrease on adult lifespan. The fecundity and egg hatching rate of female *E. grisescens* adults also significantly decreased with decreasing stress temperature. This result is inconsistent with a previous study that found certain low temperature stress promotes oviposition in female adults of *Bactrocera tau* (Walker) (Huang *et al*., 2021). The differences may be related to different insect species and different responses to temperature stress.

Low temperature stress can easily induce the production of ROS in insects, resulting in the accumulation of toxic metabolites in insects (Rojas and Leopold, 1996). Under adverse conditions, increased free radicals can lead to increased damage to many biological functional molecules. The protective enzyme system can maintain free radicals at a low level, thus preventing free radical poisoning (Ahmad and Pardini, 1990; Lu *et al*., 2014). *Tenebrio molitor* exhibited a rapid decrease in survival rate and *in vivo* protective enzyme activities as the temperature decreases in the range of -10~0°C (Liu, 2006). The defensive capacity of protective enzymes in *Harmonia axyridis* adults tends to strengthen under low temperature stress, enhancing their resistance to low temperatures (Zhao *et al*., 2014). In this study, the total protein content of female *E. grisescens* adults was significantly higher than that of male adults under each treatment. The total protein content of female adults at 15 and -8°C was significantly higher than that of other treatments, and the total protein content of male adults reached the maximum at -8°C. Under different temperature stress, the POD activity of *E. grisescens* male adults showed an upward trend with the decrease of temperature, and reached the maximum at -10°C. This result is consistent with an existing research, which showed that POD activity in *Harmonia axyridis* adults increases significantly with decreasing temperature (Zhao *et al*., 2014). This indicated that decreasing temperature could induce the increase of POD activity in male *E. grisescens* adults, so as to resist low temperature stress and improve the stress resistance of the population. The POD activity of female adults was significantly lower at 0°C compared to other treatments. The T-AOC in *E. grisescens* female adults first decreased, then increased and then decreased with the decrease of temperature. This result is consistent with the changing trend of T-AOC in *E. onukii* at 25~-10°C (Qiao *et al*., 2015). It suggests that changes in protective enzyme parameters in different insects may follow a similar trend when combating temperature stress. The CAT activity in *E. grisescens* male adults increases with the decrease of temperature, which is inconsistent with the related study on *Frankliniella occidentalis* (Pergande) after a certain low temperature stress, where CAT activity first decreases and then gradually increases (Shi *et al*., 2013), indicating that there are differences in the change trend of CAT activity in different insects. However, the research results that CAT activity in female adults decreases with decreasing temperature are consistent with research on the decreased activity of *Polyrhachis vicina* after certain low temperature stress (Liu and Ma, 2006). Under low temperature stress (except at -8°C), the SOD activity of female *E. grisescens* adults was significantly higher than that of male adults, which may be related to the increased low temperature resistance of female adults.

During the experiment, there were some deficiencies. This experiment only observed and measured the first generation of *E. grisescens* adults. Further research is needed to investigate how the second and third generations of adults respond to low temperature stress and how continuous low temperature stress affects population development.

CONCLUSION

Under low temperature stress, the longevity of male and female adults of *E. grisescens* was shortened, the number of eggs laid and the hatching rate of eggs decreased, indicating that low temperature stress had adverse effects on the population development of *E. grisescens*. At the same time, the parameters of protective enzymes also changed with the change of temperature, indicating that the uncomfortable low temperature may reduce damage to *E. grisescens* by inhibiting the activity of protective enzymes. These findings provide theoretical basis and physiological parameters for population prediction and integrated control of tea pests.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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