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Enhancement of supercooling capacity and survival by cold acclimation, rapid cold and heat hardening in *Spodoptera exigua* ☆

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ABSTRACT

Insects can increase their resistance to cold stress by prior exposure to non-lethal cold temperatures. Here, we investigated the supercooling capacity and survival of eggs, 3rd and 5th instar larvae, and pupae of *Spodoptera exigua* (Lepidoptera: Noctuidae) during CA, and responses to various pre-treatment protocols, including constant temperatures, thermoperiods, and RCH, RHH, RCH + RHH and RHH + RCH combined with thermoperiods. Only acclimated eggs demonstrated a significant decrease in SCP, from −20.7 ± 0.3 to −22.9 ± 0.3 °C, among all experimental groups compared to non-acclimated stages. Survival increased by 17.5% for eggs, 40.0% and 13.3% for 3rd and 5th instar larvae, and by 20.0% for pupae after CA. Compared to controls, survival of eggs under the conditions of thermoperiod (5:15 °C), thermoperiod (5:15 °C) + RHH, and thermoperiod (5:15, 10:20, and 15:25 °C) + RCH significantly increased. In addition, survival of 3rd and 5th instar larvae and pupae increased under the conditions of thermoperiod (5:15 °C) and thermoperiod (5:15 °C) + RCH, possibly due to the induction of heat shock proteins or cryoprotectants. However, the pre-treatments of thermoperiod + RCH + RHH and thermoperiod + RHH + RCH did not significantly enhance survival of any developmental stage. These adaptive responses may allow *S. exigua* to enhance supercooling capacity and survival in response to seasonal or unexpected diurnal decreases in environmental temperatures.

Introduction

Temperature plays a key role in the seasonal adaptation of insects. During seasonal and diel cycles, many insect species are frequently exposed to stressful low temperatures [16] that present a major challenge to survival. Insects can adopt several physiological strategies to adapt to these stressful climatic conditions. For example, insects survive subzero temperatures by either maintaining their body fluids in a liquid state at temperatures where they might be expected to freeze (freeze avoiding) or by withstanding the formation of internal ice (freeze tolerance) [2,35].

Insects can increase their resistance to cold stress when exposed to low but non-lethal cold temperatures, a process known as CA [32,40] or RCH [24,7] depending on how long they were exposed. Generally, CA requires weeks or months to achieve maximal cold-tolerance. For example, 15.5 °C for 9 days (CA) enhanced cold tolerance to 2 h sub-zero temperatures in *Psacothea hilaris* compared to the non-treatment insects [34]. In contrast to the slow CA response, RCH can be induced by chilling in hours or even minutes. For example, as little as 30 min at 5 °C increased adult survival in *Drosophila melanogaster* from 0% to >50% in response to acute cold [12]. Air temperature is in a constant state of flux, with sudden cooling or warming, and short periods of cyclical cooling and warming similar to the experimental conditions used for RCH or RHH. Survival rate significantly increased when insects were exposed to a fluctuating temperature in comparison with a constant low temperature [11,8], suggesting that survival mechanisms are preferentially induced by more natural patterns of temperature stress.

The beet armyworm, *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) is a freeze avoiding species [26], and can overwinter as larvae [38] or pupae [39,43] in temperate areas without diapause [14]. Previous studies on cold hardiness in this species have focused on SCP [26,21,15], the effect of low temperature duration on survival [26,28,21], thermoperiod and photoperiod [27], cryoprotectants induction [27,28], and RCH [36]. In fact, the ability of seasonal and diel temperature fluctuations to affect activity, survival, and cold hardiness of insects over short and longer time scales are of critical importance [3]. However, previous studies of *S. exigua* neglected the importance and the effects of seasonal

Abbreviations: RCH, rapid cold hardening; RHH, rapid heat hardening; RCH + RHH, rapid cold hardening followed by rapid heat hardening; RHH + RCH, rapid heat hardening followed by rapid cold hardening; SCP, supercooling point; CA, cold acclimation; DCA, discontinued cold acclimation.

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fluctuating temperature and superimposed diel fluctuating temperature on survival. Indeed, such results could provide helpful information for understanding the population dynamics of this species following winter and for establishing effective population monitoring and forecasting of outbreaks [2]. We hypothesized that seasonal and diel fluctuating temperature could enhance the cold hardiness of *S. exigua*.

In this study, seasonal and diel fluctuating temperatures were simulated using CA, thermoperiods, RCH, RHH, and the alternation of RCH and RHH combined with thermoperiods. The objectives were to determine (1) the effects of CA on SCP and survival of *S. exigua* and (2) the survival rates of different developmental stages following pre-treatments simulating diel fluctuations and seasonal fluctuations in temperature.

Materials and methods

Colony maintenance

Spodoptera exigua were collected from Cihui Farm (114°06′E, 30°59′N) on *Brassica oleracea* var. *botrytis* (Cruciferae: Brassica) in Wuhan City, Hubei Province, PR China during June to July, 2010. The colony was incubated at 26 ± 1 °C with a light: dark photoperiod of 12:12 h, and relative humidity of 60–80%. Honey water (10%) was provided to the adult moths through a 5 cm cotton wick inserted into the slit lid of a 20 ml plastic cup placed inside the transparent plastic container (R = 25.0 cm, H = 15.0 cm). Adult females laid eggs on the wax paper surrounding the internal face of the oviposition container. Oviposition substrates were replaced daily. Egg masses were collected from the oviposition substrates and surface-sterilized in a 5% formaldehyde solution for 20 min before being placed in plastic containers (R = 15.0 cm, H = 7.5 cm).

Newly hatched larvae were fed an artificial diet containing 35 g soybean flour, 25 g wheat bran, 5 g corn flour, 10 g sucrose, 35 g brewer's yeast, vitamin B_5, B_6 and C at 0.3, 0.4, and 3.0 g, 0.3 g choline chloride, 1 g sorbic acid, 8 g agar, and in 300 mL distilled water [22] and housed in a plastic container (R = 15.0 cm, H = 7.5 cm). Third and 5th instar larvae, pupae (<12 h old), and eggs (<12 h old) were chosen as experimental insects. Larval instar stages were determined according to Kim and Song [27].

Lower lethal temperature and time

To examine the lower lethal temperature, eggs, 3rd and 5th instar larvae, and pupae were incubated in a low temperature incubator (IL-82, Yamato Scientific Co., Ltd., Tokyo, Japan) for 24 h at a constant temperature ranging from 0 to −15 °C in 1 °C increments. After the designated exposure, specimens were removed from the low temperature to an incubator set at 26 °C with 60–80% RH and a 12 h photoperiod, and the survivals of each stage were assessed 24 h later. If larvae were able to move when they were prodded with forceps once each in the head, thorax, and abdomen [27], and if eggs and pupae were able to hatch and emerge [21], they were considered alive. The lethal times for each developmental stage were also measured. The survival rates were measured for each stage at exposure times of 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 h at each stage-specific temperature. The survival standards after warming were the same as detailed for minimum survivable temperature. Three replications and N = 90 specimens were studied in each treatment condition.

Effect of CA on SCPs

Supercooling points were measured using surface-contact thermometry following the approach developed by Carrillo et al. [6] and Hou et al. [20]. An egg (or larva or pupa) was fixed in a 1-mL polyethylene pipette tip to a copper–constantan thermocouple linked to an automatic multichannel temperature recorder (Jiangsu Senyi Economic Development Co., Ltd., Jiangsu Province, China). The pipette tip opening was plugged with cotton wool, which anchored the sensing junction of the thermocouple against the egg, larval, or pupal body. The thermocouple, together with the specimens protected by the pipette tips, was placed into a water bath (DW-40L188, Qingdao Haier Medical and Low Temperature Technology Co., Ltd., Shandong Province, China) that was cooled from room temperature with a nonlinear cooling rate of approximately 1 °C/min from 0 to −35 °C. Larval body temperatures were recorded at 1 s intervals. We ended each observation after 20 min at the minimum freeze temperature.

Acclimated stages were obtained by keeping specimens at 10 °C under 60–80% RH and a 12 h photoperiod for 7 days [4]. Third and 5th instar larvae were fed the artificial diet during incubation. Non-acclimated larvae (controls) were allowed access to artificial diet and were taken directly from the colony at 26 °C for survival tests. The SCPs of 120 acclimated and 120 non-acclimated eggs, 3rd and 5th instar larvae, and pupae were assessed for a total of 240 individual observations.

Effect of CA on survival

To determine the effect of CA on survival of *S. exigua* eggs, 3rd and 5th instar larvae, and pupae, three methods were adopted: (1) Control, each experimental stage was chosen at 26 °C, (2) CA, specimens were transferred from 26 °C to a low temperature incubator and maintained for 24 h at 20, 15, 10, 5, and finally 0 °C on successive days, and (3) DCA, stages were treated as in condition two and then transferred back to 26 °C for 24 h. Then stages in the three treatment conditions were transferred to the low temperature incubator set to the stage-specific minimum temperatures for lethal times. The survival standards after rewarming were as mentioned above. Three replications and N = 90 specimens were evaluated for each treatment.

Temperature pre-treatments

To evaluate how various acute temperature changes affect the survival, six temperature pre-treatments were used (i–vi).

i. *Constant temperature:* Incubated at 10, 15 and 20 °C for 5 days for 3rd and 5th instar larvae and pupae, and 2 days for eggs (because they hatched on the third day in pilot experiments).
ii. *Thermoperiod:* Incubated at the cyclic temperatures of 5:15 °C, 10:20 °C, and 15:25 °C (L:D 12:12 h) for 5 days for 3rd and 5th instar larvae and pupae, and 2 days for eggs. The average temperatures of the three cyclical temperature regimes were designed to be the same as the three constant temperature treatments.
iii. *Thermoperiod + RHH:* Three treatments were transferred to 26 °C (12 h) after three cyclic temperature treatments.
iv. *Thermoperiod + RHH + RCH:* Three treatments were transferred to 26 °C (12 h), and then transferred to 0 °C (12 h) after three cyclic temperature treatments.
v. *Thermoperiod + RCH:* Three treatments were transferred to 0 °C (12 h) after three cyclic temperature treatments.
vi. *Thermoperiod + RCH + RHH:* Three treatments were transferred to 0 °C (12 h), and then transferred to 26 °C (12 h) after three cyclic temperature treatments.

Each pre-treatment had a matching control where stages were reared at 26 °C. Experimental specimens in the six pre-treatments