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Diapause Induction, Color Change, and Cold Tolerance Physiology of the Diapausing Larvae of the *Chouioia cunea* (Hymenoptera: Eulophidae)

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ABSTRACT. The chalcid wasp *Chouioia cunea* Yang (Hymenoptera: Eulophidae) is one of the most dominant pupal parasitoids of *Hyphantria cunea* (Drury) (Lepidoptera: Arctiidae), an invasive pest of many forestry trees and agricultural crops. For mass rearing *C. cunea* for biological control purposes, the pupae of *Antheraea pernyi* (Guérin-Méneville) (Lepidoptera: Saturniidae) have been widely used as a substitute host in China. In this article, photoperiodic effect on diapause induction in *C. cunea* within the pupae of *A. pernyi* was investigated, and the differences in cold tolerance physiology including supercooling point, water content, and activities of three protective enzymes (Peroxidase [POD], Catalase [CAT], and Superoxide dismuase [SOD]) between diapause and nondiapause mature larvae were comparatively determined. Our results revealed that *C. cunea* possess a short-day induced larval diapause. The critical photoperiods for diapause induction in *C. cunea* were estimated to be between a photoperiod of 13:11 and 14:10 (L:D) h at 18°C, or between a photoperiod of 12:12 and 13:11 (L:D) h at 21°C or 24°C. We also found that the color of *C. cunea* diapausing larvae was taupe, while the normally developed (nondiapausing) individuals were light yellow. This body color change can be used as an indicator of diapause entry of *C. cunea* larave. The average supercooling point of diapausing mature larvae were lower than those of nondiapausing ones. There were significant differences in the activity of three protective enzymes (POD, CAT, and SOD) between diapausing and nondiapausing mature larvae.

Key Words: *Chouioia cunea*, developmental duration, diapause induction, cold hardiness

The chalcid wasp, *Chouioia cunea* Yang (Hymenoptera: Chalcidoidea) (Yang 1989), is an important endoparasitoid of several destructive defoliators located in China. *C. cunea* in Yantai City, Shandong province, has seven generations every year, overwinters as mature larvae in the host pupae (Su et al. 2004). An introduced pest species can also be controlled by the mass-rearing and release of native parasitoids in the country of introduction. The fall webworm, *Hyphantria cunea*, has been sustainably controlled by an effective gregarious pupal endoparasitoid, *C. cunea*, which is native to China(Yang et al. 2006, 2014). This Chalcidoid wasp species also was reported as pupal parasitoids of the fall webworm to South Korea (Kim et al. 2011). Because the technique of using the pupae of the Chinese oak silkworm, *Antheraea pernyi* (Guérin-Méneville) (Lepidoptera: Saturniidae), as a surrogate host to artificially breed *C. cunea* was developed in 1997 (Fig. 1), *C. cunea* has been playing an important role in biological control programs against several economically important agricultural and forest defoliators, especially the fall webworm, *H. cunea* (Wei et al. 2003). *H. cunea* is a major pest insect of many ornamental trees and shrubs as well as of several agricultural crops. Native to North America, *H. cunea* has become an invasive pest throughout Europe and Asia. Although *C. cunea* has been widely applied in biological control programs via mass rearing and releases, some physiological respects relevant to artificial rearing remain unclear. In addition, *C. cunea* maybe the typical freeze avoidant or freezing intolerant insect according to the theory of the insects cold hardiness (Salt 1961)and our preliminary study of the parasitoid. Its cold resistance and distribution were not clear. Chilling injury in the insects leads to accumulation of toxic waste (lactic acid, nitrogen waste, free radicals). Protective enzymes (POD, CAT and SOD) were found commonly in insects. They can clear the superoxide anion radical and hydrogen peroxide which insects produce for protecting organisms and cells from damage (Fridovich 1977, Rojas and Leopold 1996). The research about the enzyme activity of *C. cunea* in diapause and nondiapause state still had not been found. Therefore, in this article, the effect of photoperiod on diapause induction in *C. cunea* with the Chinese oak silkworm pupae as the alternative host was investigated. Also, developmental duration, supercooling point [SCP], water content, and activities of three protective enzymes (POD, CAT, and SOD) of *C. cunea* were determined. In addition, the article presented data on diapause induction of overwintering mature larvae under an artificial condition as well as cold tolerance physiology of diapausing and nondiapausing insects. These results may provide the theoretical basis for applying *C. cunea* as a biological control agent.

Materials and Methods

Insect Colonies. Laboratory colonies of *C. cunea* were started and established in Shenyang Agricultural University in 2007. The parasitized pupae of *H. cunea* were collected in both the infested forests and ornamental trees in Shenyang, Liaoning Province, P. R. China. These pupae were kept in the laboratory at 20–25°C. The emerged parasitoids were collected into test tubes (200 mm in length and 30 mm in diameter) sealed with cotton balls. The pupae of *A. pernyi* were selected as an alternative host to artificially propagate *C. cunea*.

Determination of Developmental Duration. The *A. pernyi* pupae with bright and green cranial roof were selected and were used as hosts. Then, newly emerged adult wasps (within 24 h) were introduced into the test tubes to oviposite. All the adult wasps were removed 36 h after the inoculation, and the *A. pernyi* pupae parasitized by *C. cunea* were maintained at 18, 21, 24, 25, and 30°C, respectively. Each treatment was repeated three times and no less than 18 individuals for each replicate. The developmental duration of each stage (egg, larva, pupa, and adult) of *C. cunea* was recorded.

Induction Diapause of *C. cunea*. The *A. pernyi* pupae parasitized by the wasps were placed into incubators (HWS-380) randomly and reared under different temperatures (18, 21, and 24°C with a variation of

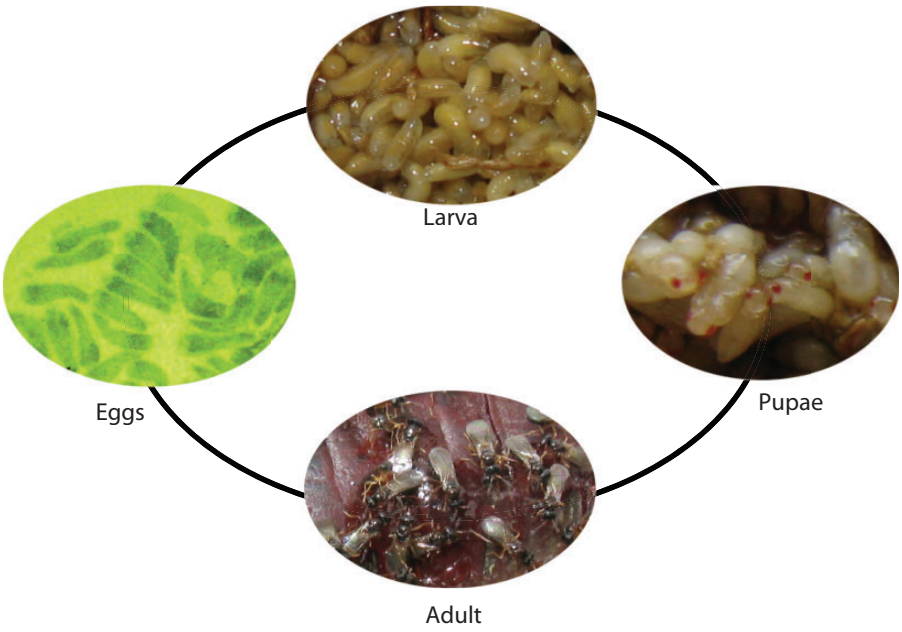

Fig. 1. Life history of *Chouioia cunea* with *Antheraea pernyi* pupae as an alternative host.

±1°C at each temperature) and photoperiods (photoperiods of 7:17, 8:16, 9:15, 10:14, 11:13, 12:12, 13:11, 14:10, and 15:9 [L:D] h). The pupae were manually covered with an opaque black cloth material to simulate scotophase. A minimum of 18 pupae were used for each treatment. Diapausing wasps could be distinguished according to their developmental duration.

Determination of Supercooling Point and Water Content. Diapausing *C. cunea* larvae, which lived in fall webworm pupa, were put into a natural environment (the hole of trees) in the middle of October to overwinter. Supercooling point and water content of the diapausing wasp larvae were measured on the 25th of each month from October 2010 to March 2011.

A diapausing larva attached to a copper-constantan thermocouple was placed in an Eppendorf tube. The tube was sealed with cotton ball and then was immersed into a refrigerated ethanol bath so that the environmental temperature could decrease at a rate of 0.5°C per minute. The temperature of the larva was recorded by a potentiometer, K-UT323 digital thermometric instrument (UNI-T TREND GROUP LIMITED, Shenzhen, China), connected to the thermocouple. The SCP was taken as the lowest temperature before freezing (indicated by the latent heat release of crystallization).Thermometer records body temperature changes per second. Data were input into computer and temperature change curve was drawn automatically When the body temperature was reduced to below 0°C and then temperature rise suddenly, the turning point of temperature curve is the supercooling point. The supercooling point and freezing point of the diapausing and nondiapausing mature larvae were determined according to the described methods by Qin and Yang (2000). Water content of the diapausing *C. cunea* was measured gravimetrically at midday and midnight. Diapausing *C. cunea* were placed in a preweighted Eppendorf tube, weighed (±0.5 µg) using a Mettler-Toledo UMX-2 microbalance before being dried for 24 h at approximately 60°C (maintained by judicious use of the HG303-4K constant temperature oven produced by experiment instrument factory, Nanjing, China), and stored for 24 h over silica gel at approximately 5°C before dry mass was determined with the microbalance.

Determination of Activities of Three Protective Enzymes. For superoxide detection, the sample of mature larvae (0.5 g) was collected from each treatment into a mortar, and pestled with Tris-HCl (50 mmol·L^{-1}) in ice-bath. The homogenate was then centrifuged at 1145(×g) for 10 min at 4°C. The supernatant fluid was diluted with Tris-HCl and transferred into a 25-ml volumetric flask. Sample solution was then used to determine SOD activity according to the method described in Beauchamp and Fridovich (1971).

For catalase detection, 0.5 g of mature larvae were ground with some quartz sand in 2–3 ml of ice-cold Phosphate buffer solution [PBS] (pH = 7.8) in a tissue mortar under 4°C. The mortar was washed two times with PBS, the crude homogenate and the elution were mixed and transferred into to a 25 ml volumetric flask. After standing at 5°C for 10 min, the mixture was centrifuged at 1145 (×g) for 15 min at 4°C in a ultracentrifuge. The supernatant fluid was left to determine CAT activity according to the method of Chance and Machly (1955).

For POD detection, the sample of mature larvae (0.5 g) was collected from each treatment into a mortar, and pestled with precooling PBS (pH = 6.0) into homogenate in ice-bath. The homogenate was then centrifuged at 644 (×g) for 10 min at 4°C. The supernatant fluid was diluted with PBS and transferred into a 25-ml volumetric flask. POD activity was monitored with guaiacol as a substrate (Simon et al. 1974).

Statistical Analysis. All data were analyzed with one-way analysis of variance and regression analysis, followed by Duncan's multiple range tests (SPSS11.5 for Windows).

Results

Developmental Duration. The developmental duration of *C. cunea* within *A. pernyi* pupae (the alternative host) were determined. At 21°C, the durations of eggs, larvae, and pupae were approximately 3, 12, 9 d, respectively. About 3 d after their emergences, newly emerged wasp adults showed up from the *A. pernyi* pupae and sought new hosts to lay eggs. The relationship between the developmental duration and temperatures could be modeled as an exponential equation ($y = 180.14e^{-0.0842\times}$; $R^2 = 0.9213$; $P < 0.01$) (Fig. 2).

Induction Diapause in C. cunea. The effect of 27 different combinations of constant temperatures and photoperiods on the diapause induction in *C. cunea* is shown in Table 1. Diapause was achieved at the combinations of three different temperatures and seven different photoperiods, but the diapause percentages were different among different combinations. The highest percentage of diapause was 100%, which was achieved at either 18°C with less than a photoperiod of 13:11 (L:D) h or 21°C with less than a photoperiod of 12:12 (L:D). There was no

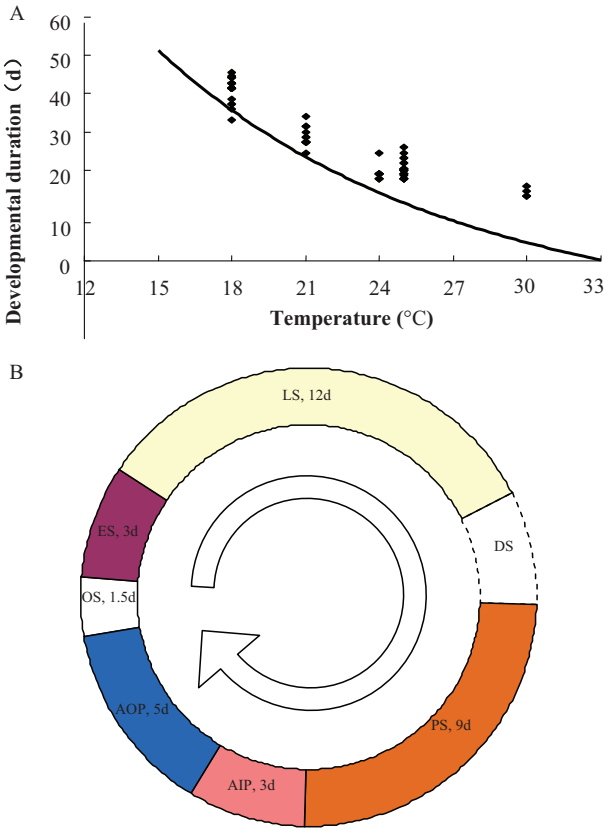

Fig. 2. Developmental duration of *Chouioia cunea* with *Antheraea pernyi* pupae as an alternative host. (A) Curve diagram of the function relationship between developmental duration and temperature of *C. cunea*. y-axis represents developmental duration (day), and x-axis represents temperature (°C). (B) The schematic of developmental periods for mass rearing *C. cunea* at 21°C. OS, oviposition stage; ES, egg stage; LS, larval stage; DS, diapause stage, the *C. cunea* entering diapause stage after the diapause induction; PS, pupal stage; AIP, adult wasps stage inside the host pupa; AOP, adult wasps stage outside the host pupa.

incidence of diapause at temperatures of 18, 21, and 24°C and photoperiods of 14:10 and 15:9 (L:D) h. These results showed that *C. cunea* is a typical long-day species, entering diapause as mature larvae. In addition, the critical photoperiods at 18, 21, and 24°C were extrapolated from the data in Table 1. At 18°C, 50% diapause rate was achieved at photoperiods of 13:11 and 14:10 (L:D) h, respectively. At 21 and 24°C, 50% diapauses rate was achieved at photoperiods of 12:12 and 13:11 (L:D) h. The incidence of diapause occurred over a slightly narrower range of photoperiod at 18°C than that at 21 and 24°C.

Color Change. In this study, further field and laboratory investigations were performed to investigate the body color of the diapausing *C. cunea* larvae. The lab results showed that the body color of the diapausing *C. cunea* was taupe, while the normally developed larvae was light yellow (Fig. 3), and all the diapause larvae of *C. cunea* observed in the field during winter were taupe in color. Therefore, this body color change can be used as an indicator of diapause entry of *C. cunea*.

Cold Tolerance of Overwintering Larvae. From 2010 October to 2011 March in Shenyang City, Liaoning Province, the monthly mean temperatures were 8.3, 1, −9.7, −17.6 −5.8, and 0.9°C, respectively. During the period of October 2010 to March 2011, the diapausing *C. cunea* mature larvae were placed in the field for the supercooling point determination. The results showed that the supercooling points of most diapausing larvae decreased with the temperature. The supercooling point of overwintering larvae in each month follows a normal distribution ($P > 0.05$). The supercooling point in January was −28.1°C,

Table 1. Incidence of diapause in *C. cunea* exposed to continuous temperature and photoperiod regimes

Photoperiod (L:D)	No. of samples	Entering diapause (%)		
		18°C	21°C	24°C
7:17	20	100 ± 0.00	100 ± 0.00	98 ± 4.58
8:16	21	100 ± 0.00	100 ± 0.00	100 ± 0.00
9:15	20	100 ± 0.00	100 ± 0.00	100 ± 0.00
10:14	23	100 ± 0.00	100 ± 0.00	100 ± 0.00
11:13	22	100 ± 0.00	100 ± 0.00	98 ± 5.31
12:12	23	100 ± 0.00	100 ± 0.00	70 ± 7.05
13:11	20	100 ± 0.00	8 ± 1.46	0 ± 0.00
14:10	22	0 ± 0.00	0 ± 0.00	0 ± 0.00
15:9	20	0 ± 0.00	0 ± 0.00	0 ± 0.00

Note: Data are presented as mean ± SD.

which was the lowest among all overwintering months, while October had the highest (–16.5°C). Duncan's multiple range tests showed that there were significant differences between each overwintering month pairs except between November and March ($P > 0.05$).

From October, the water content of overwintering larvae decreased gradually till January, then went up gradually. This tendency was similar to that of supercooling point (Fig. 4). Following linear function could describe the relationship between supercooling point (y) and water content (x): $y = -158.085 + 200.417 \times (R^2 = 0.944)$, $P < 0.01$.

Comparison of Supercooling Point and Protective Enzyme Activity Between Diapausing and Nondiapausing Mature Larvae. Our results showed that supercooling point were significantly ($P < 0.01$) lower in the diapause larvae than that in the nondiapause mature larvae (Table 2). The averages of supercooling point were −17.45°C in the nondiapause mature larvae while in the diapause larvae they were −20.75°C.

Our results also indicated that POD, CAT, and SOD activities in mature larvae varied significantly ($P < 0.01$) between the diapause and the nondiapause larvae. The POD, CAT, and SOD activities in the nondiapause larvae were 34.42 U·(g^{-1}Fw), 34.53 U·(g^{-1}Fw) and 400.43 U·ml^{-1}, respectively. In contrast, when *C. cunea* were exposed to 21°C combined with a photoperiod of (10:14) L:D h the average values of POD, CAT, and SOD activities for diapause larvae were 24.50 U·(g^{-1}Fw), 23.71 U·(g^{-1}Fw), and 230.57 U·ml^{-1}, respectively.

Discussion

C. cunea Yang is the major parasitoid of *H. cunea* (Drury), an economically important invasive pest insect. In China, the technique of using *A. pernyi* pupae as a substitute host for *C. cunea* mass rearing has been playing an important role in both mass rearing the natural enemy at large scale and in biological control of *H. cunea*. The developmental duration (from the egg to the preoviposition adult) of *C. cunea* has been reported in the past. The threshold temperature and effective accumulative temperature of *C. cunea* have been determined to be 6.14°C and 365.12 d-degrees, respectively, with *H. cunea* pupae as the host (Yang 2000), or 9.26°C and 354.05 d-degrees, respectively (Gao et al. 2008). In our study, when reared at 21°C, the duration of eggs, larvae, and pupae of *C. cunea* are determined to be approximately 3, 12, 9 d, respectively. Our study focused on developmental duration of *C. cunea* bred with the tussah moth pupae, which will guide the practice directly.

The recognition that there is an interaction between temperature and photoperiod that regulates diapause has been shown in many insect species (Eskafi and Legner 1974, Wallner 1979). The confirmation of this interaction as critical to diapause induction in *C. cunea* was shown in this study. It was the first time to investigate the phenomenon of diapause in *C. cunea*. Our results indicated that *C. cunea* is a typical short-day diapause induction species in mature larvae stage.

Cold tolerance is generally essential if arthropods inhabiting seasonally cold environments are to survive the winter. Differences in cold tolerance of the same insect species from different developmental stages have

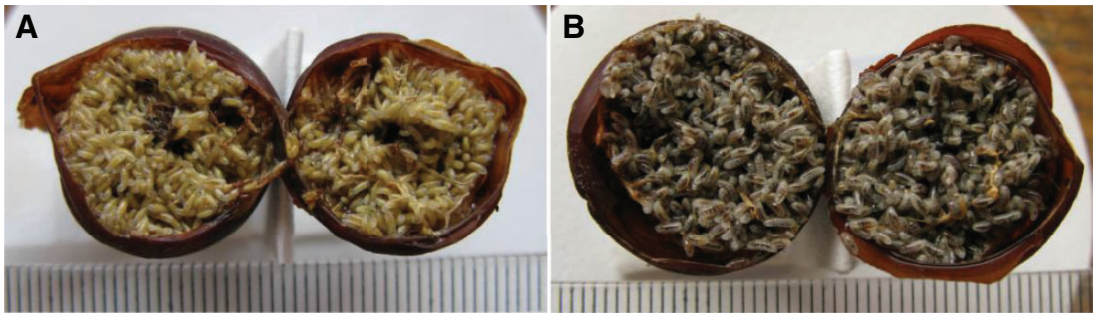

Fig. 3. Color change between normal developmental and diapausing larvae of *Chouioia cunea*. (A) Mature larval stage of the normal development (light yellow); (B) Mature larval stage of the post-diapause (taupe).

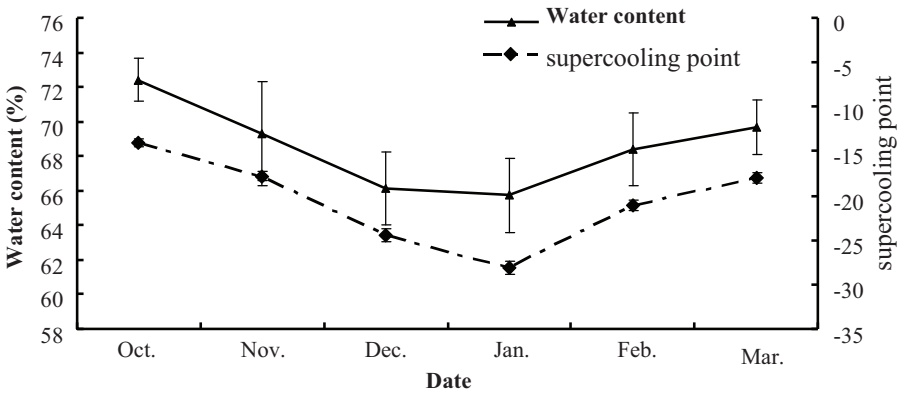

Fig. 4. Water content and supercooling point of *Chouioia cunea* over-wintering larvae. (From 2010 October to 2011 March in Shenyang City, the monthly mean temperatures were 8.3, 1, −9.7, −17.6, −5.8, and 0.9°C, respectively.)

Table 2. Protective enzyme activities of the diapause and nondiapause *Chouioia cunea* mature larvae

		No. of samples	Diapause	Nondiapause	*P*
Super-cooling capacity	Supercooling point (°C)	44	−20.8 ± 1.0	−17.5 ± 0.6	<0.001
Protective enzymes	POD/ U·(g^{-1}Fw)	33	24.5 ± 1.3	34.4 ± 1.4	<0.001
	CAT/ U·(g^{-1}Fw)	31	23.7 ± 0.8	34.5 ± 1.9	<0.001
	SOD/U·ml^{-1}	30	230.6 ± 15.8	400.4 ± 41.2	<0.001

Note: *P.* means significance testing value.

been reported in the past. The supercooling point is known to be a stage-specific parameter in some insect species (Chauvin and Vannier 1997). The supercooling point at different developmental stages of *Bactrocera dorsalis* was different (Hou and Zhang 2007). Carrillo and Cannon (2005) also found that development stages and ages within the same stage yielded numerical differences in *Plodia interpunctella* supercooling point. In this article, we obtained the similar results in *C. cunea*.

According to Shi (2007), there appeared to be a negative trend between supercooling point and water content of the diapause prepupae of *Chrysopa pallens*. Diapause prepupae with high total fats and concentration cryoprotectant in bodies had stronger supercooling capacity. The supercooling point of the diapausing mature larvae was significantly lower than those of the nondiapausing mature larvae in *C. cunea*. This agreed with the results that diapausing *Anthonomus grandis* individuals had much stronger cold tolerance than did the nondiapausing individuals (Slosser et al. 1996). A similar result was also found in *Helicoverpa armigera* (Lepidoptera: Noctuidae) in which the diapausing pupae could bear colder temperature than the nondiapause pupae in winter (Yohei et al. 2005). These results suggested that diapause of *C. cunea* may have certain relevance with cold tolerance.

Cold hardiness of overwinter larvae in *C. cunea* appears seasonal variant. The probable reason was that there exist certain relationships between diapause and cold hardiness. *C. cunea* enhanced the super cooling capacity during diapause. On the other hand, it is commonly assumed that diapause is primarily an adaptation to cold tolerance and low temperature-acclimation contributes to strengthen the ability of cold tolerance (Leather et al. 1993). Under the natural conditions, the dynamics of the super cooling capacity and cold tolerance of *C. cunea* paralleled the seasonal temperature variation by a gradual natural acclimation.

Water content of overwintering larvae of *C. cunea* also showed a seasonal variation. The insects lost water from body gradually before the arrival of low temperature. The changing water content in insects' bodies caused the variation in the concentration of other cryoprotective substances in vivo and increased the supercooling point ability of fluid. Our study showed that there was a positive linear correlation between supercooling point and water content of diapausing *C. cunea*. That is to say with the decreasing of water content, the supercooling point also decreases and vice versa. Interestingly, the conclusion we obtained is just contrary to other studies (Shi 2007). Maybe the high concentration cryoprotectant inside animals leads to the difference. A supercooling point is determined by many factors, such as antifreeze protein, low-molecular carbohydrate, lipid, glycerin, enzymes, and amino acids. Therefore, different species of insects should have different relationships between supercooling point and water content.

The important protective enzymes of defense system in insects are CAT, POD, and SOD. SOD is the first enzyme to deal with oxyradicals and responsible for catalyzing the dismutation of highly superoxide radical O_2^- to O_2 and H_2O_2. It is beneficial for repairing damaged cells. CAT and POD are also the key enzymes in antioxidant defense systems to convert the resulting free radicals H_2O_2 to water and oxygen (Hao et al. 2009). The activities of these three protection enzymes in the diapausing *C. cunea* were lower than those of in the nondiapausing individuals, which suggested that metabolic activities in *C. cunea* vivo were slow during diapause period. This result was consistent with those found in the diapausing *Chrysoperla sinica* adults, oversummering *Pieris melete,* diapausing prepupa of sawfly *Chinolyda flagellicornis* and the larvae of *Sitodiplosis mosellana* from prediapause to early diapause (Xue et al. 1997, Wang and Li 2002, Guo et al. 2006, Cheng et al. 2008). The variation of cryoprotectant in larvae of the *C. suppressalis* was adjusted by the enzyme activity (Li et al. 2002). To date, there are no data on the roles of protective enzymes and other biochemical substances in the diapause of *C. cunea* mature larvae, and the biochemical mechanism of diapause is not clear, which need to be studied in the future.

In conclusion, the physiological characteristics of *C. cunea* diapause and cold tolerance, which studied in our article, can provide a theoretical basis for better utilization in practice of *C. cunea* as a biocontrol agent.The use of the diapause characteristics could prolong the preservation time of this species in breeding process. This species distribution area could be predicted according to the cold hardiness characteristics. All of them will benefit the improvement in biological control capacity of *C. cunea*.

Acknowledgments

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