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Physiological and Endocrine Differences between Diapausing and Non-diapausing Larvae of the Pine Caterpillar *Dendrolimus tabulaeformis* (Lepidoptera: Lasiocampidae)

Rui-Dong Han[1,2], Ya-Ling Gan[1], Xue-Hua Kong[2], and Feng Ge[1,*]

[1]*State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Science, Haidian, Beijing 100080, China*

[2]*Institute of Plant-Protection, Weifang Sci-Tech Vocational College, Shouguang, Shandong Province 262700, China*

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Rui-Dong Han, Ya-Ling Gan, Xue-Hua Kong, and Feng Ge (2008) Physiological and endocrine differences between diapausing and non-diapausing larvae of the pine caterpillar *Dendrolimus tabulaeformis* (Lepidoptera: Lasiocampidae). *Zoological Studies* **47**(1): 96-102. We investigated chemical and morphological differences between diapausing and non-diapausing larvae of the pine caterpillar, *Dendrolimus tabulaeformis* (Lepidoptera: Lasiocampidae). Chemical analyses showed that both the lipid content in the whole body and the concentration of trehalose significantly differed between diapausing and non-diapausing larvae. The lipid, protein, and amino acid contents of the hemolymph of diapausing larvae were higher than those of non-diapausing larvae. The content of free fatty acids of non-diapausing larvae was 4 times higher than that of diapausing larvae. Moreover, diapausing larvae showed lower oxygen consumption than non-diapausing larvae. Differences in the anatomy of incretions according to the physiological condition (diapausing or non-diapausing) were evident, and included the number of cells in the corpora allata, the volume of the nucleus, and the internal structure of the cytoplasm. The prothoracic glands also differed between diapausing and non-diapausing larvae. These results suggest that the pine caterpillars hormonally regulate their body composition to adapt to their surroundings. http://zoolstud.sinica.edu.tw/Journals/47.1/96.pdf

Key words: *Dendrolimus tabulaeformis*, Diapausing, Non-diapausing, Corpora allata, Prothoracic glands.

Diapause is a genetically determined and endocrine-mediated dormancy that occurs at a specific developmental stage, and the expression of diapause is subject to both environmental and genetic factors. Diapause in insects represents a complex dynamic process characterized by several specific physiological and behavioral features (Tauber et al. 1986, Denlinger 1991). Many diapausing insects differ physiologically from their non-diapausing counterparts (Danks 1987). Some biochemical and molecular investigations have been conducted to describe these aspects of diapause induction and development (Li et al. 2002a b).

The pine caterpillar, *Dendrolimus tabulaeformis* Tsai et Liu (Lepidoptera: Lasiocampidae), is a serious pest of the pine, *Pinus tabulaeformis* Carr., in North China and passes through 1 generation in the Beijing area each year (Chen 1990). Pine caterpillars enter diapause as the 3rd/4th instar larvae following preconditioning of the 1st instar larva by exposure to short day lengths (Li and Gia 1989, Gia and Li 1991). Previously, we reported a difference in cold-hardiness between diapausing and non-diapausing larvae (Han et al. 2005), but the mechanism for this remains unclear.

We undertook these studies to investigate the chemical composition of the hemolymph, and the morphology and ultrastructure of the corpora allata and prothoracic glands of diapausing and non-dia-

*To whom correspondence and reprint requests should be addressed. Tel :86-10-64807130. Fax: 86-10-64807099.
E-mail:gef@ioz.ac.cn

pausing larvae of the pine caterpillar to determine what factors may be associated with differences in cold-hardiness between the 2 larval states. This knowledge may allow elucidation of mechanisms to explain this phenomenon in the pine caterpillar.

MATERIALS AND METHODS

Insect collection

Full-grown larvae prior to cocooning were collected from a forest located in the suburbs of Huairou County, Beijing City (40°54'N, 116°37'E). Larvae were allowed to form cocoons, and adults emerged under natural conditions. Eggs were then used in all experiments from those insectary-reared females, and the resulting larvae were fed fresh pine needles of *P. tabulaeformis*.

Conditions for inducing diapausing and non-diapausing larval stages

Larvae were exposed to a temperature of 27°C with a photophase: scotophase cycle of either: 8-L: 16-D (8 h of light: 16 h of dark) to induce diapause, or 16-L: 8-D to maintain diapause-free development (Gia and Li 1991). Because most of the pre-diapause larvae were 4th instars, only newly molted individuals entering the 4th instar stage were used throughout the experiments (Han et al. 2005).

Determination of water and lipid contents

The dry weights were obtained of the 2 larval groups, diapausing and non-diapausing, each with 15 individuals. Larvae were individually weighed on an electronic balance (with a sensitivity of 0.1 mg, Sartorius, R200D.A.G., Göttingen, Germany), kept for 24 h at 65°C in an oven, and re-weighed to obtain the dry weight.

The water content was estimated as the ratio of the difference (fresh weight (FW) - dry weight (DW)) divided by the FW, .i.e., water content = (FW - DW)/Fw x 100% (Guo 1979).

Disassociated larvae were then used for the lipid analysis. Dried insects were pulverized with a mortar and pestle, and approximately 100 mg of the resulting homogenate was extracted with 15 ml of 1: 1 chloroform: methanol as described by Tuskes and Brower (1978).

Determination of fatty acid, trehalose, and free amino acids in hemolymph

Hemolymph collection

Larval hemolymph was collected from *D. tabulaeformis* by puncturing a proleg with a needle, and the exudate was collected in an ice-cold bleeding solution. The hemolymph was immediately centrifuged at 2000 x*g* for 5 min to remove hemocytes.

Chemical analysis

Free fatty acid was determined by a 1-step extraction colorimetric assay (Wei 1979). Trehalose was measured by gas liquid chromatography as described by Goto (1993). Free amino acids were assayed with an amino acid analyzer (Hitachi L-8800, Tokyo, Japan) as described by Goto et al. (1998). The protein concentration was determined by a bicinchoninic acid assay (Pierce, Rockford, IL).

Determination of oxygen consumption

The metabolic rate of *D. tabulaeformis* larvae was manometrically measured as oxygen consumption with a Gilson respirometer (Gilson Medical Electronics, IGRP-14, Middleton. WI, USA). Respiratory vessels of 10 ml volume were used with sodium calcite to absorb carbon dioxide (abs. CO_2) with a small piece of wet cotton to maintain a constant water vapor pressure. The recorded value of oxygen consumption of each individual at rest at 27°C was obtained from an average of three 0.5 h readings (Kalushkov 2001).

Ultrastructure of the corpora allata and prothoracic glands

Diapausing and non-diapausing larvae were dissected to remove the corpora allata and prothoracic glands. These tissues were fixed in 4% glutaraldehyde for 3 h, rinsed 3 times with 0.2 M buffered sodium cacodylate for 30 min, then fixed with 1% osmic acid for 2 h, rinsed 3 times in 0.2 M buffered sodium cacodylate for 30 min, gradually dehydrated in ethanol, and embedded in Epon812. The corpora allata and prothoracic glands of diapausing and non-diapausing larvae were cut into sections with an LKB-V ultramicrotome (Sweden) and examined in a H-300 (Hitachi, Japan) electron microscope.

Statistical analyses

One-way analysis of variance (ANOVA) (SAS 6.12, SAS Institute, Cary, NC) was used to analyze differences in the measured metabolic parameters between diapausing and non-diapausing larvae of *D. tabulaeformis*. The difference between means was compared with Tukey's multi-range test (SAS Institute 1996) at $p < 0.05$. Test data were transformed prior to Tukey's test where appropriate, to satisfy assumptions of normality; that is, the percent data (i.e., lipid and water contents) were arcsine square root-transformed and the other measured metabolic parameters were all log-transformed.

RESULTS

Metabolic parameters

The lipid content of diapausing larvae was higher than that of non-diapausing larvae, while the water content was lower. The trehalose content of diapausing larvae (1.94 ± 0.34 mg/g) was higher than that of non-diapausing larvae (0.36 ± 0.12 mg/g). Protein and amino acids in hemolymph of diapausing larvae were higher than those of non-diapausing larvae. The concentration of free fatty acids of non-diapausing larvae was 4 times higher than that of diapausing larvae. Diapausing larvae showed lower oxygen consumption (respiration intensity) than non-diapausing larvae. There were significant differences ($p < 0.05$) in the above metabolic parameters between diapausing and non-diapausing larvae (Table 1).

The higher level of free fatty acids in non-diapausing larvae is consistent with higher metabolic rates, and the correspondingly higher lipid content and lower metabolic rate in diapausing larvae are consistent with higher energy storage compared to non-diapausing larvae (Table 1).

Anatomical differences in the corpora allata

The corpora allata are a pair of small oval glands consisting of closely interdigitated cells, surrounded by a thin stromal sheath within which are scattered tracheoles and axons. There is an apparent increase in the nuclear volume of corpora allata cells of non-diapausing larvae. The cytoplasm is rich in free ribosomes and dense mitochondria, but has less endocrease. There are also many cisternae consisting of smooth endoplasmic reticula in the cytoplasm. Spaces among the cells are clearly visible (Fig. 1). In diapausing larvae, the corpora allata are small in volume. The cytoplasm surrounding the nucleus contains very few mitochondria, a few irregularly distributed ribosomes, a few compact granules, and some rough endoplasmic reticula and axons. Intermembranous creases are formed among cells (Figs. 1A, B).

Anatomy of the prothoracic glands

The prothoracic glands are located among the tracheae and in the prothoracic spiracles of *D. tabulaeformis*. They are divided into 2 segments: the anterior segment is shorter and thicker, with larger gland cells and an unevenly thick lumen; and the posterior segment is thinner and longer, and the

Table 1. Chemical composition and oxygen consumption by *Dendrolimus tabulaeformis*

	Diapause state	
Content	Diapausing	Non-diapausing
Lipid (%)	13.02 ± 1.33 a	8.08 ± 0.69 b
Trehalose (mg/g)	1.94 ± 0.34 a	0.36 ± 0.12 b
Free fatty acid (μmol/ml)	232 ± 20 b	1101 ± 13 a
Protein (mg/ml)	20.56 ± 1.20 a	14.75 ± 0.85 b
Amino acid (μmol/ml)	4595 ± 45 a	3889 ± 13 b
Water content (%)	79.50 ± 1.34 a(30)b	83.19 ± 0.60 a (45) a
Oxygen consumption (μl/g · min)	11.52 ± 6.37 b	24.17 ± 1.02 a

Different lowercase letters (a, b) for data in a row indicate a significant difference by Tukey's test at $p < 0.05$.

lumen is evenly thin and reaches the abdomen.

In the glands of diapausing larvae of *D. tabulaeformis*, the basement membrane (B in the figures) of gland cells is dense, and there are many invaginated cells in a compact state under the tunica intima (Figs. 2A, B). In the cell plasma, saccules have a wide range of inclusions, but saccules are sparse, mitochondria (M in the figures) are visible, and there are fewer rough endoplasm reticula (ER in the figures) (Fig. 2B). Nuclei (N in the figures) have large segments, and the nuclear membrane border is clear (Figs. 2A, B).

In glands of non-diapausing larvae, the basement membrane of gland cells is loose and the tunica externa is thick (Figs. 2C, D). There are many saccules close to the tunica intima. As the secreted products of gland cells are exported to the lumen of the gland by exocytosis, there are fewer inclusions in the saccules (Fig. 2D). The cell nucleus is spherical. There are numerous mitochondria, a mass of rough endoplasmic reticula, fewer smooth endoplasmic reticula, and many highly electron-dense glycogen particles in the plasma (Fig. 2D). Others have suggested that there may be a protein-transporting molting hormone synthesized by ribosomes of gland cells (Panov 1970, Joplin et al. 1993).

DISCUSSION

Cold-hardiness and diapause are both essential components of winter survival for most insects in the temperate zone, but in many insects, it is not clear how these two are related. Diapausing insects, in general, have stronger tolerances to low temperatures, compared to non-diapausing insects (Tauber et al. 1986, Denlinger 1991). In a previous study, our results suggested that the cold-hardiness of diapausing larvae is greater than that of non-diapausing larvae of pine caterpillars of *D. tabulaeformis*. The SCP and survival rate at the beginning of exposure to low temperatures significantly differed between diapausing and non-diapausing larvae (Han et al. 2005). Šlachta et al. (2002) also reported that the level of chilling tolerance is much lower in non-diapausing (reproducing) individuals of *Pyrrhocoric apterus* than in diapausing individuals. Pullin et al. (1991) suggested that suppression of metabolism during diapause may have a direct role in increased cold-hardiness. High metabolic demands of tissues of non-diapausing insects or those at an early stage of diapause may be hard to meet at low temperatures due to inhibited activities of enzymes. This might partly explain the causes of low-temperature mortality in insects whose metabolic rate is not lowered by diapause.

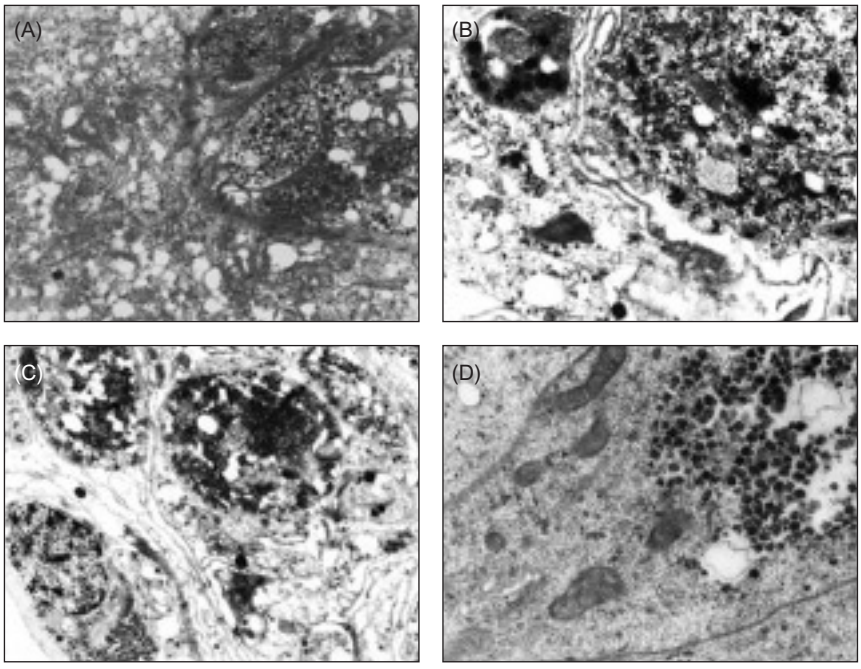

Fig. 1. Corpora allata cells of diapausing (A, B) and non-diapausing larvae (C, D). Scale bar for A and C = 0.6 μm, B and D = 0.3 μm.

In this study, chemical analyses indicated that differences between diapausing and non-diapausing larvae could account for the cold-hardiness of the former. Trehalose, the major insect blood sugar, is utilized by many overwintering insects as a cryoprotectant, and there is a close relationship between cold hardiness and trehalose accumulation in insects (Storey and Storey 1988, Ding et al. 2003). The water content in the whole body significantly differed and was lower in diapausing than in non-diapausing larvae of the pine caterpillar, while the reverse was found regarding trehalose. This result is consistent with trehalose functioning as a cryoprotectant in diapausing pine caterpillars.

Lipids and glycogen are 2 major forms of energy reserves, and patterns of utilization of these energy substrates can differ during diapause (Han et al. 1998). Our results suggest that diapausing larvae have the ability to reserve energy in the form of lipids. This has also been suggested to be the case in other diapausing insects (Li et al. 2002b, Ding et al. 2003), which suggests that some lipids in non-diapausing larvae are hydrolyzed into glycerol and free fatty acids (Kukal 1988). Moreover, protein and amino acid contents are greater in diapausing larval blood than in non-diapausing larvae, which means that diapausing pine caterpillar larvae reserve nutrition to maintain the development of diapause (Denlinger et al. 1991). Furthermore, the respiration rate also differs between diapausing and non-diapausing larvae. Metabolism is reduced in diapausing larvae to less than 1/2 that of non-diapausing larvae (Šlachta et al. 2002).

The morphology and ultrastructure of the corpora allata and prothoracic glands differ in diapausing compared to non-diapausing larvae. In this study, the number of cells in the corpora allata, the volume of the nucleus, and the internal structure of the cytoplasm differed under the 2 different physiological conditions. The corpora allata of non-diapausing larvae are in an active state, while those of diapausing larvae are in an inactive state. The morphology and ultrastructure of the corpora allata are obviously related to their physiological activity. The prothoracic glands also differed between diapausing and non-diapausing larvae. The smooth endoplasmic reticula are where steroids are synthesized in adrenocortical cells of animals. Thus, some have deduced that the molting hormone is synthesized by smooth endoplasmic reticula of the prothoracic gland. However, after years of study, it is now known that smooth endoplasmic reticula are not absolutely predominant in prothoracic cells. Beaulaton (1986) observed that smooth endoplasmic reticula in prothoracic gland cells of Antheraead contained mega-mitochondria. Hence, there is inadequate morphological evidence that the smooth endoplasmic reticula of prothoracic gland are where the molting hormone is synthesized, i.e., the multivesicular body carries out the function of the

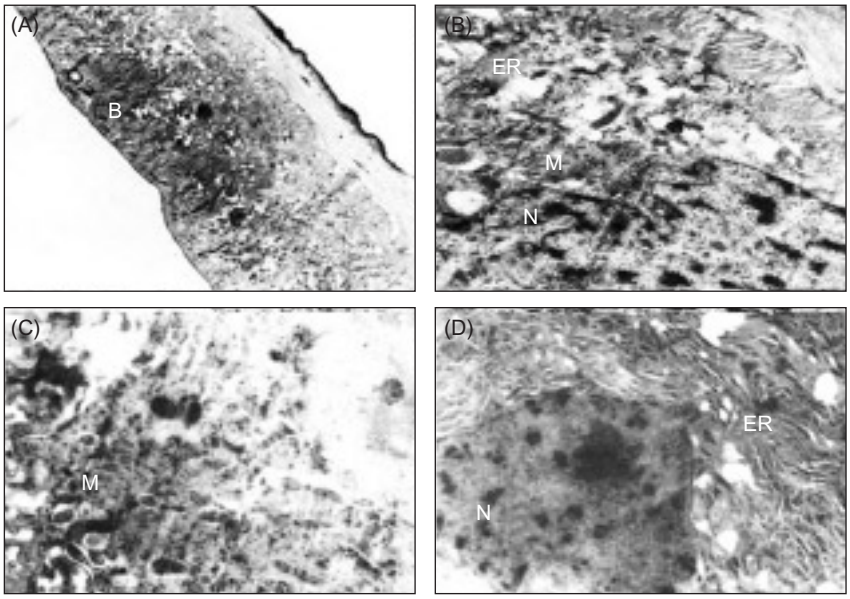

Fig. 2. Corpora prothoracic gland cells of diapausing (A, B) and non-diapausing larvae (C, D). Scale bar in A = 2 μm, B = 0.5 μm, C = 0.3 μm, D = 0.5 μm. B, basement membrane; M, mitochondria; ER, endoplasm reticula; N, nuclei.

ecdysone. There are masses of alveoli in the plasmaof prothoracic gland cells, which can also be observed in the integument of the multivesicular body. Therefore, it can be concluded that alveoli in the plasma of prothoracic glands are at least related to intracellular transportation and secretion out of the cell. According to our observations of the morphology of pine caterpillars, metabolism in the plasmaof diapausing prothoracic gland cells is slow, and the secretion capacity of gland cells which secrete the molting hormone is reduced, while many saccules appeared in non-diapausing larval prothoracic glands, and there is secretion activity of gland cells indicating a large amount of molting hormone is being produced there. This observation is consistent with the results of McDaaniel et al. (1976), but differs from the results of Joplin et al. (1993). There are fewer rough endoplasmic reticula in prothoracic gland cells of diapausing larvae of *D. tabulaeformis*, while in non-diapausing larvae, prothoracic gland cells are extremely rich in rough endoplasmic reticula. We considered that in active prothoracic glands, the appearance of abundant endoplasmic reticula is closely related to the storage and release of secretion material from vesicles. How the small number of smooth endoplasmic reticula relates to differences in cellular morphology regarding regulation of diapause through various physiological and biochemical processes in prothoracic gland cells still needs to be studied further.

In our study, we analyzed differences in chemical components and morphology of glands between diapausing and non-diapausing larvae. We inferred that both contribute to the induction of a diapausing or non-diapausing state and consequently to differences in cold-hardiness.

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