Degeneration of host prothoracic glands caused by *Campoletis chlorideae* polydnavirus*

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Abstract The development of last instar *Helicoverpa armigera* prothoracic glands was investigated first; then the effects on the prothoracic glands of *Helicoverpa armigera* was studied by injection with calyx fluid and polydnavirus (PDV) from the endoparasitoid *Campoletis chlorideae*. Results showed that 3 female equivalents of calyx fluid or 4 female equivalents of PDV induced degeneration of host prothoracic glands. 24 h after calyx fluid or PDV injection the ultrastructure of the gland cells showed a significant decrease in intercellular channel system, while an increase in the number of round mitochondria, lysosomes and whorled figures, the organelle of some cells has degenerated and only organelle debris remained in the cells. These results suggest that calyx fluid or PDV cause the inactivation and degeneration of host prothoracic glands.

Keywords: ***Helicoverpa armigera*, *Campoletis chlorideae*, polydnavirus, calyx fluid, prothoracic glands.**

Some endoparasitic ichneumonids and braconids have a kind of special virus, called polydnavirus (PDV). PDVs are unusual in having multiple covalently closed circular double-stranded DNA[1~3], and they are critical in disrupting host immune system and regulating host development. Because of the special genome and their roles of both pathogen and mutualist, the research on them has always been the top issue in insect physiology and virology. PDVs replicate in the calyx epithelial cells of the lateral oviduct and are released into the lumen of the oviduct where they constitute calyx fluid together with other substance. When a female wasp oviposits into a host, the calyx fluid and venom are also injected into the host. PDVs enter the host and express in host hemolymph and fat body, etc., suppress host immune reaction, regulate host endocrine system, change the physiological status of the host to ensure the successful development of parasitoid. Prothoracic glands (PGs) are one of the most important endocrine organs of insect. Under the stimulation of prothoracicotropic hormone (PTTH) which is produced by the brain[4,5], PGs secrete α-ecdysone, which is converted to β-ecdysone in a number of other tissues[6]. β-ecdysone elicits a diverse array of physiological and biochemical responses so that the insects can grow, develop and culminate ecdysis and metamorphosis. Previous studies show that the PDV of *Campoletis sonorensis* (CsIV) can bring its host (*Heliothis virescens*) a wide variety of effects, including a reduction in growth, feeding, ecdysone titer[7] and degeneration of host PGs[8]. The PDV of *Cardiochiles nigariceps* causes its host *H. virescens* a develomental arrest and prothoracic inactivation without *H. virescens* pupating, but the prothoracic glands remain their structural integrity. At the same time, the ecdysone titer in the hemolymph of *H. virescens* is significantly lower than that of normal larva[9], while the PDVs of *Cotesia congregata* have no effect on the PGs of its host *Manduca sexta*[10]. So, we know that in different systems the effects of PDVs on PGs are different.

Campoletis chlorideae, a hymenopteran, ichneumonid parasitoid, is the predominant natural enemy of cotton pest *Helicoverpa armigera* in North China, mainly parasites low instar larvae. The parasitoid oviposits egg into the body of the host, where the egg develops with its host. Our former research demonstrated that in the oviduct of the female wasp, there are abundant PDVs, termed CcIV. CcIV can significantly inhibit the encapsulation of the haemocytes; CcIV transcripts ranging from 0.6 kb ~ 7 kb can be detected in the haemocytes of the host 1 d and 2 d post parasitization. Among the total 11 tran-

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scripts, the 1.0 kb gene was most abundantly expressed[11]. But whether CcIV affects the PGs of *H. armigera* remains to be determined. Since PGs are very small especially in low instar larvae, and attached to many tissues, it is difficult to get integral PGs, so we used the last instar larvae to study the effects of calyx fluid and PDVs on the PGs of *H. armigera* by means of injection in vitro.

1 Materials and methods

1.1 Insect culture

H. armigera and *Pseudaletia separata* larvae were reared on artificial diets[12,13] at (25 ± 1)℃ and a 15 h light (L):9 h dark (D) photoperiod. Since *C. chlorideae* were easy to rear when *P. separata* were used as host insects, *P. separata* were used for maintaining the colony of *C. chlorideae* in laboratory. For *C. chlorideae* oviposition, the late 2nd or early 3rd instar host larvae were stung by mated female wasps for 1～2 times. The parasitized host larvae were kept under the same temperature and photoperiod. Adult wasps were fed with a 10% honey-water solution.

1.2 The development of normal *H. armigera* PG cells

The last (5th)-stadium *H. armigera* larvae were staged according to Webb and Dahlman[14]. Chronologically, these stages are new (N), slender (S), digging (D), burrow-digging (BD), cell formation (CF) and pharate pupa (PP). At new stage, the head capsule is wider than the thorax and abdomen; at slender stage, the body and head capsule widths are approximately equal; digging stage is characterized by extensive feeding and the larvae dig holes in the diet; the main characteristic of burrow-digging is the cessation of feeding and the presence of a red frass, and the larvae begin wandering; at cell formation stage, the larvae form a cell; and at the stage of pharate pupa, the body length of *H. armigera* becomes shortened and the abdominal segment becomes distinct. Besides these characteristics, some others such as pigmentation, feeding behavior and tactile response were also used as references.

Prothoracic glands were dissected under an Olympus stereomicroscope from the last instar *H. armigera* larvae. The PGs were placed on slides in Pringle's saline[15]. Ten glands from each stage were measured and photographed under a converting microscope.

1.3 Collection of calyx fluid and PDV

The ovaries of female wasps were dissected in Ringer's solution by gently pulling the ovipositor from the abdomen. The calyces were punctured and the contents were diffused into Ringer's solution. This preparation was centrifuged at 3000 r/min for 3 min (Beckman, microfuge™ 11) to precipitate the eggs and tissue debris, and the supernatant was collected. The pellet was washed twice with Ringer's solution by centrifugation. The supernatant was combined and used as calyx fluid.

As for virus purification, the calyx fluid of about 100 ovaries was transferred onto a 25%～65% continuous sucrose gradient, and centrifuged at 48000 r/min for 6 h at 4℃ (Hitachi, P50). The virus band was collected, resuspended in Ringer's solution, and repelled by centrifugation at 48000 r/min for 30 min[1]. The resuspended virus pellet was stored at −70℃.

1.4 The effect of calyx fluid and PDV on the structure of host PG

Calyx fluid or PDVs were injected into each anaesthetised larva from the proleg. Each larva was injected 3 female equivalent of calyx fluid or 4 female equivalent of PDVs, the volume of which was 6 μL and 4 μL respectively. Normal larvae and those injected with the same amount of Ringer's were used as controls. Ten glands were removed from each group at 12 h, 24 h, 36 h, 48 h post injection, and the size of cells was measured and photographed.

The PGs were further observed under a transmission electron microscope (TEM) Hitachi-600. PGs used for TEM observation were removed 24 h after injection and treated by routine methods.

2 Results and analysis

2.1 Size and morphological development of the last instar *H. armigera* PGs

The PG of *H. armigera* is a Y-shaped organ consisting of linear skeins of cells; every gland is composed of about 60 round or ovoid cells. The PG morphology of the last instar larvae does not change much except the increase in volume. Fig. 1(a) shows a normal PG of the last instar *H. armigera* at BD-stage. The mean PG cell size of the last instar *H.*

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armigera is shown in Table 1.

Table 1. Mean PG cell size of each stage of the last instar *H. armigera* larvae

	Larva stage						
	N	S	D1	D2	BD	CF	PP
Cell diameter[a)] (μm)	40.5±0.3a	40.1±0.3a	44.8±0.3b	47.5±0.3c	69.4±0.6d	67.4±0.6e	63.9±0.6f

a) Data in the table are means ± SE ($n = 550$), means followed by the same letter are not significantly different at the 5% level by the LSD test.

The results show the cell size is small at N phase, but it is a bit bigger than S phase. During D1 phase, cell size does not increase much, but from D2 to BD stage, the cell size increases greatly, but from then on the size of the cells decreases slightly.

2.2 Effect of calyx fluid and PDVs on the structure of the last instar *H. armigera* PGs

Comparison of the normal BD phase *H. armigera* PGs with those injected with Ringer's shows the cell size change was not significant, and at the same time, Ringer's injection had no effect on larva development, but after injection with calyx fluid, BD phase larvae stopped development and could not get ecdysis for the remainder of the time. By the time of 12 h after injection, cell size began to reduce (Fig. 1 (b)), at 24 h after injection, the cell size further reduced and began to shrink (Fig. 1 (c)). By the time of 48 h after injection, some cells degenerated (Fig. 1 (d)). Under the light microscope, other tissues such as brain, middle gut, trachea, neuron ganglion, etc. did not indicate any morphological degeneration after calyx fluid injection. Calyx fluid or PDV injection has the same effect on PGs. For BD-staged PGs, the cell size changed after Ringer's or calyx fluid injection (Table 2).

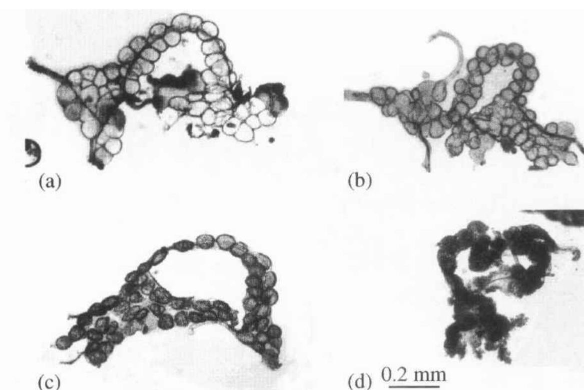

Fig. 1. Prothoracic glands of the last instar *H. armigera* larvae. (a) Prothoracic glands of the normal BD phase *H. armigera* larvae, (b) 12 h after injection with calyx fluid, (c) 24 h after injection with calyx fluid, (d) 48 h after injection with calyx fluid.

Table 2. Effect of calyx fluid on the PG cell size of the last instar *H. armigera* larvae

Hours after injection	Cell diameter[a)] (μm)		
	Uninjected	Ringer's injected	Calyx fluid injected
0	69.4±0.9a	69.2±0.7a	69.6±0.5a
12	67.4±0.8a	66.7±0.6a	63.7±0.5b
24	65.3±0.7a	64.5±0.9a	56.5±0.7b
36	64.0±0.8a	64.1±0.8a	42.9±0.6b

a) Data in the table are means ± SE ($n = 220$), means followed by the same letter are not significantly different at 5% level by the LSD test.

Further observation under the TEM indicated that PG cells were surrounded by a fibrous basal lamina; the peripheral intercellular channel system (ICS) which was formed from the folded invaginations of the plasma membrane was very prominent in the control PG cells (Fig. 2 (a)). In the cytoplasm of the cells there were many elongated mitochondria (Fig. 2 (c)). The irregular nucleus contained polydisperse heterochromatin spread in the cytoplasm, and also there were many transporting sacs, maybe containing ecdysone secreted by the PG cells (Fig. 2 (a)). In contrast, for the controls, the ICS of the gland cells from calyx fluid injected larvae reduced significantly (Fig. 2 (b)), many mitochondria turned round-shaped, and the number of the lysosomes increased and their volume grew larger, seemed containing organelle debris (Fig. 2 (d)); and the whorled figures might contain undigested autophagous vacuoles, increased both in number and volume compared with those of the controls (Fig. 2 (b), (f)). In some cells, the ICS had already disappeared, the organelle had degenerated, and only the granular particles remained (Fig. 2 (e)); no nucleolus was found in calyx fluid or PDVs injected PG cells, and neither PDV was found in the injected cells.

3 Discussion

Under the light microscope, PG cells in BD phase were the largest, the ICS of these cells was most prominent and the width and depth were the biggest. In the cytoplasma there were many transporting sacs. All these indicated that the PG cells are most actively secreting ecdysone at this stage. These

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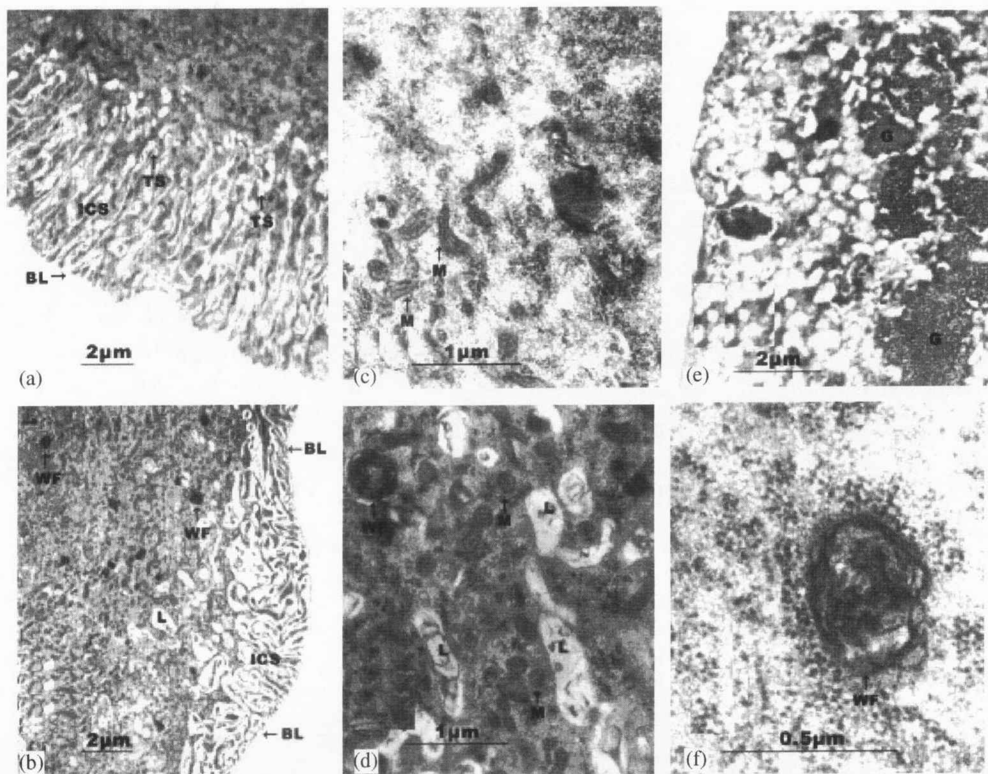

Fig. 2. The ultrastructure of the prothoracic gland cells from the normal and calyx fluid injected last instar *H. armigera* larvae. (a) The prothoracic gland cells from normal larvae. PG cells are surrounded by a fibrous basal lamina (BL), the peripheral intercellular channel system (ICS) formed from the folded invaginations of the plasma membrane, the mitochondria (M) and many transporting sacs (TS) in the cytoplasm. (b) ICS of the prothoracic gland cells from calyx fluid injected larvae. (c) Mitochondria (M) of the prothoracic gland cells from normal larvae. (d) Mitochondria (M) and lysosomes (L) of the prothoracic gland cells from calyx fluid injected larvae. (e) The ICS and granules (G) of degenerated organelle of some prothoracic gland cells from calyx fluid injected larvae. (f) Further enlargement of the whorled figure of the prothoracic gland cells from calyx fluid injected larvae.

findings agree well with that of previously reported[16]. Zhu et al. have revealed that the peak ecdysteroid concentration occurs on the fifth day of pupal life[17]. It is likely that the PG cells in BD phase secrete ecdysone most abundantly, and then the ecdysone is transported and converted to ecdysteriod. All of these processes take time, so the time when the PG cell size became the largest is ahead of the peak time of ecdysteriod concentration.

Some species of parasitoids have well adapted to the metamorphosis of their hosts during the long period of evolution, so they could survive under different host physiological states, such as egg-larva endoparasitoids, while some others could only develop within hosts at certain physiological status, such as larva endoparasitoids. For the latter, they must regulate host endocrine physiology so as to accelerate or reduce host development. In this process, PDV is the main contributor. Many studies have indicated that PDV can modulate host developmental periods in a number of ways, for example, increasing juvenile hormone titer through reducing juvenile hormone esterase activity[18], or reducing ecdysone levels through inhibition of the release of PTTH[9] or by affecting PG directly so as to prolongate larval stage[7,19], while other species could shorten larval stage by reducing juvenile hormone titer[20]. CcIV can cause the degeneration of host PG cells, maybe by which to reduce the ecdysone titer so as to delay the development of the host, and in this way the progenies of the parasitoids could accommodate themselves to the physiological status of their hosts.

Calyx fluid and PDV have the same effects on host PG cells, so it was assumed that the degeneration was virus-induced, since in calyx fluid there are ovarian protein, etc. in addition to PDVs. The expression peak of CcIV (1 ~ 2 days after parasitization)[11] was consistent with the degeneration time of the PGs, which elucidated that it was the PDVs that caused the changes of PGs, though the mechanism is not clear at present.

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The effects of CcIV on the PGs of *H. armigera* resemble those of the CsIV on *H. virescens* PGs[8], such as the disappearing of the ICS, the rounding of the mitochondria, the number increasing of the lysosomes, no PDVs existing in the cells, etc., but the phenomena such as the myelin figures, the vacuolation of the endoplasmic reticulum, etc., which occurred in the CsIV injected host PG cells, were not observed in our system. In addition, we discovered that in the injected *H. armigera* PG cells, the organelle of some cells degenerated, and part of fat body of the larvae turned black. The reason why the fat body turns black may be that CcIV affected the fat body, and then affected the PG cells through fat body. Since fat body is the primary synthesis site of protein, fatty acid and PG stimulation required lipoprotein[21], and also the site of 20-monoxygenase metabolism of ecdysone which turns α-ecdysone to β-ecdysone. Fat body turning black may cause host nutrient deficiencies and endysone titer decrease.

The fact that CcIV affected the structure of the PG cells indicated that it might disrupt a process or the processes that are vital for maintaining the integrity of the cells. No CcIV found in the injected *H. armigera* PG cells indicated that CcIV could not penetrate cell membrane to enter the cells. It is possible that the CcIV was expressed in other *H. armigera* tissues, and then the products affected PG cells. Some existing studies indicated that the genes of *Microplitis demolitor* polydnavirus (MdBV) and CsIV encode cysteine-rich proteins[22,23], and the cysteine-rich proteins and peptides have been separated from animal venoms, such as those of carnivorous snails, scorpions, spiders and snakes. In many of the proteins or peptides, the cysteine residues maintain conformational stability that supports protein functional activities[24,25]. Li et al. found that the CsIV protein targets host haemocytes directly so as to suppress host immune reaction[26]. Strand et al. demonstrated that the mRNA of MdBV was expressed in granular cells and plasmatocytes, the primary classes of haemocytes involved in the host defense reaction[22]. The gene of CcIV also encodes cysteine-rich protein (our unpublished data), but whether its protein has the same function remains to be determined. However Varricchio et al. demonstrated that the PDV of *Cardiochiles nigriceps* was expressed in the PGs of parasitized *Heliothis virescens*[27], and Falabella et al. found a 0.6 kb transcript of *Toxoneuron nigriceps* PDV in parasitized *Heliothis virescens* PGs[28]. No matter in what way did the CcIV exert its role, it changed the physiological status of its host through affecting PG cells, so that *H. armigera* could not get ecdysis; in the mean while it did not affect so many tissues and organs so that the host does not die soon, which ensures the development of parasitoid progeny. It might be the results of coevolution among species.

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