A THORACIC FACTOR STIMULATES REPLICATION OF *MICROPLITIS DEMOLITOR* BRACOVIRUS

by

PENG JIANG

(Under the Direction of Michael Strand)

ABSTRACT

Polydnaviruses (PDVs) are an unusual family of symbiotic DNA viruses, associated with parasitic wasps. The genome of PDVs is integrated in the wasp's genome as a provirus and the virus only replicates in the ovary of female wasps. To date, PDVs have been described from two families of parasitic wasps: Ichneumonidae and Braconidae. PDVs bud through the calyx cell membrane into the oviduct lumen and are injected into host insects during parasitization. Prior studies show that PDVs are integrated into the genomes of every male and female of relevant wasp species. The formation of PDV virions in pupal wasp ovaries coincides with the appearance of melanization patterns in the pupal cuticle. *Microplitis demolitor* bracovirus (MdBV) is symbiotically associated with the braconid *Microplitis demolitor*. In this study, I characterized the timing and regulation of MdBV replication. My results indicated that MdBV replication begins one day after *M. demolitor* pupal ecdysis. A series of ligation experiments indicated that MdBV replication is stimulated by a factor produced in the pupal thorax. Radioimmunoassays further indicated that ecdysteroid titer of *M. demolitor* decreased from 5 ng in day one female pupa to 3 ng in day three female pupa.

INDEX WORDS: Polydnavirus, *Microplitis demolitor* bracovirus, Replication

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Chapter 1. Introduction and Literature Review

1.1 Biology of parasitoid wasps

The number of extant species of insects is estimated at between six and ten million and potentially represents over 90% of the metazoan life forms on Earth (Chapman, 2006). Parasitoids are insects that develop during their immature stages on the surface or inside the body of a host and are free living as adults. There are four insect orders that are particularly renowned for this type of life history. They are the Hymenoptera, Diptera, Coleoptera and Strepsiptera. By far, the majority of parasitoids are in the order Hymenoptera. About 20% of known insect species are parasitic wasps (Godfray *et al.*, 1994). Many parasitic wasps are important biological control agents of numerous agricultural pests (Quicke *et al.*, 1997).

Hymenoptera is divided into two suborders, Symphyta and Apocrita. Within the hymenopteran suborder Apocrita, among the largest and best-known subgroups are the ichneumon wasps (superfamily Ichneumonoidea). There are more than 100,000 parasitic wasp species in the superfamily Ichneumonoidea, which is divided into two families, the Braconidae and Ichneumonidae (Stoltz and Vinson, 1979). About 50,000 species of Braconidae exist around the world, which are grouped into 45 subfamilies and 100 genera. The oldest ichneumonoid fossils are from the early Cretaceous, about 140 million years ago (Rasnitsyn, 1983). For the Braconidae, the oldest fossils are from about 93 million years ago (Basibuyuk *et al.*, 1999). Several parasitoid groups in the Hymenoptera arose about 160 million years ago.

Hymenopteran parasitoids often have unique life cycles. Most braconids are primary parasitoids (both external and internal) of predominantly larval stage Coleoptera, Diptera, and

Lepidoptera. Around one week after oviposition into the host body, the braconid larva will emerge and make a cocoon outside the host's body. For braconids like *Microplitis demolitor*, the wasp larva molts to the pupal stage approximately 24 h after spinning a cocoon. The newly formed pupa is white in color with orange eyes and fully formed legs and antennae. Thereafter, the body darkens to a black color, followed by emergence as an adult.

The reproductive system of female Hymenoptera consists of a pair of ovaries, which are connected to a median oviduct by a pair of lateral oviducts. The ovarioles are a series of long slim egg tubes in which oocytes develop. At the posterior end of the ovariole, the calyx is connected to the lateral oviduct. Once eggs have been developed completely, the mature eggs are moved into the calyx. To help the parasitoid's development, many factors used by wasps, such as Polydnaviruses (PDVs), venom and virus-like particles, are all injected into the hosts along with eggs.

1.2 Polydnaviruses

During the 1960's, George Salt found that parasitic wasp eggs gained protection when they passed through the calyx region of the female wasp's reproductive tract (Salt, 1965). In 1967, Rotheram determined that the blue translucent particles which can protect parasitoid eggs were enveloped with virus-like particles (Rotheram, 1967). In 1975, Vinson determined that these virus-like particles consisted of protein, complex sugars and DNA (Vinson and Scott, 1975). In 1984, these viruses well recognized as new family named the polydnaviridae (Stoltz *et al.*, 1984).

About 30,000 parasitic wasp species in the subfamilies of Braconidae and Ichneumonidae carry Polydnaviruses (PDVs). PDVs are double-stranded DNA viruses with circular genomes. PDVs associated with braconids are named bracoviruses (BVs), and PDVs associated with

ichneumonids are called Ichnoviruses (IVs) (Stoltz, 1993). BVs occur in eight subfamilies (Adeliinae, Cardiochilinae, Cheloninae, Dirrhoponae, Mendesellinae, Khoikhoiinae, Miricinae and Microgastrinae) of braconid wasps, and IVs are found associated with ichneumonid wasps in two subfamilies (Campopleginae and Banchinae) (Webb and Strand, 2005). The genome and life cycle of BVs and IVs are similar, however, BVs and IVs do not come from the same ancestor (Webb, 1998; Whitfield, 2002).

Studies of polydnaviruses over the past years have shown that the genetic information for replication of these viruses is coded by the wasp genome, instead of the DNA that is packaged into virions. There are two hypotheses concerning the origin of polydnaviruses. One possibility is that polydnaviruses evolved from viruses, but are no longer viruses. During the evolution of these virus and parasitoid associations, the wasps may first have vectored insect pathogenic viruses during oviposition. After that, while the parasitoid fed on virions during growth, the viral genes integrated into wasp chromosomes gradually. Wasps thereafter only maintain viral genes in their genome to benefit wasp reproduction (Beckage and Gelman, 2004). Another hypothesis is that PDVs are novel immunosuppressive organelles in parasitic wasps (Federici and Bigot, 2003).

The functions of PDVs mainly affect host development and suppress host immunity. First, PDVs are the main factor affects host development. *Campoletis sonorensis* uses symbiotically associated PDV to target prothoracic glands in host *Heliothis virescens* and finally induce the destruction of these glands (Dover et al., 1988). Another example is that injection of *Cotesia congregata* PDV into *Manduca sexta* larva induces abnormally high levels of JH in hemolymph so that host metamorphosis is suppressed (Beckage and Riddiford, 1982). Besides affecting host development, PDVs also exhibit immunosuppressive activities. It was found that

PDV from *Microplitis demolitor* suppresses the spreading of plasmatocytes which are essential in cellular immunity (Strand and Noda, 1991). Furthermore, injection of *Microplitis demolitor* bracovirus or *Cotesia congregate* bracovirus into *Pseudoplusia includens* or *M. sexta*, respectively, significantly decreased PO activity in the host hemolymph. As many PDV gene families have been identified, the functions of these genes have been studied in depth.

1.3 PDVs integration and replication

Replication of PDVs occurs only in female wasps in a region of the reproductive tract called the calyx. Virions accumulate to high density in the lumen of the calyx, and wasps inject a quantity of this virus into hosts when laying their eggs. Most PDV-carrying wasp species parasitize the egg or larval stage of insects in the order Lepidoptera (moths and butterflies). PDVs do not replicate in the wasp's host but do express genes whose products cause severe physiological alterations. PDVs genomes have two forms: a linear proviral form which is integrated into the parasitic wasp genome and an episomal form which occurs after replication. The episomal form of PDVs is packaged into nucleocapsids. PDVs are only transmitted vertically as an integrated provirus (Webb and Cui, 1998). Afterward, a portion of the proviral genome is packaged into viral particles.

The encapsidated genome of PDVs consists of multiple circular DNA segments that are packaged into nucleocapsids surrounded by a single envelope (BVs) or two envelopes (IVs) (Webb, 1998). The single envelope of BVs appears to be assembled within the nucleus. The inner envelope of IVs seemed to be assembled in the nucleus of calyx cells, which the outer envelope is acquired by budding through the plasma membrane of calyx cell into the oviduct lumen. PDV virions from different wasp species may contain one or several nucleocapsids. The sizes of BVs genomes range from 125 to 600 kbp. BVs virions vary in length and package only a

single genome segment per capsid (Albrecht *et al.*, 1994). *Microplitis demolitor bracovirus* (MdBV) contains 15 segments that range from 3.6 to 34.3kb (Beck and Strand, 2007).

1.4 Ecdysteroids in parasitic wasp development

Insects have endocrine glands, which are tissues that secrete chemical messagers which are transported to other body parts by hemolymph. These endocrine glands include the prothoracic glands which produce ecdysteroids, the corpora cardiac, and the corpora allata, which produce juvenile hormone. The first step in the study of the endocrine control of insect molting was taken in 1922. Stefan Kopec found a factor in the brain of the gypsy moth *Lymantria dispar* that is important for metamorphosis. This factor was defined as prothoracicotropic hormone (PTTH). In 1954, Butenandt and Karlson found another endocrine hormone---ecdysone, which controls insect molting. They purified 25 mg of the hormone from 500 kg of *Bombyx mori* pupae, and it had two forms, α- and β-ecdysone. α-ecdysone is now referred to as ecdysone, and β-ecdysone is named 20-hydroxyecdysone (20-HE) (Karlson, 1996). Ecdysone is the first insect hormone to be structurally identified. Ecdysone is mainly found in the immature insect stages, and was thought to only be produced by prothoracic glands. In target tissues, ecdysone is hydroxylated to the active form 20-HE. Ecdysteroid hormones are one of the most common hormones in insects, crabs and some plants.

The specific hormones are involved in female insect reproduction. In hemimetabolous insect orders, the production of vitellogenin relies on juvenile hormone (JH) (Chawanji *et al.*, 2005). JH are a group of acyclic sesquiterpenoids that regulate development, reproduction, diapause and polyphenisms (Riddiford, 1994). JH was discovered in 1965, and the molecular structure solved in 1967. JH is produced in the corpora allata in the head of insects and dispersed throughout the hemolymph to act on responsive tissues. JH regulates the formation of new

endoplasmic reticulum in the fat body, and as a result, vitellogenin production was affected. In many holometabolous insects, both JH and 20-HE are involved in regulating the rate of vitellogenin production. Control of vitellogenesis by 20-HE is different among different insect species. In the silkworm, *Bombyx mori*, vitellogenin synthesis coincides with a rise of 20-HE (Kawamura *et al.*, 2001). In contrast, in the Indian meal moth, *Plodia interpunctella*, vitellogenin synthesis coincides with a decrease of ecdysteroid titer (Siaussat *et al.*, 2008).

It is already known that prothoracic glands are a main source of ecdysone. However, recent studies show that other tissues in the insect body can also excrete ecdysteroids, such as reproductive tissues, epidermis oenocytes, and embryos (Maeda *et al.*, 2008). In 1992, Webb and Summers reported that 20-HE stimulates replication of CsIV. In vitro culture of *C. sonorensis* ovaries with 20-HE showed that CsIV replication was regulated by the ecdysteroid titer. The active ecdysteroid concentration was between 0.1 μg/ml and 2.0 μg/ml. In 2003, Marti *et al.* measured the ecdysteroid titer of ovaries at different pupal stages of *Chelonus inanitus*, a parasitic wasp belonging to the family Braconidae (Marti *et al.*, 2003). However, the ecdysteroid titer data from Marti's article did not support Webb's hypothesis. Ecdysteroids increased immediately after the pupal ecdysis, especially for 20-HE. From *C. inanitus* pupal stage 3 to 6, when the *Chelonus inanitus* bracovirus viral DNA began to replicate, 20-HE titer had decreased and continued to drop.

1.5 Summary

Many parasitic wasps are important biological control agents of numerous agricultural pests. Parasitic wasps may also provide new biopesticides, because the mechanisms which the parasitoid manipulate their host's physiology and biochemistry are well known. Biology and development vary with different endoparasitic wasp species. Most parasitic species have high

reproductive capacity and develop rapidly. First, adult female wasp lays her egg inside the host and it hatches. Second, wasp larva continues to develop and then makes cocoon outside the host body. Finally, after ecdysis and pupa development in the cocoon, adult wasp emerges into the environment to continue the life cycle. Usually, a wasp larva hatches from an egg and develops through several instars before forming a pupa. However, some parasitic wasps undergo a process called polyembryony during development, such as *Copidosoma spp*. (Chalcidoidea, Encyrtidae). When an egg is inserted into a host, it can divides and gives rise to hundreds of larvae.

During oviposition, PDVs are injected into the host body along with the eggs. Two families of parasitic wasps contain this kind of virus. Ichnoviruses associated with ichneumonids, while bracoviruses associated with braconids. Ichnoviruses reported that evolved from ascoviruses, based on phylogenetic analyses of major capsid and 4L proteins of ascoviruses and related proteins in *Campoletis sonorensis* virions (Webb and Cui, 1998). On the other hand, bracoviruses may be derived from an ancestral nudivirus. Studies showed that complementary DNAs derived from braconid wasp ovary genes encode subunits of a viral RNA polymerase. The structural components of bracoviruses particles are similar to nudiviruses (Bezier *et al.*, 2009).

Although the evolution of BVs and IVs are different, the life cycle of BVs and IVs are the same. The replication of BVs occurs only in the calyx region of the ovary of female parasitic wasps and begins in the early pupal stage. Nucleocapsids are assembled in calyx cell nuclei and acquire envelopes later (Pasquier-Barre *et al.*, 2002). IVs acquire a second membrane because they bud from calyx cell, while BVs are single enveloped and released through cell lysis (Belle *et al.*, 2002). Virions accumulate to high densities in the female oviducts and transferred to the parasitoid's hosts with eggs during oviposition. Then the viruses infect host tissues, and the expression of viral gene could be detected several hours later after parasitization.

PDV genomes consist of multiple circular double-stranded DNA segments and lipid envelopes. The lipid envelope of the capsid is obtained in the nuclei of calyx cells. Ichnovirus genomes usually have more than 20 DNA segments. The *Campoletis sonorensis* ichnovirus (CsIV) genome is composed of 22 segments. These 22 segments encode about 248 kb of unique sequence. BV genomes have fewer but larger genome segments than IVs. BV virions vary in length, and the genome ranges from 125 to 250 kbp. The *Glyptapanteles indiensis* BV has 13 segments which from 11 to 30 kbp (Chen *et al.*, 2003). The *Cotesia congregate* BV has 20 segments ranging from 10 to 20 kbp (Dibhajj *et al.*, 1993).

Past research has focused on the function of polydnavirus, but the replication mechanism is seldom studied. The main goal of this dissertation is to find out if there is a signal factor responsible for the initiation of BV replication in the braconid wasp, *Microplitis demolitor*. Firstly in Chapter 2, I defined four *M. demolitor* pupae developmental stages. MdBV amounts were quantified through quantitative real time PCR at each pupal stage. The initiation time point of MdBV replication was identified. In Chapter 3, we did a series of ligation experiments to testify whether MdBV replication is stimulated by an exogenous factor and determined at which pupal stage the factor is sent to the ovary. After we made sure that this factor exists in the thorax of *M. demolitor* pupae, extract of the thorax was made to purify the factor. In 1992, Webb and Summers reported that polydnavirus replication in *Campoletis sonorensis* was stimulated by 20-HE (Webb and Summers, 1992), however, they did not measure the ecdysteroid titer. To determine whether 20-HE correlated with MdBV replication, ecdysteroid titers were measured on different *M. demolitor* pupal stages by radioimmunoassay for chapter 4. In general, my specific objectives in this research are:

- Quantify of MdBV amounts during pupal development.

- Determine when MdBV replication begins.
- Assess whether MdBV replication is stimulated by an exogenous signal.
- Determine at which *M. demolitor* pupal stage the signal is sent to the ovaries.
- Identify which tissue or organ releases this exogenous signal.
- Identify this signal.

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Chapter 2. Quantify MdBV amounts in different *M. demolitor* pupal stages

2.1 Introduction

As noted in Chapter 1, thousands of wasp species in the Braconidae and Ichneumonidae carry PDVs. Previous studies indicate that PDV replication begins during the pupal stage in both braconids and ichneumonids (Norton and Vinson, 1983; Marti *et al.*, 2003). However, the prior timing of replication can vary among species. Therefore, the first goal of my study of MdBV replication is to determine precisely when virion formation begins during the pupal stage of female *M. demolitor*. Within the cocoons, the larval appearance persists for approximately 24 h, and the eyes become progressively orange. During molting, the larval skin is eliminated; the pupa is uncolored except for the eyes and ocelli. At this stage, the 'white pupae' has a fully formed body with legs and antennae. Afterward, the most prominent developmental feature is stripes of melanized of the cuticle on the thorax and abdomen. These stripes of melanization could be a good marker for precise characterization of the pupal developmental stages.

Prior studies show that development of the ovary is strictly correlated with the pigmentation pattern of the pupa (Albrecht *et al.*, 1994). Wyler and Lanzrein recorded the virus replication processes in *Chelonus inanitus*. They found that calyx cells begin to differentiate shortly after pupation, and the extent of non-viral DNA and integrated proviral DNA amplified correspondingly. After this, calyx cells increase in size, and the nuclei become large. From *C. inanitus* pupal stage 1 to stage 2, the ovary develops from a small compact structure into a complex organ (Wyler and Lanzrein, 2003). In *C. inanitus* pupal stage 2, the oviduct and calyx region are covered by an ovarial shell. The calyx is separated from the oviduct by a thin

epithelial layer. Elongated cells were seen on the edge of the calyx region. In *C. inanitus* pupal stage 3, cells in the calyx show large nuclei. Some nuclei at the lower part of the calyx become extremely large. DNA content in the calyx increased, and empty envelopes could be identified. Between pupal stage 4-6, the number of cells with large nuclei increased to occupy the majority of the calyx region. Oocytes began to appear in the calyx, which indicated the presence of calyx fluid (Marti *et al.*, 2003a). After the mature virions pack the nucleus, the nuclear membrane and the plasma membrane lyse one after another. In the ovary of 1-day-old adult female, the lower outer part of the calyx is filled with virions. The latest stages of calyx cells are located close to the oviduct lumen, while younger stage calyx cells move to the ovaries.

The initiation of BV replication begins in the early pupal stage (Pasquier-Barre *et al.*, 2002). Release of bracovirus virions from the calyx cell nuclei into the oviduct has several steps. First, virions and cytoplasmic organelles are mixed together. Second, electron microscope studies showed calyx cells with disintegrated nuclear cell membranes. Third, this disintegrated calyx cell is surrounded by phagocytic epithelial cells (Wyler and Lanzrein, 2003). One possibility could be that the debris from disintegrated calyx cell was removed, so the calyx fluid only consists of virions.

To analyse the timing of PDVs replication by a molecular method, Pasquier-Barre *et al.* did a quantitative PCR experiment. The PCR product of EP1 sequence of CcBV was obtained by extracting DNA from wasp ovaries. The excision of EP1 sequence leaving an empty locus on the wasp chromosome is measured. The results showed that the onset of EP1 replication occurs during the *C. congregate* day 4 pupae. The amount of PCR product increased dramatically from day 4 to day 5 pupae and remained high level after pupal day 5. This result may indicate that the CcBV replication begins from pupal day 4. However, traditional quantitative PCR is not as

accurate as quantitative real time PCR which could detect the DNA copy number after each PCR cycle. In 2003, replication of non-viral and viral DNA in the course of pupal-adult development was measured by quantitative real time PCR. This quantify measurement was carried out with primers specific for integrated and excised CiBV viral segment CiBV12. In this aim we propose to measure MdBV virion amounts using a similar approach (Marti *et al.*, 2003b; Savary *et al.*, 1999).

The braconid wasp *Microplitis demolitor* carries *Microplitis demolitor* bracovirus (MdBV) and parasitizes the larval stage of *Chrysodeixis includens*, *Trichoplusia ni*, and other moth species in the family Noctuidae. The MdBV genome consists of 15 DNA segments which range from 3,611bp (segment A) to 34,334 bp (segment O). The majority of genes encoded by MdBV consisted by the ptp, vankyrin, tRNA, egf-motif and glc gene families (Rivkin *et al.*, 2006). Beck *et al.* assessed the relative amount of these segments by quantitative real time PCR. Calyx fluid from two day old female wasps was analyzed. Results showed that segment C was the least abundant MdBV DNA, and used as a standard value of 1. Segment J (13.7kb), O (34.3kb), H (11.2kb), N (17.4kb) and B (6.3kb) are 13.3-, 8.8-, 7.3-, 5.4- and 4.9-fold than segment C. The rest of segments are from 1.6- to 4.1-fold than segment C. After converting these values to percentages, segment J (20.3%), O (13.5%), H (11.1%), N (8.2%) and B (7.4%) accounted for about 60% of the viral DNAs. Picogreen quantitation indicated that 98.7±8.0 (SD) ng of MdBV DNA. If standardize one equivalent of calyx fluid to equal 100 ng of viral DNA, there are 1.73×10^{8} copies of segment C, 7.30×10^{8} copies of segment D, 8.64×10^{8} copies of segment B and 25.25×10^{8} copies of segment J. While segment D has a median copy numbers among these 15 segments.

In this chapter, we sorted the *M. demolitor* pupal developmental stages according to melanization patterns on their body and described the morphological changes on different pupal stages. The beginning of the pupal stage was defined as the time just after pupal ecdysis. *M. demolitor* female pupae ovaries of 0 h, 12 h, 24 h, 36 h, 48 h, 60 h, 72 h and adults were dissected, and the MdBV virion amount on each pupae stages were measured.

2.2 Materials and Methods

Insects. *Microplitis demolitor* (Hymenoptera, Braconidae) is a larval endoparasitoid of *Chrysodeixis includens* (Lepidoptera, Noctuidae) larvae. Adult female wasps have a pair of short antennae compared to adult male wasps. *M. demolitor* was reared by parasitizing four instar *C. includes*. Wasp larvae develop during 1 week, then they emerge and spin cocoons on the back of the caterpillars. Males usually emerge early than females.

C. includens larvae were fed on artificial diet. *M. demolitor* and *C. includens* were reared at 27 ˚C under a 16 hour light: 8 hour dark photoperiod as previously described (Strand *et al.*, 1997).

Determination of *M. demolitor* pupal stages. The silk of cocoons was cut and the *M. demolitor* pupae were carefully removed. Pupae were sorted according to their melanization pattern. The different stages were defined from day 0 to day 4 after pupal ecdysis (day 0) as shown in Fig. 1. Female pupae were identified by the body color, pattern of melanization stripes on the body or presence of developing ovaries.

MdBV collection. Calyx fluid was collected from different pupal stages of *M. demolitor*. by established methods (Beck et al., 2007). In brief, reproductive tracts were dissected from *M. demolitor* pupae in phosphate buffered saline. MdBV virions were collected by centrifugation of

the calyx fluid at 4˚C for 20 minutes at 16,000 × g and resuspended in phosphate buffered saline. The MdBV purified from one wasp was referred to as one wasp equivalent.

MdBV viral genomic DNA isolation. MdBV was resuspended in 100 µl of reaction buffer (0.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, 10 mM Tris–HCl pH 7.5) containing 2 µl DNase I (Ambion) and incubated 30 minutes at room temperature to eliminate any non-viral DNA. The virions were then collected by recentrifugation and washed with Pringle's saline (154 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$ and 22 mM D-glucose). To extract the genomic DNA from MdBV, purified viral particles were resuspended in PBS with 2 % sarcosyl and 0.5 mg/ml Proteinase K at 62°C for 1 hour. The MdBV genomic DNA was purified by phenol:chloroform extraction and isopropanol precipitation (Beck et al., 2007).

Relative quantitative real-time PCR. Total DNA was extracted from the male and female *M. demolitor* pupal reproductive organs. Relative quantitative real time PCR (rqRT-PCR) was conducted using Elongation Factor 1 alpha (EF1α) gene (GenBank: DQ538655.1) to represent *M. demolitor* genomic DNA; using Segment D to represent MdBV DNA. Specific primers are listed below:

EF1α gene forward primer: 5'-ATTGAAGGCCGAGCGTGAAC-3',

EF1α gene reverse primer: 5'-CCGAGGGTGAAAGCAAGGAG-3';

Segment D forward primer: 5'-TGGTGGATTATTGAACGAGATGTA-3',

Segment D reverse primer: 5'-GAAAAGATTGGGAAGATTGGATAG-3'.

In a 10 µl PCR reaction, there was 1 µl cDNA, 500 nM primers and 5 µl iQ SYBR Green Supermix (Bio-rad). Reactions were run on a Rotor Gene 2000 Real-Time PCR Cycler (Corbett Research) for 45 cycles with denaturation at 94°C 20 seconds, annealin g at 55°C for 20 seconds

and extension at 72℃ for 20 seconds. Data were analyzed by the ΔΔCt method. The transcriptional level of male pupae of *M. demolitor* was standardized as 1.

2.3 Results

Morphological changes in the ovary and calyx region in *M. demolitor* pupal-adult development.

As noted in the Introduction, the appearance of MdBV virions in the ovaries of *M. demolitor* pupae coincides with the melanization patterns on the new adult cuticle. As the pupae developes, the melanization patterns become predominant and deeper, and the MdBV viron amount increases as well. To determine the time point when MdBV replication begins, the silk of the cocoons was cut using ophthalmologic scissors and pupae were removed from cocoons. Pupae were sorted by their body melanization patterns. Photographs of pupae at different developmental stages are showed in Figure 2.1.

The course of *M. demolitor* pupal-adult development is presented in Figure 2.1. At stage 1, the head, thorax and abdomen of *M. demolitor* pupae are white except the compound eyes which are light red. At stage 2, the head and abdomen of wasp pupae are still white. Slight black vertical stripes are observed on the dorsal side of thorax. The color of the compound eyes become deep red and ocelli turn red. At stage 3, the head and thorax turn to dark black. Slight yellow transverse stripe patterns are shown on the abdomen The compound eyes and ocelli are black. At stage 4, the whole *M. demolitor* body turns black, and the color of forelegs, midlegs and hindlegs are dark yellow. The color of 1-3 abdominal segments are dark yellow. After stage 4, *M. demolitor* pupae will become adults.

The course of the morphological changes occurring in the ovary and calyx region is also recorded and presented in Fig. 2.1. At stage 1, the ovary is a small, compact organ. Two separate

ovarioles can be seen clearly. The diameter of a stage 1 ovary is about 220 μm. At stage 2, the ovaries were the same shape, but the ovary size become large. The diameter of a stage 2 ovary is about 290 μm. At stage 3, the bluish MdBV virions in the calyx region of the ovary can be seen under microscope with 4 × magnification. The diameter of the ovary is about 360 μm. At stage 4, mature oocytes are visible in the oviduct, some passing through the calyx region. The diameter of stage 4 ovary is about 410 μm. After stage 4, the ovary becomes mature with a diameter about 450 μm.

Based on our observations of pupal ovaries at 6 h intervals over a 72 h period, we estimated that MdBV replication began between 24 h and 36 h after pupal ecdysis at 27˚C, which coincides with the color of compound eyes turning deep red. The appearance of MdBV virions in ovaries of wasp pupae coincides with the appearance of melanization patterns on the new adult cuticle during stage 2.

Quantify MdBV amounts on different *M. demolitor* pupal developmental stages.

For this objective, we used relative quantitative real time polymerase chain reaction (rqRT-PCR) to compare different virion amounts at different pupal developmental stages. In this assay, we chose MdBV segment D as an indicator of the MdBV abundance. The reason we chose Segment D to represent MdBV is because segment D has a medium abundance among the 15 MdBV segments (Beck and Strand, 2003), When we did rqRT-PCR, segment D can represent MdBV virion amounts precisely. On the other hand, we chose three genes to represent *M. demolitor* genomic DNA: *Microplitis demolitor* elongation factor 1 alpha (EF1α) gene (GenBank: DQ538655.1), *Microplitis demolitor* wingless (wg) gene (GenBank: DQ538598.1) and *Microplitis demolitor* partial long-wavelength rhodopsin (LW-Rh) gene (GenBank: AJ536001.1). After rqRT-PCR examination, elongation factor 1 alpha (EF1α) represented *M.*

demolitor genomic DNA better than other two genes, so EF1α was used as an *M. demolitor* genomic DNA control.

We measured replication of MdBV in the course of pupal-adult development (Fig. 2.2). The lower part of the ovary containing only the calyx region and oviducts was removed for *M. demolitor* genomic DNA and MdBV viral DNA isolation. RqRT-PCR results showed that on stage 1, as the calyx region is not yet separated from the reproductive part of the ovary, there is no MdBV replication in the ovary. On stage 2, 24 h after the pupal ecdysis, the MdBV began replication. We saw the calyx region is separated from reproductive part, while the viscous sticky bluish particles cannot be watched under 4 times magnification microscope. 36 h after pupation, the MdBV amount increased to 100 relative virion abundance, viscous sticky bluish particles can be watched under 4 times magnification microscope. In 48 h ovary of *M. demolitor* pupae, MdBV virions raised stable, reaching about 200 relative virion abundance. From 48 h to 60 h, the virion amount increased to 500 relative virion abundance sharply. After 72 h until adult, the MdBV virion amount keep stable, excised circular viral segments are not further amplified.

Determine the specific startup time point of MdBV replication.

The initiation of MdBV viral DNA replication coincided with the pupal morphological changes. We observed that 36 h after pupal ecdysis, viscous sticky bluish particles can be observed in the calyx region of *M. demolitor* ovary. According to the results of rqRT-PCR, we can conclude that the initiation of MdBV viral DNA replication occurred between 12 h and 24 h after pupal ecdysis.

2.4 Discussion

Under our raring conditions, adult female of *M. demolitor* live for one month and lay eggs until they die. It was found that the calyx fluid collected from one *C. inanitus* female

contained 150- 250 ng viral DNA at adult stage and approximatedly 0.1 ng viral DNA is injected along with each egg (Marti *et al.*, 2003b). For *M. demolitor*, the amount of virus present in the ovary of adult female would be sufficient for ovipositions among the whole life of a female. Southern blotting showed that 0.005 equivalent to 0.02 equivalent MdBV is injected along with each eggs (Strand *et al.*, 1992).

In the ichneumonid *Campoletis sonorensis* and the braconid *Chelonus inanitus* (a species belonging to a different braconid subfamily), replication of PDVs have been shown during the late pupal adult development (Albrecht *et al.*, 1994; Webb and Summers, 1992). Although the association between wasps and viruses is proved independently among the braconid and ichneumonid, these studies showed that replication of PDVs between the two polydnavirus genera follows a similar pattern. Furthermore, it was reported that the all bracoviruses derived from the same virus which associated by an ancestor braconid wasp, so the common replication pattern among bracovirus might be the same.

From the beginning of stage 2, proviral segment D showed more amplification than EF1α. After stage 2, the entire proviral cluster is specifically amplified. During adult stage, the episomal form of viral DNA in the female ovary maintains a certain amount. In baculoviruses, palindromic structures have been reported to the origins of replication (Pearson *et al.*, 1992; Ahrens *et al.*, 1995; Kool *et al.*, 1995). In CiBV, several inverted repeats have been demonstrated on all viral segments (Wyder *et al.*, 2002). Furthermore, it is conceivable that some of the palindromic structures might induce the origins of replication. A large palindromic structure was found in the EP1 circle of the CcBV (Savary *et al.*, 1997).

Another way of viral replication is the rolling circle mechanism. In this process, a large circular viral molecule should be exist which containing an origin of replication. For example,

for CcBV, it was showed that at least two viral segments and downstream sequences is contained in a large molecule, and this large molecule is amplified before the excision of individual segments (Pasquier-Barre *et al.*, 2002).

2.5 References

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Figure 2.1. *Microplitis demolitor* pupal-adult development. Photographs of the different pupal stages used in the analysis are represented. The pupal stages are defined according to their body melanization. At stage 1, the head, thorax and abdomen of *M. demolitor* pupae are white except the compound eyes which are light red. At stage 2, the head and abdomen of wasp pupae are still white, slight black vertical stripes are observed on the dorsal side of thorax, the color of the compound eyes and ocelli are deep red. At stage 3, the head and thorax turn to dark black, slight yellow transverse stripe patterns are shown on the abdomen, the compound eyes and ocelli are black. At stage 4, the whole body is black. The color of 1 to 3 abdominal segments are dark yellow. The course of the morphological changes occurring in the ovary and calyx region is also presented.

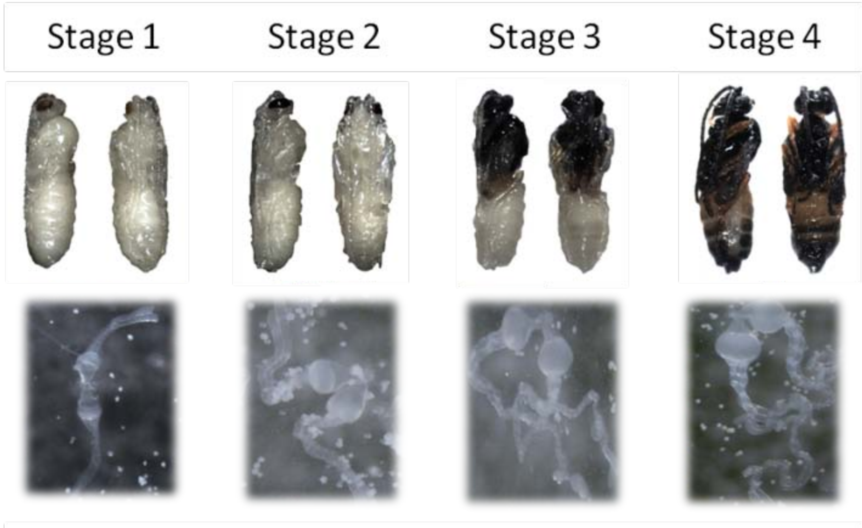
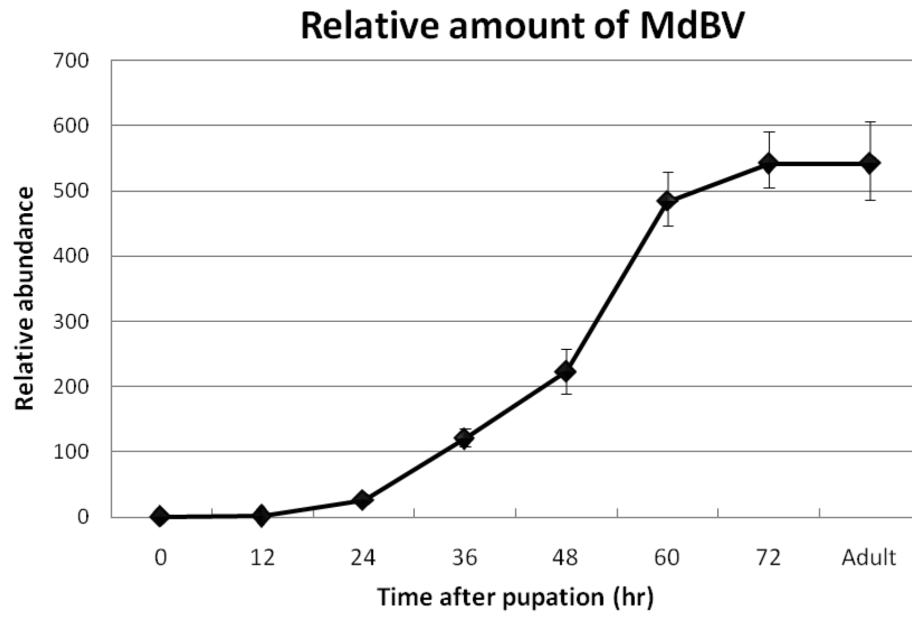

Stage 1
Stage 2
Stage 3
Stage 4

Figure 2.2. Relative abundance of *Microplitis demolitor* bracovirus on different pupal stages. Virion amounts on different pupal developmental stage are measured by rqRT-PCR. MdBV segment D is an indicator of the MdBV DNA segments; elongation factor 1 alpha (EF1α) represents *M. demolitor* genomic DNA. Data were analyzed by the ΔΔCt method.


Relative amount of MdBV
Relative abundance
700
600
500
400
300
200
100
0
0
12
24
36
48
60
72
Adult
Time after pupation (hr)

Chapter 3. Ecdysteroids in the course of *Micropitis demolitor* pupal–adult development

3.1 Introduction

Ecdysteroids are insect molting and sex hormones that consist primarily of ecdysone and 20-hydroxyecdysone (20-HE) (Karlson, 1996). Ecdysone is the first insect hormone to be structurally identified, while 20-HE is the hydroxylated form of ecdysone and is the true molting hormone in inducing a molt. In most insects, 20-HE is involved in the female reproduction and the variations in the vitellogenesis. Prothoracic glands are the main but not the only source of ecdysteroids (Chino *et al.*, 1974). Even though the prothoracic glands degenerate in adult insects, ecdysteroids are still presenting in their hemolymph. It was reported that ecdysteroids can also be produced by the larval and pupal sheaths of the male testes (Delbecque *et al.*, 1990). In many female insects, ecdysteroids can also be produced by the follicle cells in the ovaries (Maeda *et al.*, 2008). Ecdysteroids from ovaries may be released into the hemolymph and activate the synthesis of yolk proteins. Furthermore, ecdysone plays an important role in mosquito's oogenesis (Uchida *et al.*, 1998). After being released from the mosquito ovary, ecdysone then converted to 20-HE. Prior studies showed that large doses of 20-HE (5-10 μg) could activate ovaries of unfed female mosquito (Hagedorn *et al.*, 1975; Spielman *et al.*, 1971; Uchida *et al.*, 1998).

In 1992, Webb and Summers reported that 20-HE stimulates polydnavirus replication (Webb and Summers, 1992). They reported that the initiation of *Campoletis sonorensis* ichnovirus (CsIV) replication can be blocked by blocking endogenous hormone release. Moreover, in vitro culture *C. sonorensis* ovaries with 20-HE showed that CsIV replication was

up regulated by the ecdysteroid titer. The ecdysteroid concentration required to stimulate replication was between 0.1 μg/ml and 2.0 μg/ml. From these results, the author reached four conclusions: 1, CsIV replication could be prevented by ligation. 2, CsIV replication is regulated by a head or thorax signal. 3, 20-HE induces replication. 4, the 20-HE responsiveness of the ovary might be regulated.

In this chapter, I used in vivo injection and in vitro culture *M. demolitor* pupal ovary with 20-HE to test whether this hormone could stimulate replication of MdBV. Furthermore, ecdysteroid titers were measured on different *M. demolitor* pupal developmental stages by radioimmunoassay.

3.2 Materials and Methods

Insects. *M. demolitor* was reared and staged as described in Chapter 2. *C. includes* was also reared as described in Chapter 2.

In vivo injection. For this study, 0 h and 12 h *M. demolitor* female pupae (prior to the initiation of viral DNA replication) were ligated between thorax and abdomen. 12 h after these ligation time points, 0.2 μl 1× Phosphate buffered saline (PBS), 50ng/μl, 5ng/μl and 0.5ng/μl 20-HE was injected into the ligated pupal abdomen. Ligated pupal abdomen injected with Phosphate buffered saline alone were referred to as mock-infected control.

In vitro ovary culture. *M. demolitor* ovaries were isolated from 0 h to 12 h female pupae (prior to the initiation of viral DNA replication) and cultured individually in vitro with 50ng/μl, 5ng/μl and 0.5ng/μl 20-HE in sealed 96 well microtiter plate. Culture conditions are TC100 insect medium plus 10% Fetal Bovine Serum (FBS) at 27˚C.

Radioimmunoassay. Modified procedures for in vitro tissue ecdysteroid production and the ecdysteroid radioimmunoassay (RIA) were followed (Riehle and Brown, 1999). 0 h, 24 h, 48 h,

72 h and 96 h *M. demolitor* female pupae were homogenized in 500 μl methanol. As controls, male pupae on the same time point were prepared similarly. For each experiment, triplicates of two pupal bodies were placed in 0.65ml tube. The anti-ecdysteroid rabbit serum (AS 4919, a gift from P. Poncheron, Université P. et M. Curie, Paris, France) recognizes ecdysone and 20-hydroxyecdysone equally (Porcheron *et al.*, 1989), as verified with our RIA. For RIA, each tube contained 50 μl of a stock [23,24-^{3}H(N)]ecdysone solution (=[^{3}H]ecdysone; 12,000–13,000 counts/minute (cpm)/50 μl; Perkin-Elmer, Boston, MA), 50 μl of antiserum diluted to 1:35,000–45,000 (final dilution for bound to free [^{3}H]ecdysone cpm ratio (Bound/Free=1), and 50 μl of sample or 20-hydroxyecdysone standard. Triplicate tubes were set up for each of the 20-hydroxyecdysone standards (1, 5, 10, 25, 50, 100, 250, 500, and 1000 pg). After overnight incubation at 4 °C, bound and free radiolabeled ecdysone were separated by the ammonium sulfate method, and pellets in tubes were dispersed in water and scintillation fluid and counted in a scintillation counter (Beckman). For each RIA, a standard curve was plotted from the averaged Bound/Free (Y-axis) and log values for the 20-hydroxyecdysone standards (X-axis). The quantity of immunoreactive ecdysteroids in samples was calculated from a regression equation for the linear portion (10–250 pg) of the standard curve; samples were diluted when necessary to stay within this range. Sample values reported for each tissue treatment are presented as "ecdysteroid ng", because the secreted ecdysteroid species are unknown, and the values are means of triplicate treatments from three experiments (N=9).

3.3 Results

In vivo injection and in vitro ovary culture with 20-hydroxyecdysone.

I did a series of in vivo 20-HE injection experiments and in vitro ovary culture with 20-HE experiments to test whether 20-HE could induce replication of MdBV. For the in vivo

injection experiments, 0 h, 12 h, 24 h, 36 h, 48 h old *M. demolitor* pupae were ligated between the thorax and abdomen, so the ovaries were isolated from the source of ecdysteroids. Twelve h after ligation, 2 μl of three different concentrations of 20-HE (50ng/μl; 5ng/μl; 0.5ng/μl) were injected into different time point ligated abdomens. Until 96h after pupal ecdysis, however, the ligated abdomens with 20-HE injection showed no evidence of maturation.

I also cultured *M. demolitor* ovaries with 20-HE in vitro. 12 h old *M. demolitor* pupal ovaries were dissected and cultured in TC100 insect medium plus 10% Fetal Bovine Serum (FBS) in 96 well microtiter plates. Twelve h after dissection, three different concentrations of 20-HE (50ng/μl; 5ng/μl; 0.5ng/μl) were added into culture wells. Again, there are no virions were observed in the ovaries cultured with different 20-HE concentrations.

Ecdysteroid titers in the course of *M. demolitor* pupal-adult development.

In vitro application of 20-HE has been proposed that this hormone regulates CsIV replication (Webb and Summers, 1992). However, no ecdysteroid measurements have been made to support this hypothesis. To get an overview of the ecdysteroid changes in the course of *M. demolitor* pupal–adult development, ecdysteroids of male and female pupae in 100% methanol fractions were analysed. Results showed that ecdysteroid titers in female *M. demolitor* were higher than in males. From 0 h female pupae to stage 2 female pupae, the ecdysteroid titer is maintained at 4.6 ng per pupa. After stage 3 until stage 4, the ecdysteroid titer drops to 2.8 ng per pupa. For *M. demolitor* male pupae, after pupal ecdysis, the ecdysteroid titers increased rapidly after the pupal molting, reached a peak at stage 1 and then dropped continuously (Fig. 3.1).

I also cut the female abdomen on different pupal developmental stages, and the ecdysteroid titers were measured. Results showed that the ecdysteroid titer of female abdomens had the same amount as the female whole-body. To assess whether ligation affected the

ecdysteroids of *M. demolitor* female pupae, 0 h, stage 1, stage 2 and stage 3 *M. demolitor* female pupae were ligated between the thorax and abdomen. 12 h after each ligation time point, ligated abdomen was cut to measure the ecdysteroid titer. Radioimmunoassay results showed that the ecdysteroid titers of ligated abdomen were higher than immediately cut abdomen on each time point. This may indicate that the ovary of *M. demolitor* pupae could generate ecdysteroids in the absence of the prothoracic gland. Overall, the ecdysteroid titers of 0 h, stage 1 and stage 2 ligated abdomens were higher than stage 3 ligated abdomen (Fig. 3.2).

3.4 Discussion

Until now, the precise mechanism for the control of viral replication is unknown. Webb and Summers thought that the hormonal regulation of a viral 'replicase' which would control the excision or copy of the integrated virus to produce an extrachromosomal, superhelical form. Ecdysteroids increased immediately after the pupal molt; thereafter ecdysteroids decreased. Before our study, there is the only study data on ecdysteroid quantification in pupal-adult development of a polydnavirus-carrying wasp which is the *Chelonus inanitus* (Marti *et al.*, 2003). In the braconid *C. inanitus*, the ecdysteroid titers and the CiBV amounts were measured during pupal developmental stage. However, the titer data do not support Webb's hypothesis, since at *C. inanitus* pupal stage 3b, when excised viral DNA first appears, ecdysteroid titer has already decreased and continues to drop, whereas CiBV replication is rise up to pupal stage 6. Furthermore, ecdysteroid titers were also highest just after pupation in pupae of an ichneumonid ectoparasitoid which does not carry polydnaviruses (Gelman *et al.*, 2000).

For our experimental data, comparing MdBV titers during the *M. demolitor* pupal developmental stages, the ecdysteroid titers showed the opposite trend. The data were obtained with whole body homogenates because of collection of young pupae haemolymph is difficult.

From *M. demolitor* pupal stage 1 to stage 4, the amount of MdBV increased while the ecdysteroid titer decreased. Furthermore, in vivo injection and in vitro ovary culture with 20-HE did not show any positive results. Thus, a specific role of ecdysteroids in regulating polydnavirus replication appears not true. It is conceivable that the in vitro 20-HE treatment accelerated calyx cell differentiation and thus indirectly caused an increase in viral DNA.

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Figure 3.1 Ecdysteroids in whole-body extracts of *M. demolitor* female and male pupae from pupal stage 1-4 and just after pupal ecdysis. Designation of stages is according to my description in Chapter 2. Ecdysteroids in the 100% methanol are expressed in 20-HE equivalents per insect as determined with the Gilbert antibody. Data are means ± SE of three determinations.

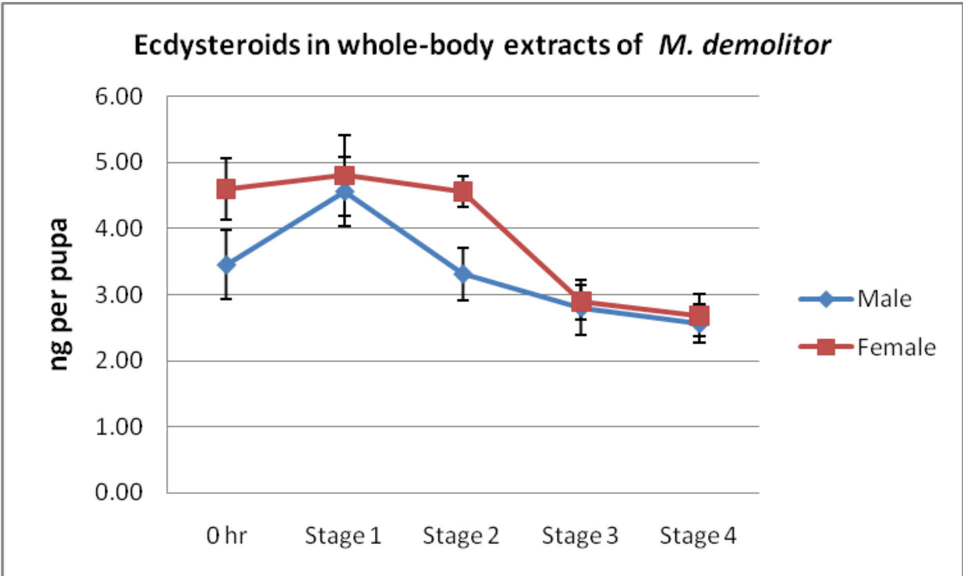
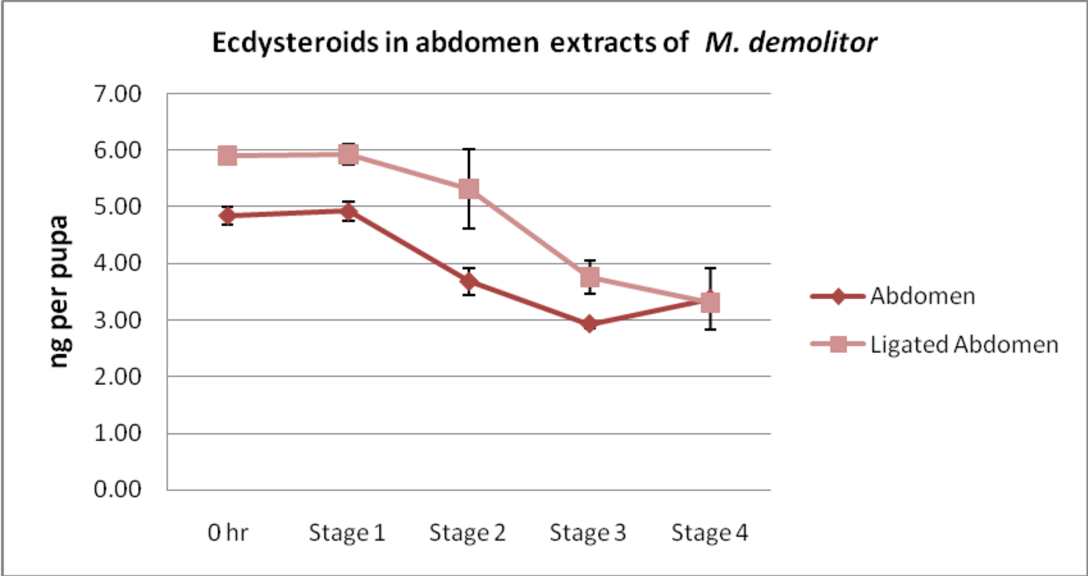

Ecdysteroids in whole-body extracts of *M. demolitor*
6.00
5.00
4.00
3.00
2.00
1.00
0.00
ng per pupa
0 hr
Stage 1
Stage 2
Stage 3
Stage 4
Male
Female

Figure 3.2 Ecdysteroids in abdomen and ligated abdomen extracts of *M. demolitor* female pupae from stage 1-4 and just after pupal ecdysis. For ligated abdomen measurement, pupae were ligated between the thorax and abdomen on each pupal stage. 12 h after each ligation time point, ligated abdomens were cut to measure the ecdysteroid titer. Designation of stages is according to my description in Chapter 2. Ecdysteroids in the 100% methanol are expressed in 20-HE equivalents per insect as determined with the Gilbert antibody. Data are means ± SE of three determinations.



Ecdysteroids in abdomen extracts of *M. demolitor*
ng per pupa
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
0 hr
Stage 1
Stage 2
Stage 3
Stage 4
Abdomen
Ligated Abdomen

Chapter 4. Assess whether MdBV replication is stimulated by an exogenous signal

4.1 Introduction

To assess whether MdBV replication is stimulated by an exogenous signal, we conducted a series of ligation experiments. Ligation is a classic experimental. In 1894, Bataillon did the first insect experiment when he ligated the silkworm moth larvae to show that the abdomen region pupated depending on the time and place silkworm larvae were ligated (Bataillon, 1894). In 1917, Kopec published 'Experiments on the metamorphosis of insects'. In this paper, the author ligated insects between the head and thorax to isolate the brain, corpora allata and corpora cardiaca from the posterior parts to prevent metamorphosis. In 1934, the father of insect physiology, Vincent Wigglesworth did the same kind of experiment by using the blood-sucking bug *Rhodnius prolixus* (Hemiptera) to determine the critical period for hormone release from the brain, which revealed that brain cells were the source of the brain hormone (Wigglesworth, 1934). In 1952, Williams revealed the relationship between the *Hyalophora cecropia* pupal diapause and the function of brain and prothoracic glands by ligation experiment (Williams, 1952). In a word, ligation is a functional and classic experimental method to isolate the target organs from exogenous signals.

There are many glands in the insect body, such as corpus allata, corpus cardiaca and prothoracic gland, which are tissues that specialize in the secretion of chemical messengers. These glands excrete different hormones for endocrine control of molting, growth and other processes. The corpus allata (CA) is located in the posterior region of the insect head (Lloyd *et al.*, 2000). Most insect orders contain a paired CA which is located ventrally. Specifically, in the

higher Diptera, the pair is fused into one and is located dorsal to the aorta. The CA contains intrinsic glandular cells and neurosecretory cells (Hodkova *et al.*, 2001). The function of CA is mainly involved in development and reproduction. The CA is the major organ of Juvenile hormone (JH) synthesis and release. Because JH is not stored in the CA, CA activity is regulated both neurally and through the release of neurosecretory hormones (Weaver and Audsley, 2009). The corpus cardiaca (CC) is a paired or single structure that lies posterior to the insect brain (Veelaert *et al.*, 1998; Clynen *et al.*, 2001). Both extrinsic and intrinsic neurosecretory cells could be found in the CC. The CC is composed of two lobes; the storage lobe derived from extrinsic cells and the glandular lobe derived from intrinsic cells. The CC was originally considered to be the organ for PTTH release in all insects. Many studies showed that the CC is the major organ in insects which could release a large number of neuropeptides. The CC also releases an ovarian ecdysteroidogenic hormone in mosquitoes, adipokinetic hormone, several neuroparsins and myotropins (Gade *et al.*, 2008). In 1940, Fukuda demonstrated that the ecdysone secreting organ was the prothoracic gland. In double ligation experiments with last instar silkworm moth larvae, Fukuda observed that the larval portions ligated anterior to the prothoracic gland pupated, while the posterior did not. When the prothoracic gland was implanted into the posterior portions, it pupated again. PTTH which is released by the corpus cardiaca activates the prothoracic glands to produce the ecdysteroid molting hormone (Marchal *et al.*, 2010; Ishizaki and Suzuki, 1994). Ecdysteroids are a generic name for a group of related steroid hormones which include ecdysone and 20-hydroxyecdysone. In 1992, Webb and Summers reported that 20-hydroxyecdysone could stimulate the replication of *Compoletis sonorensis* ichnovirus (Webb and Summers, 1992).

In this chapter, I used the ligation method to determine whether MdBV replication is stimulated by an exogenous factor and find out at which pupal stage the factor is sent to the ovary. After we make sure that this factor exists in the thorax of *M. demolitor* pupae, extract of the thorax was made to purify the factor. In vivo injection and in vitro ovary culture experiments were conducted to examine the activity of thorax extract.

4.2 Materials and Methods

Insects. *M. demolitor* was reared and staged as described in Chapter 2. *C. includes* was also reared as described in Chapter 2.

MdBV collection. Calyx fluid was collected from the female wasps by established methods (Beck and Strand, 2007). Reproductive tracts were dissected from *M. demolitor* in phosphate buffered saline. MdBV virions were collected by centrifugation of the calyx fluid at 4°C for 20 minutes at 16,000 × g and resuspended in phosphate buffered saline. The MdBV purified from one wasp was referred to as one wasp equivalent.

MdBV viral genomic DNA isolation. MdBV was resuspended in 100 μl of reaction buffer (0.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, 10 mM Tris–HCl pH 7.5) containing 2 μl DNase I (Ambion) and incubated 30 minutes at room temperature to eliminate any non-viral DNA. The virions were then collected by recentrifugation and washed with Pringle's saline (154 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$ and 22 mM D-glucose). To extract the genomic DNA from MdBV, purified viral particles were resuspended in PBS with 2 % sarcosyl and 0.5 mg/ml Proteinase K at 62°C for 1 hour. The MdBV genomic DNA was purified by phenol:chloroform extraction and isopropanol precipitation (Beck and Strand, 2007).

Relative quantitative real-time PCR. Relative quantitative real time PCR (rqRT-PCR) was conducted using Elongation Factor 1 alpha (EF1α) gene (GenBank: DQ538655.1) to represent

M. demolitor genomic DNA and Segment D to represent MdBV DNA. Specific primers are listed below:

EF1α gene forward primer: 5'-ATTGAAGGCCGAGCGTGAAC-3',

Reverse primer: 5'-CCGAGGGTGAAAGCAAGGAG-3';

Segment D forward primer: 5'-TGGTGGATTATTGAACGAGATGTA-3',

Reverse primer: 5'-GAAAAGATTGGGAAGATTGGATAG-3'.

In a 10 µl PCR reaction, there was 1 µl cDNA, 500 nM primers and 5 µl iQ SYBR Green Supermix (Bio-rad). 18S ribosomal RNA was used for endogenous control. Reactions were run on a Rotor Gene 2000 Real-Time PCR Cycler (Corbett Research) for 45 cycles with denaturation at 94˚C 20 seconds, annealing at 55˚C 20 seconds and extension at 72˚C 20 seconds. Data were analyzed by the ΔΔCt method. The transcriptional level of EF1α at time point 0 was standardized as 1.

Thorax extract. First, head and abdomen of 24 h to 36 h *M. demolitor* female pupae were removed. One hundred female thoraces were collected and put into a 1.5ml eppendof tube with water. After grinding, sonication and centrifugation, the supernatant of the thorax tissue was transferred into a new tube and frozen in liquid nitrogen for 30 min. The frozen extract was then lyophilized overnight. Finally, we dissolved the lyophilized powder in PBS and diluted to a 0.25 wasp equivalent concentration (Fig. 3.2).

In vivo injection. For this study, 0 h and 12 h *M. demolitor* female pupae (prior to the initiation of viral DNA replication) were ligated between the thorax and abdomen. Twelve h after these ligation time points, 0.2 µl 1× Phosphate buffered saline (PBS), 0.25 equivalent, 0.025 equivalent, 0.0025 equivalent and 0.00025 equivalents of the thorax extract was injected into the

ligated pupal abdomen. Ligated pupal abdomens injected with PBS alone were referred to as the control.

In vitro ovary culture. *M. demolitor* ovaries were isolated from 0 h to 12 h female pupae (prior to the initiation of viral DNA replication) and cultured individually in vitro with a thorax extract prepared from 24 h to 36 h pupae (posterior to the initiation of viral DNA replication) in a sealed 96 well ELISA microtiter plate. Culture conditions were TC100 insect medium plus 10% Fetal Bovine Serum (FBS) at 27˚C.

4.3 Results

Assess whether MdBV replication is stimulated by an exogenous signal.

Using the insect ligation experiments described above, we ligated *M. demolitor* pupae at the head or abdomen to see if parasitic wasp metamorphosis is related to an exogenous signal from the head or thorax. First, 0 h, 12 h, 24 h and 36 h *M. demolitor* female pupae were ligated between the head and thorax (Fig. 4.1A). Until 96 h after pupal ecdysis, the development of the thorax and abdomen were recorded. We observed that the thorax and abdomen can develop normally for each time point. Second, 0 h, 12 h, 24 h and 36 h *M. demolitor* female pupae were ligated between the thorax and abdomen (Fig. 4.1B). Until 96 h after pupal ecdysis, the development of the abdomen was recorded. Twenty four h, 36 h ligated abdomens developed normally, while zero h and twelve h ligated abdomens did not stimulates pupation. I concluded that a factor from the thorax instead of head and this exogenous signal is sent to the abdomen between 12 h and 24 h.

To assess whether MdBV replication is stimulated by an exogenous signal, 0 h, 12 h, 24 h, 36 h *M. demolitor* female pupae were ligated between the head and thorax. Until 96 h after pupal ecdysis, *M. demolitor* ovaries were dissected to see if MdBV virions formed. The results

showed that bluish virus particles existed in the calyx region of ovaries, which indicated that head ligation cannot isolate the exogenous signal. Second, 0 h, 12 h, 24 h, 36 h *M. demolitor* female pupae were ligated between the thorax and abdomen, until 96 h after pupal ecdysis, ovaries were dissected to see if the MdBV virion existed. The results showed that there were no bluish virus particles in 0 h and 12 h ligated abdomen ovaries after 96 h after pupal ecdysis. On the other hand, 24 h and 36 h ligated abdomen ovaries showed bluish MdBV virus particles. These results demonstrated that there was an exogenous signal from the thorax of *M. demolitor* which stimulated replication of MdBV between 12 h and 24 h after pupal ecdysis.

In vivo injection and in vitro culture *M. demolitor* pupae ovaries with thorax extract.

To prove if this exogenous signal exists in the thorax, we made a *M. demolitor* female pupal thorax extract and did a series of in vivo injections and in vitro culture experiments. Because of we cannot identify which organ or tissue in the thorax releases the signal, *M. demolitor* pupal thorax extracts were made as described in the materials and methods (Fig. 4.2).

To test the activity of the thoracic extract, I did a series of in vivo injection experiments. First, 0 h and 12 h *M. demolitor* female pupae (prior to the initiation of viral DNA replication) were ligated between the thorax and abdomen. Twelve h after ligation, 0.2 μl 1× PBS, 0.25 equivalent, 0.025 equivalent, 0.0025 equivalent or 0.00025 equivalents of the thorax extract was injected into the ligated pupal abdomen. Until 96 h after pupal ecdysis, we checked the development of each abdomen, however, no abdomens showed any development.

I then performed a series of in vitro ovary culture experiments to determine if any thoracic extract could induce MdBV replication. Ovaries were isolated from 0 h to 12 h pupae (prior to the initiation of viral DNA replication) and cultured individually in vitro with a thoracic extract prepared from 24 h to 36 h pupae (posterior to the initiation of viral DNA replication) in

96 well microtiter plate. Culture conditions were 117 µl TC100 insect medium plus 10% Fetal Bovine Serum (FBS) at 27˚C. 3 µl 1× PBS; 0.25 equivalent male thorax extract; 0.25 equivalent, 0.025 equivalent, 0.0025 equivalent and 0.00025 equivalent female thorax extract was added into the each microtiter plate well (Fig. 4.3). I then recorded the percentage of ovaries that contained calyx fluid 96 h after pupal ecdysis. Experimental results showed that the thoracic extract dose dependently stimulated MdBV replication.

Determine which *M. demolitor* developmental stage the signal is sent to the ovary.

Zero h, 12 h, 24 h, 36 h, 48 h, 60 h and 72 h pupae were ligated between the thorax and abdomen. As the results showed before, 12 h ligation stopped the calyx fluid from forming while 24 h ligation did not. I then used rqRT-PCR to compare the virion amounts among different ligated time points. I dissected pupal ovaries until 96h after pupal ecdysis for each ligation time point and extracted viral DNA. As before, I used Segment D to represent MdBV viral DNA and EF1α gene to represent *M. demolitor* genomic DNA (Fig. 4.4). RqRT-PCR results showed that replication of MdBV began between 12 h and 24 h. At 96 h, 24 h to 72 h ligated abdomen, the MdBV virion amount increased stably. According to the ligation time point and rqRT-PCR results, I concluded that the exogenous signal is sent to *M. demolitor* ovary between 12 h and 24 h after *M. demolitor* pupal ecdysis.

Characterization of the thoracic factor that stimulates MdBV replication.

After finding the location of this factor, I initiated studies to characterize its chemical properties. Boiling of the thoracic extract eliminated its biological activity (Fig. 4.5). In contrast, fractionation using filter columns suggested that activity was in a fraction with a mass between 30 to 50 kD (Fig. 4.6).

4.4 Discussion

At the end of this objective, I concluded that MdBV replication is regulated by an exogenous signal which comes from *M. demolitor* pupal thorax. For the onset of CcBV replication, studies revealed that an amplification of EP1 virus sequences precedes the excision of the individual circles. There are several fragments correlate to the EP1 integrated form were amplified. When CcBV begins replication, a large amplified molecule can be observed before the EP1 circle. This may indicate that coamplification precedes the excision of individual circles. Following excision, a specific product can be obtained by using PCR analysis, and this product corresponds to the amplification of an abundant molecule. After that, the double-stranded DNA circles could be amplified by a rolling-circle mechanism (Pasquier-Barre *et al.*, 2002). The level of amplification is about ten times higher in some specific cells in the wasp ovaries. It would be interesting to test whether the off-size fragments used to diagnose the integration of MdBV in *M. demolitor* genomic DNA or the integration of other PDVs in relevant parasitic wasps (Fleming and Summers, 1991; Gruber *et al.*, 1996; Xu and Stoltz, 1991).

In ichnoviruses (IVs), small virus circles are generated from larger circles through another kind of recombination pathways. Through this mechanism call 'nesting', large and small circles are both found in the virus particles. The CsIV genome contains many cross-hybridizing segments, such as small segments R and M are found within a larger segment W (Cui and Webb, 1997). Chromosomal amplifications in *Drosophila* salivary glands and ovaries have been studied (Bostock, 1986; Osheim *et al.*, 1988; Spradling, 1981). In the ovary of *Drosophila*, two clusters of chorion genes were amplified in specialized cells prior to their expression in oogenesis. Chromosomal amplification is achieved through additional rounds of replication. The highest

level of amplification is observed in the center region which containing chorion genes (Spradling, 1981).

After find out that the thorax factor is a protein between 30 kD and 50 kD. For future experiment, we could use High-performance liquid chromatography (HPLC) to further separate and purify the signal factor. Then we could use peptide sequencing and mass spectrometry analyses to sequencing this protein factor. After synthesize cDNA and subcloning, we could testify the activity of the pure protein factor by in vivo injection or in vitro ovary culture.

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Figure 4.1. Ligation on *M. demolitor* pupae. Figure A. Ligation between head and thorax. 0 h, 12 h, 24 h and 36 h pupae were ligated between head and thorax. Until 96 h after pupal ecdysis, the thorax and abdomen of all ligated pupae developed normally (left below picture). Figure B. 0 h, 12 h, 24 h and 36 h pupae were ligated between thorax and abdomen. Until 96 h, 24 h, 36 h ligated abdomen developed normally (right below picture), while 0 h and 12 h ligated abdomen did not develop (middle below picture).

A

B

12h ligation

24h ligation

Figure 4.2. Process of making *M. demolitor* female pupal thorax extract. First, head and abdomen of 24 h to 36 h *M. demolitor* female pupae were removed. 100 female thoraces were collected and put into a 1.5ml micro tube with water. After grinding, sonication and centrifugation, the supernatant of the thorax tissue was transferred into a new tube and froze in liquid nitrogen for 30min. Then the frozen extract was lyophilized overnight. Finally, the lyophilized powder was dissolved in PBS and dilute to a 0.25 wasp equivalent concentration.

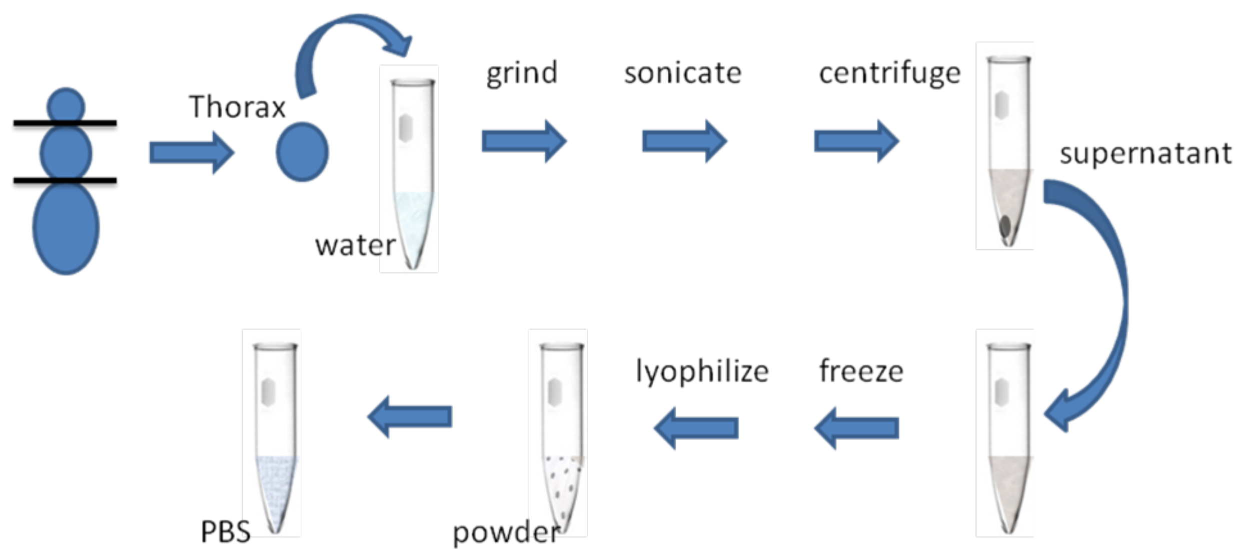

Thorax
water
grind
sonicate
centrifuge
supernatant
lyophilize
freeze
PBS
powder

Figure 4.3. Percentage of 12 h *M. demolitor* pupal ovary wakened by thorax extract. Ovaries were treated with 1× PBS; 0.25 equivalent male thorax extraxt; 0.25 equivalent, 0.025 equivalent, 0.0025 equivalent and 0.00025 equivalent female thorax extract and cultured in the 96 well ELISA microtiter plate. Until 96 h after pupal ecdysis, percentage of wakened ovary was recorded. Culture conditions are TC100 insect medium plus 10% Fetal Bovine Serum at 27˚C.

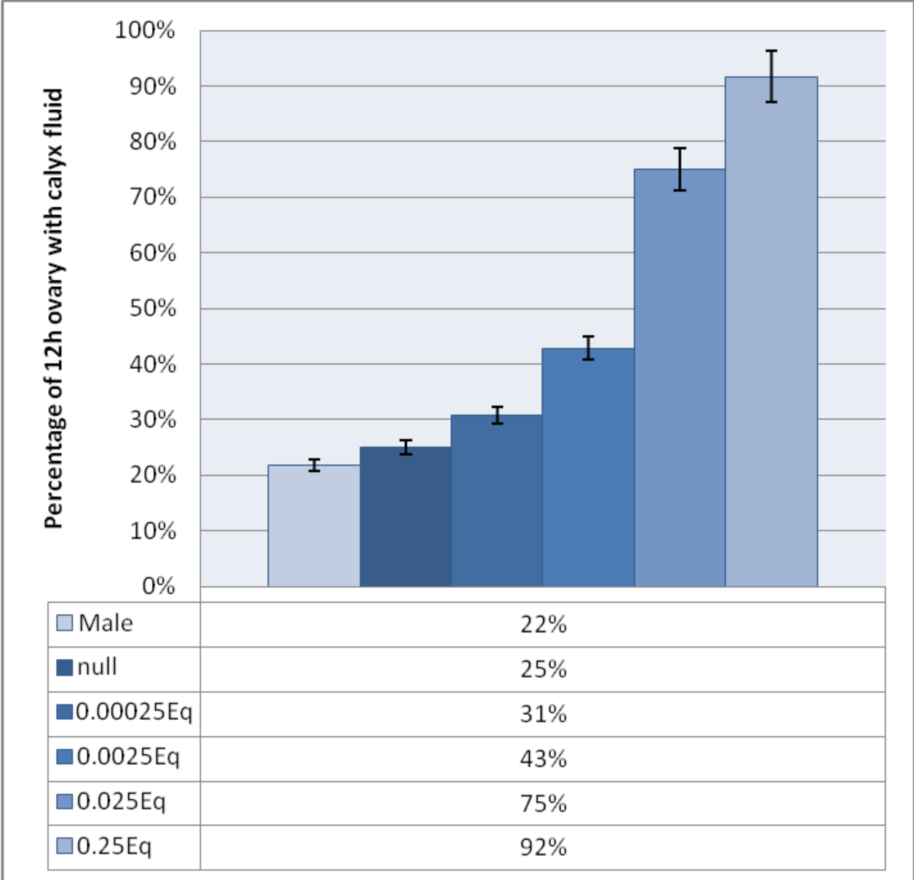

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Percentage of 12h ovary with calyx fluid
Male
null
0.00025Eq
0.0025Eq
0.025Eq
0.25Eq
22%
25%
31%
43%
75%
92%

Figure 4.4. Relative MdBV abundance during different ligated pupal stages. Female pupae of *M. demolitor* were ligated between head and thorax at 0 h, 12 h, 24 h, 36 h, 48 h, 60 h and 72 h. Until 96 h after pupal ecdysis, ovaries were dissected for each ligation time point and extract viral DNA for rqRT-PCR. MdBV segment D is an indicator of the MdBV DNA segments; elongation factor 1 alpha (EF1α) represents *M. demolitor* genomic DNA. Data were analyzed by the $\Delta\Delta$Ct method.

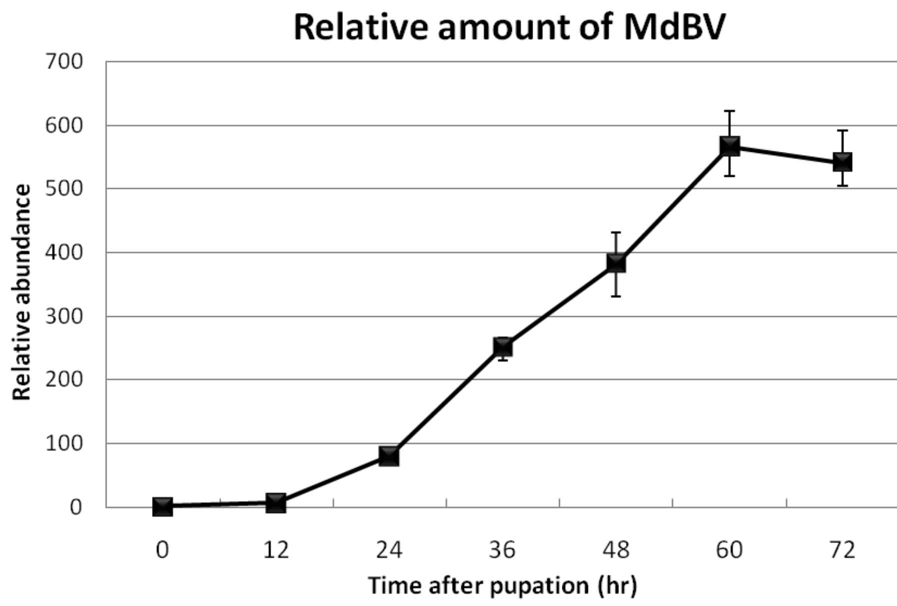
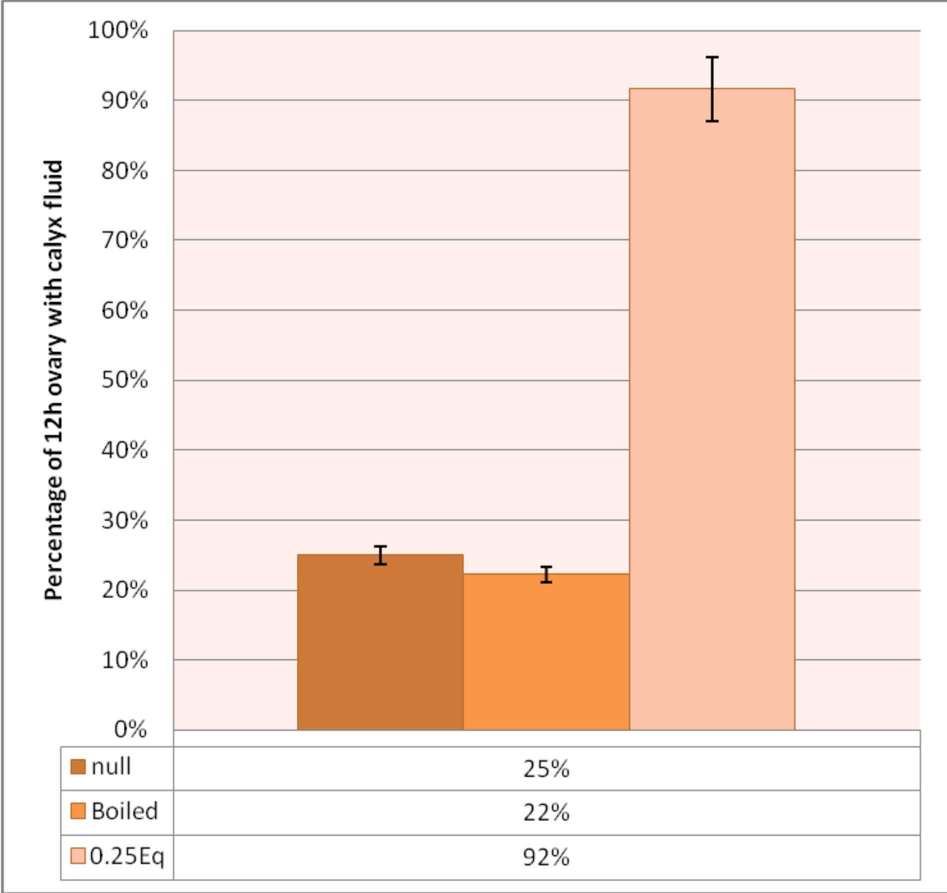
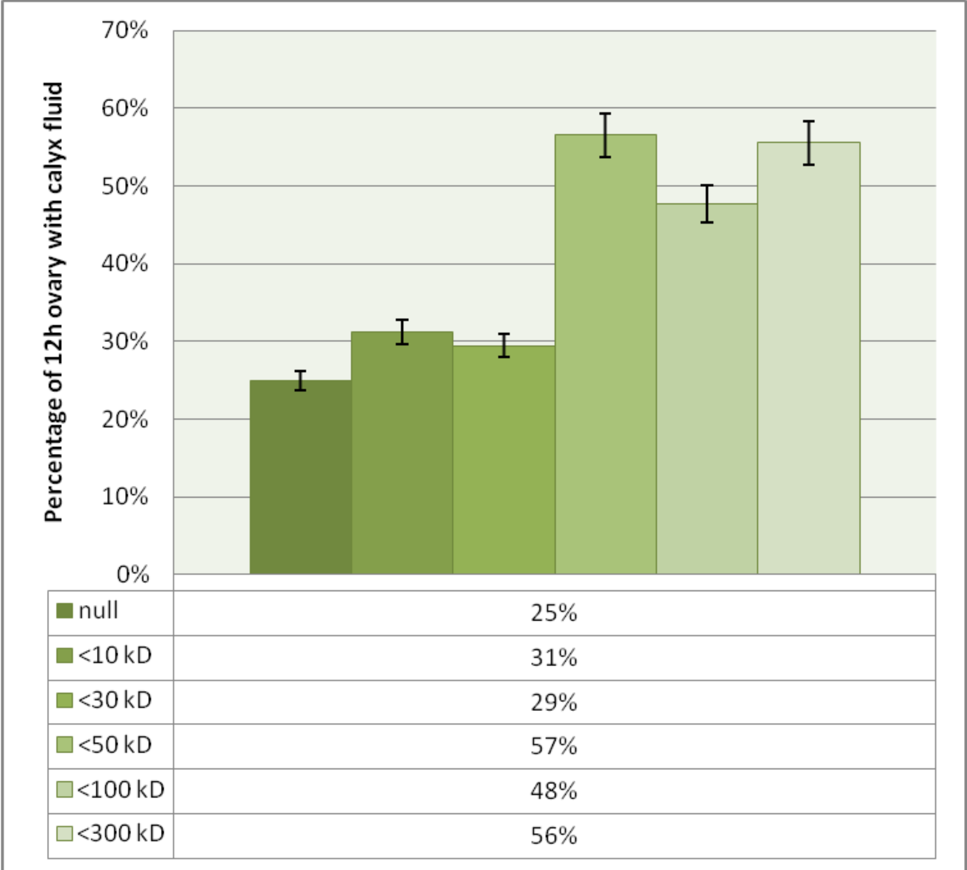

Relative amount of MdBV
Relative abundance
700
600
500
400
300
200
100
0
0
12
24
36
48
60
72
Time after pupation (hr)

Figure 4.5. Percentage of 12 h *M. demolitor* pupal ovary wakened by female thorax extract boiled or not. Ovaries were treated with boiled and 0.25 equivalent thorax extract. Each ovary was cultured in the 96 well microtiter plate. Until to 96 h after pupal ecdysis, percentage of wakened ovary was recorded. Culture conditions are TC100 insect medium plus 10% Fetal Bovine Serum at 27˚C.



100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Percentage of 12h ovary with calyx fluid
null
25%
Boiled
22%
0.25Eq
92%

Figure 4.6. Percentage of 12 h *M. demolitor* pupal ovary wakened by different molecular weight filtrate cuts thorax extract. Ovaries were treated with thorax extracts after different molecular weight filter cuts and while cultured in the 96 well microtiter plate. Until to 96 h after pupal ecdysis, percentage of wakened ovary was recorded. Culture conditions are TC100 insect medium plus 10% Fetal Bovine Serum at 27˚C.


70%
60%
50%
40%
30%
20%
10%
0%
Percentage of 12h ovary with calyx fluid
null
25%
<10 kD
31%
<30 kD
29%
<50 kD
57%
<100 kD
48%
<300 kD
56%

Chapter 5. Conclusions

There are two forms of the *Microplitis demolitor* bracovirus (MdBV) genome: a linear proviral form which is integrated into the *M. demolitor* genome and an episomal form which occurs after replication (Beck *et al.*, 2007; Beck and Strand, 2005; Beck and Strand, 2003). MdBV exists in the calyx region of ovaries in the endoparasitic wasp *Microplitis demolitor*. Virions accumulate to high density in the calyx and wasps inject 0.005 - 0.02 equivalent of MdBV into hosts when laying their eggs (Strand *et al.*, 1992). MdBV does not replicate in the wasp's host but express genes whose products cause severe physiological alterations.

In this study, I examined whether there is an exogenous signal factor responsible for the initiation of MdBV replication in *M. demolitor*. First, because prior studies showed that PDV replication correlates with pigmentation of wasp pupae (Stoltz and Vinson, 1979; Albrecht *et al.*, 1994), I defined *M. demolitor* pupae developmental stages according to their body melanization patterns. Furthermore, ovaries were dissected at each pupal stages, and obverse that ovary development and the appearance of MdBV bluish virions correlate with the wasp pupae melanization patterns. Second, MdBV episomal virion amounts were quantified by quantitative real time PCR to find out when MdBV began to replicate during the pupal stage. According to my observation under 4 × microscope, the MdBV bluish virion appeared on stage 3. However, quantitative real time PCR results showed that the viral replication began on late pupal stage 1. The polydnavirus viral amounts have been measured in the parasitic wasp *Chelonus inanitus* and *Campoletis sonorensis*, and results showed that polydnavirus replication in these two species began in the early pupal stages (Webb and Summers, 1992; Marti *et al.*, 2003).

The initiation of replication of Polydnaviruses has only been studied by Webb and Summers. They believe that the replication of *Campoletis sonorensis* ichnovirus is regulated by the hormone 20-HE (Webb and Summers, 1992). Correspondingly, I assume that ecdysteroids could regulate the replication of MdBV. In vivo injection and in vitro ovary culture with 20-HE experiments were done and ecdysteroid titers of different *M. demolitor* pupae developmental stages were measured. However, results did not support my hypothesis that 20-HE could stimulate MdBV replication both in vivo and in vitro. Although not reported in the experimental results, the activity of PTTH, bombyxin or methoprene (juvenile hormone analog) was also checked both in vivo and in vitro, and none of the reagents stimulated replication of MdBV.

Excluding ecdysteroids, I assumed whether MdBV replication is stimulated by an factor. My ligation experiments showed that onset of MdBV replication is regulated by an exogenous factor from *M. demolitor* pupal thorax. With ligation between thorax and abdomen during pupal stage 2, the ovary developed normally and MdBV virions could be measured at 96 h after pupal ecdysis. Virions were not observed when ligation on pupal stage 1. I also measured the MdBV virion amounts in ligated abdomen by rqRT-PCR. Results showed that the exogenous signal is sent to the ovary between 12 h and 24 h after pupal ecdysis. To prove whether the MdBV replication is regulated by a factor from *M. demolitor* thorax, pupal thorax extract was made to test its activity. In vitro ovary culture experiment showed that crude thorax extract could induce MdBV replication. Next step, I proved that the thorax factor is a protein. Molecular weight filters were used to separate and purify the active factor, which is between 30 to 50 kD.

Overall, my thesis characterizes the *Microplitis demolitor* pupae developmental stages and MdBV virion amounts during pupation. My data also provide evidence that replication of MdBV began on late pupal stage 1. Furthermore, a protein factor from *M. demolitor* pupal thorax

instead of 20-hydroxyecdysone could regulate the replication of MdBV. For future experiment, we could use high-pressure liquid chromatography (HPLC) to further separate and purify the thorax factor. Then we could use peptide sequencing and mass spectrometry analyses to sequencing this protein factor. After synthesize cDNA and subcloning, we could test the activity of the pure protein factor by in vivo injection or in vitro ovary culture.

5.1 References

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Appendix

List of Abbreviation

20-HE	20-hydroxyecdysone
BV	Bracovirus
CA	Corpus allata
CC	Corpus cardiaca
CiBV	Chelonus inanitus bracovirus
CcBV	Cotesia congregate bracovirus
CsIV	Campoletis sonorensis ichnovirus
EF1α	Elongation Factor 1 alpha
FBS	Fetal Bovine Serum
IV	Ichnovirus
JH	Juvenile hormone
MdBV	Microplits demolitor bracovirus
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDV	Polydnavirus
PTTH	Prothoracicotropic hormone
RqRT-PCR	Relative quantitative real time PCR
RIA	Radioimmunoassay
VLP	Virus-like particle