CHAPTER 1

Introduction

1.1 Historical Background

Diapause is a state of developmental arrest that is common among insects and is under the control of hormonal interactions (Denlinger, 2002). In most multivoltine species, diapause occurs in response to environmental cues which is referred to "facultative diapause", but in univoltine species, diapause is genetically determined to occur at a certain stage in each generation regardless of the environmental factor; this is referred to as "obligatory diapause" (Denlinger, 1985). In insects that undergo a complete metamorphosis, diapause can occur in any stage of their growth and is regulated by the neuroendocrine system (Singtripop *et al*., 1999).

In general, diapause is controlled by two types of factors, endocrine and environmental factors. The subesophageal ganglion (SG), which is connected to the brain, is where diapause hormone and pheromone biosynthesis activating neuropeptide (DH-PBAN) is synthesized. DH-PBAN is an important insect neuropeptide that is reportedly involved in diapause regulation. DH-PBAN induces embryonic diapause by inhibiting ovarian development in *Bombyx mori* and terminates pupal diapause by activating the prothoracic gland (PG) to synthesize ecdysone *Helicoverpa* spp. Moreover, DH-PBAN stimulates sex pheromone biosynthesis in female moths for male attraction (Denlinger *et al*., 1985; Zhang *et al*., 2004b). In *B. mori*, DH stimulates the transcription of the trehalase gene in ovaries, and thereby increases trehalase activity which facilitates higher accumulation of glycogen in eggs, a prerequisite for diapause initiation during which sufficient reserves are sequestered for survival during the diapause period and for post-diapause development (Shiomi *et al*., 1994; Su *et al*., 1994; Hahn and Denlinger, 2011). Although, DH-PBAN genes have been widely studied in more than 30 insect species spanning four orders (Choi *et al*., 2010), there is no previous knowledge of the DH-PBAN genomic structure or gene expression profile in

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the bamboo borer, *O. fuscidentalis* Hampson, a member of the lepidopteran family Crambidae.

In addition to endocrine factors, environmental factors are crucial to the control of diapause in many temperate insect species (Tauber *et al*., 1986; Danks, 1987; Hodek & Hodková, 1988; Hodek, 2002). Diapause termination by photoperiod and high-temperature were reported in *Calliphora vicina* (Vinogradova, 1974) and *Lucilia sericata* (Tachibana and Numata, 2004). In the zygaenid moth, *Pseudopidorus fasciata*, the larvae are highly sensitive to photoperiod, and both diapause induction and termination are dependent on whether the night-lengths exceed the critical night length or not, i.e., 10.5 h for diapause induction and 10 h for diapause termination (Wei *et al*., 2001; Li *et al*., 2003). Furthermore, the expression of the DH gene has been shown to be temperature dependent in *B. mori* and *Helicoverpa* spp. In addition, the environmental signal of low temperature acts on the brain to release a factor that regulates the SG to synthesize and secrete FXPRLamide peptides into the hemolymph and act on the PGs to synthesize ecdysone, and ecdysone then directly causes the pupae to break diapause in *Helicoverpa* spp (Zhao *et al*., 2004).

The bamboo borer, *O. fuscidentalis*, is a univoltine lepidopteran insect that experiences an annual severe dry season in Northern Thailand, Laos and Myanmar. The fifth instar larvae enter diapause and remain inside the internode of bamboo culm for nine months, from September until the following June each year. Preliminary observations show that the inside of a bamboo shoot is completely dark. There is practically no fluctuation of humidity with the lowest relative humidity of the year being 95% and the highest 100%. The lowest monthly average temperature is above 20 °C with the daily temperature change inside a bamboo shoot following that of the outside with a delay of 1 h or less. Thus, pre-diapause preparation and diapause development of the bamboo borer larvae occur in a relatively stable environment. Pupation of individual larvae of a single colony appears to occur synchronously because development of adults from pupae in an individual internode is well-synchronized. This indicates that the break of diapause must be environmentally regulated. Photoperiod is a common environmental cue to break diapause. The bamboo culm wall is more than 1 cm thick and it may be impossible that light would pass through the wall because the

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light permeability of the wall is about $1x10^{-21}$%/cm (Singtripop *et al*., 1999). In addition, photoperiod is likely to play an important role in the termination of larval diapause in *O. fuscidentalis* because adult female moths lay egg cluster on newly grown bamboo shoots in early August, the entrance hole for the first instar larvae is left on the bamboo shoot. The size of the hole increases continuously until pupation occurs inside the bamboo shoot in the middle of June. This suggests that the light intensity may increase as the hole size increases (Singtripop, T., unpublished data). However, the mechanism for breaking larval diapause by photoperiod remains poorly understood. Thus, studying the roles of both endocrine and environmental factors may help us to understand the mechanism of larval diapause regulation in *O. fuscidentalis*.

Insect molting and metamorphosis are regulated by the interplay of two hormones, 20-hydroxyecdysone (20E) which triggers the successive molts throughout the life cycle and juvenile hormone (JH) which determines the developmental stage of the newly molted insect (Yamanaka *et al*., 2013). In insects, JH is synthesized by the corpora allata and contributes to maintain larval growth (Gilbert *et al*., 2000). 20E is converted from ecdysone produced by the prothoracic glands and triggers larval molting and metamorphosis with the larval-pupal transition. Higher levels of JH during larval stages prevent metamorphosis, whereas lower levels or an absence of JH at the end of the larval or pupal stages allows 20E to promote metamorphosis. JH also regulates female reproductive maturation in adult insects (Dubrovsky, 2005; Riddiford, 1996, 2012).

The molecular mechanisms underlying the actions of 20E have been elucidated first in the fruit fly, *Drosophila melanogaster*, and confirmed in various other insects. In a cell, 20E binds to a heterodimeric receptor comprised of the ecdysone receptor (EcR) and ultraspiracle (USP), both of which are transcription factors belonging to the nuclear receptor superfamily (Hiruma and Riddiford, 2009; Thummel, 1996). The liganded 20E-EcR-USP complex binds to ecdysone response elements (EcREs) located in the promoter regions of target genes and directly activates the transcription of the early ecdysone-inducible genes such as E74, E75 and Broad-Complex (BR-C), most of which encode transcription factors (King-Jones and Thummel, 2005). These factors, in turn, induce a secondary response by repressing some of the early genes and activating the expression of tissue-specific genes.

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The mechanism mediating the JH response is a fascinating question. Recently, some studies have reported that JH can induce the transcription of a large number of genes *in vivo* or *in vitro* (Zou *et al*., 2013; Willis *et al*., 2010; Beckstead *et al*., 2007), and specific JH-response elements (JHREs) in some JH-regulated genes have been identified (Li *et al*., 2011). However, little is known about the crosstalk between JH and 20E in insect larvae. The molecular mechanism underlying the actions of JH remains largely unknown. JH has been shown to bind with a heterodimer comprised of the JH receptor methoprene-tolerant (Met) and its binding partner Taiman (Tai)/Ftz-F1-interacting steroid receptor coactivator (FISC)/steroid receptor coactivator-1 (SRC-1); both of these proteins are transcription factors that belong to the basic-helix-loop-helix/Per-Arnt-Sim (bHLH-PAS) family (Jindra *et al*., 2013; Dubrovsky and Bernardo, 2014). The JH-Met-Tai complex binds to JH response elements (JHREs) located in the promoter regions of the target genes and induces their transcription. In the red flour beetle, *Tribolium castaneum*, Met plays a similar key role in JH action during the larval-pupal metamorphosis and precocious development of adult structures, indicating that Met is involved in antimetamorphic JH signaling (Konopova and Jindra, 2007; Parthasarathy *et al*., 2008). In the larval stage, Met inhibits expression of BR-C that promotes pupal development (Minakuchi *et al*., 2009). BR-C, one of the 20E-induced early transcription factors, is expressed specifically during larval-pupal metamorphosis under the control of 20E and JH (Bayer *et al*., 1996; Zhou and Riddiford, 2002; Riddiford *et al*., 2003). BR-C is necessary for metamorphosis and the role of BR-C in the larval-pupal transition has been studied in *D. melanogaster* (Zhou and Riddiford, 2002), *B. mori* (Uhlirova *et al*., 2003; Wang *et al*., 2010), and *T. castaneum* (Suzuki *et al*., 2008; Parthasarathy *et al*., 2008).

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Larval diapause is an arrested period of post-embryonic development characterized by a major shutdown in metabolic activities and is under the control of hormonal interactions (Denlinger, 2002). A high juvenile hormone titer in the hemolymph induces and maintains larval diapause in several lepidopteran insects. The ecdysteroid titer in hemolymph is low during larval diapause and increases after diapause termination. During diapause in those species, a high level of JH suppresses the ecdysone secretory activity of prothoracic gland (PG) and thereby inhibits pupal metamorphosis (Bean and Beck, 1980, 1983).

In several lepidopteran insects such as the Southwestern corn borer, *Diatraea grandiosella*, (Chippendale and Yin, 1979), the European corn borer, *Ostrinia nubilalis*, (Bean and Beck, 1980, 1983) and the rice stem borer, *Chilo suppressalis* (Agui & Hiruma, 1977), larval diapause is induced by a high juvenile hormone titer in the hemolymph that is maintained throughout diapause. During the long larval diapause in *O. fuscidentalis*, the PG exhibit ecdysone secretory activity, although the activity is low and the ecdysteroid titer in the hemolymph is very low (Singtripop *et al*., 1999). An application of a juvenile hormone analogue (JHA) to diapausing *O. fuscidentalis* larvae induces pupation by increasing the ecdysteroid titer in hemolymph. However, diapausing *O. fuscidentalis* larvae induce pupation after brain removal and treatment with 1 µg JHA, indicating that brain is not the primary target of the applied JHA. In the JHA-treated diapausing *O. fuscidentalis* larvae, the secretory activity of JHA-donor PG was increased at 10 days after JHA treatment (Singtripop *et al*., 2000). When PG from JHA-treated larvae were dissected and transplanted to another non-treated larval, the hemolymph ecdysteroid titer in this larvae exhibited a small increase within 16-18 days after transplantation (Singtripop *et al*., 2008). This indicates that JHA does not activate the ecdysteroid biosynthesis in PG directly but may stimulate through other tissues. In addition, recent studies have shown that treatment with exogenous diapause hormone (DH) can break larval diapause of *O. fuscidentalis* by increasing levels of ecdysteroid in the haemolymph (Subta, P., unpublished data) correlated with *OfDH-PBAN* mRNA level during the development of *O. fuscidentalis* (Suang *et al*., 2015). This suggested that *OfDH-PBAN* transcript may be involved in regulating larval diapause termination. Consequently, it may be possible that JHA may directly stimulate the subesophageal ganglion by activating the subesophageal ganglion to synthesize DH-PBAN and released into the hemolymph act on the prothoracic gland to release ecdysone, and have an effect on larval diapause termination. Anywise, the mechanism for breaking larval diapause by JHA is still unknown. In this study, the effect of JHA and 20E on the expression of *OfMet*, *OfDH-PBAN,* ecdysone receptor genes and ecdysone inducible genes in SG and PG are examined both *in vivo* and *in vitr*o. Therefore, knowledge of expression pattern of *OfMet*, *OfDH-PBAN,* ecdysone receptor genes and ecdysone inducible genes may help us to better understand the mechanism of larval diapause termination in *O. fuscidentalis*.

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In addition, the cloning and sequencing of the *O. fuscidentalis* DH-PBAN precursor cDNA (*OfDH-PBAN* cDNA) using reverse transcriptase polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) strategies were described. The expression profile of *DH-PBAN* mRNA in the SG and other tissues during the developmental stages is examined using the combined method of quantitative real-time PCR. Furthermore, the effect of photoperiod on *OfDH-PBAN* gene expression is examined which is a key factor for the termination of larval diapause.

1.2 Objectives

1.2.1 To study the molecular characterization and gene expression of *OfDH-PBAN* in subesophageal ganglion of the bamboo borer

1.2.2 To examine the expression level of *OfDH-PBAN* mRNA in other tissues and in developmental profile of the bamboo borer

1.2.3 To study roles of photoperiod on *OfDH-PBAN* mRNA expression in the bamboo borer

1.2.4 To investigate the effect of JHA and 20E on *OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA expression in subesophageal ganglion and prothoracic gland of the bamboo borer

1.3 Literature Review

1.3.1 Diapause

Diapause is an arrest in development accompanied by a major shutdown in metabolic activity. Unlike a simple quiescence that is an immediate response to an unfavorable environmental condition, diapause is a genetically programmed response that occurs at a specific stage for each species. Sometime, the insect enters diapause at this particular stage in each generation regardless of the environmental conditions (e.g., pharate first instar of the gypsy moth, *Lymantria dispar*). Thus, developmental program is referred to as an obligatory diapause. Much more frequently, the decision to enter diapause is determined by environmental factors, usually daylength, received by that individual or its mother at an earlier developmental stage. This is referred to as a

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facultative diapause (Denlinger, 1985). Insect enter diapause as a mechanism to bypass adverse conditions such as low temperatures, drought, or a lack of food. It can either be an obligatory part of an insect's life cycle, or 2 facultative and triggered by certain environmental cues such as short photoperiod and low temperatures (Tauber and Tauber, 1976). Insects exposed to short day-length during a photosensitive stage will be programmed to enter diapause (Denlinger, 2002). This is then followed by a phase where the insect prepares to enter hibernation, often by building up nutrient stores and increasing protective measures to withstand adverse environmental conditions (Denlinger, 2002; Tauber and Tauber, 1976). Survival of diapausing insects is due to decreased metabolism and the reserves of lipids, carbohydrates, protein and amino acids, which replace the lack of food intake of diapausing insects and sometimes confer protection from external cold (Denlinger, 1985).

Development can effectively be interrupted at many points, as evidenced by the rich diversity of developmental stages used by different species for diapause. Embryonic diapauses have been documented for nearly all possible stages of embryonic development, ranging from early blastoderm formation to pharate larvae. Larval diapauses are most common in late instars, but early instar diapauses are not at all uncommon. Pupal diapause is well documented and a few species diapause as pharate adults. Not surprisingly, it appears to be no instances where development is halted once pharate adult development has been initiated. The most prevalent cases of adult diapause involve newly emerged adults that have not yet reproduced, but there are also examples of diapause interrupting periods of reproduction. Most commonly, insect enter diapause only once during their life cycle, especially those living at higher latitudes. They may extend their development over several years, and thus spend successive winters in diapauses of different stages. Some long-lived adults may go through multiple diapauses to coordinate egg laying with favorable conditions (Danks, 1987; Saunders, 2002).

The term "diapause" perhaps falsely suggests a period of arrest in which development comes to a complete halt. The period of diapause should not be regarded as a simple stop and restart of development. Andrewartha (1952) originally coined the term "diapause development" to refer to the ongoing progression of events that occurs

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during diapause and eventually result in termination of diapause. It is evident that diapause is a dynamic process, as evidenced by characteristic changes in the utilization of energy reserves, systematic changes in patterns of oxygen consumption, changes in responsiveness to environmental stress and hormones, and distinct patterns and changes in gene expression. A progression of events must transpire before diapause can be terminated. In some cases, development is not completely halted. There are several examples in which developmental processes continue during diapause, albeit at much slower rates such as ovarian maturation during the adult diapause of *Culex pipiens* (Readio *et al*., 1999) and embryonic development during diapause in the pea aphid, *Acyrthosiphon pisum* (Shingleton *et al*., 2003). In some cases, there can be profound differences even within the body of a single individual. For example, during pupal diapause in the tobacco hornworm, *Manduca sexta*, most tissues of the body are arrested, but the testicular stem cells continue to undergo mitotic division (Friedlander and Reynolds, 1992). These unique events of diapause, coupled with prediapause distinctions in diapause-programmed individuals, suggest that it is perhaps most appropriate to view diapause as an alternate developmental pathway.

1.3.2 Hormonal control of diapause

The juvenile hormone (JH) and ecdysteroid, two of the major families of insect hormones that direct insect development, metamorphosis, and reproduction, are intimately involved in regulating diapause. The JH, which are isoprenoids secreted by the corpus allatum (CA), maintains the juvenile characters during the premetamorphic molts, while the steroid hormones from the prothoracic gland (PG), ecdysone and related compounds, dictate the decision to molt. In turn, the CA and PG are regulated by both neural and humoral factors from the brain. Brain neuropeptides governing the CA can exert either a stimulatory (allatotropins) or an inhibitory action (allatoinhibins) on the CA (Hiruma and Kaneko, 2013). The dominant regulator of the PG is the brain neuropeptide prothoracicotropic hormone (PTTH). These hormones, together with diapause hormone (DH), a unique neuropeptide that regulates the embryonic diapause of the commercial silkworm, are the key hormonal regulators of insect diapause (Denlinger, 1985). In certain situations, the presence of one or more of these hormones

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promotes diapause while in others it is the absence of a certain hormone that causes diapause.

Hormonal basis for embryonic diapause

Embryonic diapauses are common among the Orthoptera, Hemiptera, Lepidoptera and Diptera (especially species of *Aedes mosquitoes*), but there are reports from other orders as well. The diverse stages in which embryonic diapauses occur suggest a potentially rich diversity of regulatory schemes. The great diversity of diapausing stages among insect eggs demonstrates the potential of the embryo for the evolution of developmental arrest (Yamashita and Suzuki, 1991). In many insect species, including the silkworm, embryonic diapause intervenes prior to maturation of the sensory and neuroendocrine system. In such cases, the embryo has a strong reliance on maternal determinants for diapause regulation. No information is available on hormonal factors that are produced in the early embryos to regulate diapause, although ecdysteroids and juvenile hormone accumulate during oogenesis and are carried over to eggs that are laid. A regulatory scheme linking ecdysteroids and juvenile hormone to embryonic diapause seems likely in the more advanced embryos, but such a role has not been established. Much attention about the hormonal mechanism of embryonic diapause is focused on the maternal determinants of diapause in the silkworm, *Bombyx mori* (Yamashita and Hasegawa, 1985).

The most intensely studied embryonic diapause is that of *B. mori*. Classic experiments, begun in the 1950s by Fukuda (1952), firmly set the stage for a wealth of papers documenting the regulatory mechanism controlling the early embryonic diapause in this commercially important species. Diapause in *B. mori* differs from that of most model species. While both temperature and daylength are key components of the diapause decision, the specifics are reversed as compared to other diapause models. High temperature and long daylength prompt diapause induction, while low temperature and short daylength lead to nondiapause development. Diapause induction in this species is under strict maternal control. The photosensitive stage occurs far in advance of the actual diapause stage, exposure of developing female embryos and larvae to diapause-

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inducing conditions will cause the female to produce eggs that are destined to enter diapause. This effect is mediated by DH, a neuropeptide released by the female adult during her period of egg maturation. DH acts upon the ovarioles, leading to the production of diapause-destined eggs. After being oviposited, the silkworm embryos enter a diapause characterized by a G_2 cell cycle arrest (Nakagaki *et al*., 1991). Once diapause has been established, this cell cycle arrest can be broken only after the embryos have been chilled for 1-3 months at 5 ºC. Following this mandatory chilling period, development can be initiated when the embryos are transferred to a higher temperature.

As early as 1926, ovary transplantation experiments indicated that the factor controlling diapause induction is bloodborne (Umeya, 1926). Later studies by Fukuda and Hasegawa identified the brain and subesophageal ganglion as the tissues controlling the release of the diapause-inducing factor now known as DH, and bioassays performed by injecting nondiapause-type individuals with tissue extracts showed DH activity in subesophageal ganglion and brain extracts.

Hormonal basis for larval and pupal diapauses

Larval diapauses are best known from the Lepidoptera, but there are good examples of larval (or nymphal) diapauses in other orders as well. Diapause in the last larval instar is reported most frequently, but some larvae diapause in earlier instars or as prepupae. Pupal diapause has been examined extensively in Lepidoptera and the higher Diptera, but appears to be rare in other taxa. It is indeed the true pupal stage that is usually involved in diapause, but occasional reports indicate a diapause late in pharate adult development. Larval and pupal diapauses share much in common. Central to both is a failure to progress to the next metamorphic stage. This suggests that one of the key endocrine events regulating such a diapause is likely to be a failure of the prothoracic gland to produce the ecdysteroids needed to initiate the next molt. Invariably, this has proven to be a key element of larval and pupal diapauses. One might also predict a shutdown until the end of diapause in the production of prothoracicotropic hormone (PTTH), the hormone from the brain that stimulates the prothoracic gland to produce ecdysone. This may often be the case but not always. The other

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major metamorphic hormone, JH, may or may not play a role, depending on the species (Agui *et al*., 1979; Denlinger, 2002).

Larval diapause frequently intercedes at the end of larval life, just before the onset of pupation and metamorphosis, but it is not at all uncommon in earlier instars as well. Common to most examples of larval diapause is a shutdown in the brain-PG axis. In the absence of ecdysteroids from the PG, the larva fails to initiate the next molt. The failure of the brain to release ecdysteroids can usually be directly attributed to the brain's failure to release PTTH (Richard and Saunders, 1987). In a number of species, JH may also play a role. For example, in the southwestern corn borer, *Diatraea grandiosella*, the JH titer remains elevated throughout diapause, and the diapause can be terminated only when the JH titer drops (Yin and Chippendale, 1973). In some other species such as the European corn borer, *Ostrinia nubilalis*, the JH titer is high in early diapause but then declines and remains low throughout the remainder of diapause (Gelman *et al*., 1992). The high titer of JH prevents the brain from releasing the PTTH which stimulates ecdysteroid synthesis.

Pupal diapause is the consequence of a shutdown in the brain-PG axis. Thus, in the absence of ecdysteroids from the PG the progression of adult differentiation is halted. At the termination of diapause, ecdysteroids are again released, triggering adult development. In *Hyalophora cecropia* a period of chilling is required before the brain can stimulate the PG to release ecdysteroids (Williams, 1946). Pupal diapauses can usually be quickly terminated with an injection of 20-hydroxyecdysone (20E). The effective dose of 20E required for diapause termination changes with the progress of diapause in pupae of *Sarcophaga argyrostoma* (Gibbs, 1975) and *Mamestra configurata* (Bodnaryk, 1977).Usually the absence of ecdysteroids can be attributed directly to a failure of the brain to release the neuropeptide PTTH that needed to stimulate the PG to synthesize ecdysteroids, for example in the flesh fly, *Sarcophaga cressipalpis* (Denlinger, 1981). Recently, it was shown that the pupal diapause of moths in the agriculturally important Heliothis-Helicoverpa complex also can be terminated

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with DH (Zhang *et al*., 2004b), the hormone known to induce silkworm diapause. Thus, DH elicits two quite opposite effects on diapause in different moth species.

Hormonal basis for adult diapause

The central feature of adult diapause is a cessation in reproduction. For females, this implies an arrest in oocyte development, and in males the most conspicuous feature is their failure to mate with receptive females. The status of the testes is not a consistently reliable indicator of diapause in males (Pener, 1992). In some cases, the testes are underdeveloped, while the testes may be full of mature sperm cysts in other cases. In both males and females, the accessory glands are usually reduced in size during diapause. Although a period of long-distance flight may precede the entry into diapause, once the adults arrive at their diapause site, flight muscles typically degenerate for the duration of diapause and then regenerate when diapause is terminated. Adults remain fairly inactive in diapause, but some local movements may be noted. Mating may occur either before the entry into diapause or after diapause has been completed. When mating occurs prior to diapause, the males usually die without entering diapause and only the female bridge the diapause period. In cases where both sexes diapause, mating usually occurs only after diapause has been terminated.

Most of the early work on the hormonal control of adult diapause focused on JH. The pioneering work on adult diapause, initiated in the late 1950s by Jan de Wilde and his colleagues in the Netherlands, utilized the Colorado potato beetle as their model system (de Kort, 1990). They observed that the JH titers in diapause-programmed *Leptinotarsa decemlineata* decrease after adult emergence, remain low throughout diapause, and then rise after termination of diapause. Further evidence supporting the role of JH in adult diapause was provided by the responsiveness of diapausing adults to JH, histological studies of the CA, and experimental manipulations of the CA. The application of synthetic JH and JH analogs to diapausing females prompts egg-laying (although such periods are not sustained). The CA from diapausing *L. decemlineata* is small and inactive. Surgical removal of the CA induces a diapause-like state in long-day beetles (those not programmed for diapause): they stop feeding, leave the food, and

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burrow into the soil. Chemical destruction of the CA in long-day beetles with precocene II elicits the same diapause-like response. In nondiapausing adults, cauterizing the pars intercerebralis, the region of the brain that controls CA activity, also induces a diapause-like state. This same surgery performed with diapausing adults blocks the beetle's ability to become reproductively active when placed under long daylengths (the environmental signal used to promote reproduction). Severing the axons between the pars intercerebralis and the CA does not appear to alter the diapause decision, thus suggesting that the CA in this species is under hormonal, rather than neural, control.

1.3.3 The bamboo borer

Biology of the bamboo borer

The bamboo borer, *O. fuscidentalis,* is found in Northern of Thailand, Laos and Myanmar. It enters diapause for 9 months from September to the following June. The mature larvae are a favored food for mountain tribe people, and they have recently become popular in urban areas as well. They are sold in markets almost throughout the year, except for the months of July-September, indicating that there are no larvae in the field in those months. The food plant of bamboo borer larvae is young bamboo shoot. The bamboo borer has one year life's cycle (Fig.1.1). Adults usually appear in August, in mid wet season, and lay egg clusters on young bamboo shoots in this season. Newly hatched larvae feed on the inner pulp of internodes. After complete growth in September, the mature larvae enter diapause and remain in this stage until the following May before they pupate and become adult in wet season (Singtripop *et al.*, 1999).

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Preliminary observations showed that larvae in Chiang Mai Province feed on at least 5 different bamboo species, *Dendrocalamus membranaceus* Munro, *D. hamitonii* Nees & Arn, *Bambusa nutans* Wall. ex Munro, *B. blumeana* Schult and *Gigantechloa albociliata* Kurz. When compare the nucleotide sequences of the cytochrome C oxidase subunit 1 (COI) region of mitochondrial DNA in larvae collected from the five different bamboo species, results verified that those larvae belong to the same species, *O. fuscidentalis*. In addition, mature larvae were

collected each month and their body weight, head capsule width, protein, fat content and hemolymph ecdysteroid titer were measured. Body weight progressively decreased during the 9 months. The growth curve of the head capsule width conformed to Dyar's law. Fat content fluctuated during this period whereas protein levels remained constant until March, then significantly increased. During this period, the hemolymph ecdysteroid concentration remained low. These results confirm that the bamboo borer larvae enter diapause at the end of feeding period of the fifth (last) larval instar and the larval diapause lasts in June (Singtripop *et al*., 1999).

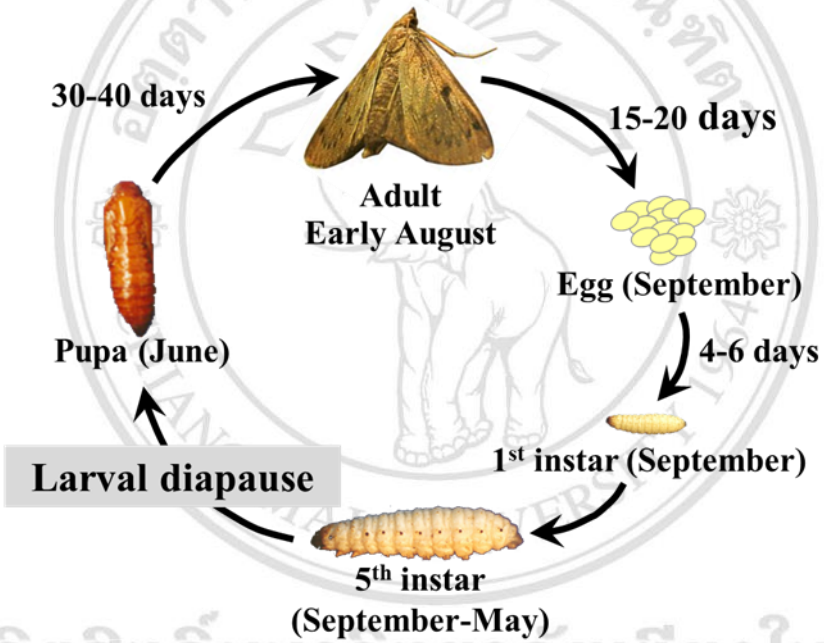

Figure 1.1 Life's cycle of the bamboo borer

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Larval diapause in the bamboo borer

Larval diapause is a developmental arrest and best known from the Lepidoptera, but this is good example of larval diapause in other orders as well. Diapause in the last larval instar is reported most frequently, but some larval diapause in earlier instars or as prepupae, which may occur in response to period of drought that reduces the food supply. The availability of food may be profoundly influenced by seasonal rhythms (Denlinger, 1986). Rain provides a wealth of new food resources for many phytophagous insects. The long diapause

is important in maintaining synchrony between the insect life cycle and the phenology of the host plants in the tropics. Bamboo produces new shoots in the wet season and the shoots become hard by the end of the wet season. Therefore, the long period of larval diapause in *O. fuscidentalis* appears to be well adapted to the recurring annual dry-wet season (Singtripop *et al*., 1999).

Changing of environmental conditions may not affect diapause development in the bamboo borer. Preliminary observations show that the inside of a bamboo shoot is completely dark (light permeability of the bamboo wall is approximately $1x10^{-21}$% cm^{-1}). There is practically no fluctuation of humidity with a lowest relative humidity of 95% and a highest of 100% throughout the year. The lowest monthly average temperature is >20 °C, with the daily temperature change inside a bamboo shoot adhering to that of the outside with a delay of 1 h or less. Thus, pre-diapause preparation and diapause development of the bamboo borer larvae occur in a relatively stable environment (Singtripop *et al*., 2002a).

In *O. fuscidentalis*, juvenile hormone analogue (JHA) simulated the prothoracic glands and thereby induced pupal metamorphosis, indicating that the larvae enter diapause after the switch in responsiveness of prothoracic glands to JH and after pupal commitment (Riddiford, 1978; Riddiford *et al*., 1980). Larvae moved when disturbed, showing that they are not prepupae. In addition, the frosted frass is excreted by larvae in two or three days after 20E injection. Frosted frass is the last fecal material excreted by lepidopteran last instars before gut purge (Nijhout and Williams, 1974). This observation indicates that *O. fuscidentalis* larvae enter diapause after they cease feeding but before they purge their gut contents, probably around the onset of the wandering stage (Singtripop *et al*., 2000)

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Endocrine mechanisms underlying JH- or 20E- mediated termination of larval diapause in the bamboo borer

1) The breaking of larval diapause by 20E

In the many cases in which diapause results from a hormone deficiency, a diapause-like state can be induced by surgically extirpating the relevant endocrine gland, and conversely, diapause can be readily terminated by implantation of an active gland or by injection or application of the missing hormone (Denlinger, 1985).

When diapause larvae of the *O. fuscidentalis* are treated with more than 1 µg 20E, they are active moved on the day of injection and the following day if touched, but became motionless 2 days after the injection. Between one and two days after the injection, they produced one or two pieces of frosty feces, and occasionally their hindgut is partially ruptured outside. Three to four days after becoming motionless, the larvae produced a tanned pupal cuticle beneath the larval cuticle but do not shed the old larval cuticle. The changes in the responses of larvae to exogenous 20E are examined in order to estimate the progress of diapause development. The responsiveness of larvae declined gradually from September to November when larvae are least responsive to 20E, and then decrease markedly from January to February. This indicated that the intensity of diapause increases from September to November and terminated gradually thereafter. Sensitivity of larvae to 20E is at the same level from September to December, and increases remarkably from December to January. The increases in the responsiveness and sensitivity in the latter half of diapause may be brought about by respective mechanism (Singtripop *et al*., 2002b).

2) The breaking of larval diapause by JHA

In *O. fuscidentalis*, Singtripop *et al*. (2000) clearly demonstrated that JHA terminated larval diapause by increasing the ecdysteroid titer in hemolymph. However, the brain is not involved directly in the activation of the PG by JHA. In the JHA-treated diapausing *O. fuscidentalis* larvae, the

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secretory activity of JHA-donor PG was increased at 10 days after JHA treatment (Singtripop *et al*., 2000) and the hemolymph ecdysteroid titer in the JHA-recipients exhibited a small increase 16-18 days after transplantation, indicating that JHA did not activate the ecdysteroid biosynthesis in PG directly (Singtripop *et al*., 2008).

An application of JHA to diapausing larvae induced the production of a pupal cuticle under the larval cuticle. Repeated applications of JHA induced complete pupae. After topical treatment of diapausing larvae with 1 μg of JHA, the larvae turn brown and develop a hard and pigmented cuticle, which indicated pupation of these larvae (Fig. 1.2). Treatment of diapausing larvae with a lower amount of JHA (0.1 μg) yields larvae that become inactive before formation of the brown cuticle (G0). The body colour then changes from creamy to light yellow and these larvae are designated as G1. The next day, the dorsal epidermis becomes light brown (designated G2) as a result of the deposition of a pigmented pupal cuticle beneath the old larval cuticle. Approximately 1 day later, the entire body becomes brown (G3). The body colour turns darker and the integument hardens a further 2 days after G2, and this stage is designated G4. Approximately 3 days after deposition of the pupal cuticle (G2), pupae enter stage G5. None of the G5 larvae shed the old cuticle. If the old cuticle is removed with forceps, the treated insects are found to possess evaginated appendages, such as antennae, compound eyes and mandibles. They also have forewings covered with a tanned cuticle, hind wings with almost no tanned cuticle, and legs with a tanned cuticle. The larval prolegs with crochets disappear. These morphological characteristics indicate that the animals with tanned cuticles are complete pupae (Singtripop *et al*., 2000).

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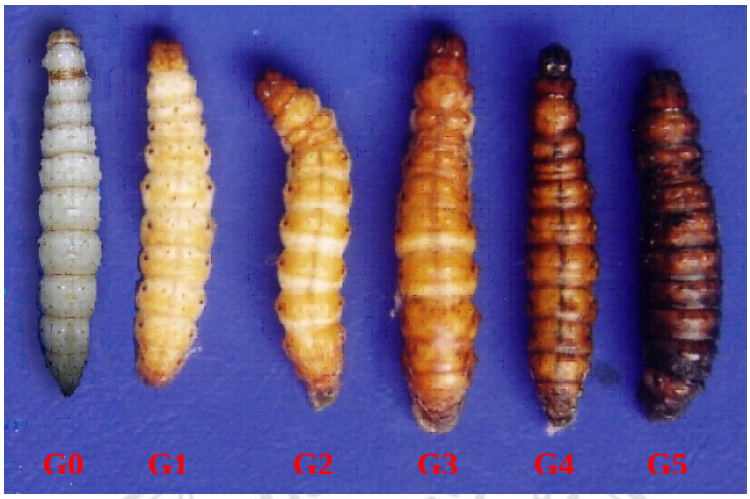

Figure 1.2 Pupal metamorphosis induced by S-methoprene (JHA) treatment of diapausing larvae of *O. fuscidentalis*. Larvae were treated once with 1 μg JHA by topical application. Progression of pupal metamorphosis graded from 1 to 5 (G1-G5) (Singtripop *et al*., 2000).

1.3.4 Diapause hormone and pheromone biosynthesis activating neuropeptide

Diapause hormone and pheromone biosynthesis activating neuropeptide (DH-PBAN) is an important insect neuropeptides that is reportedly involved in diapause regulation. DH-PBAN induces embryonic diapause by targeting developing ovaries in *B. mori* and terminates pupal diapause by activating the prothoracic gland (PG) to synthesize ecdysone in *Helicoverpa spp*. Moreover, DH-PBAN stimulates sex pheromone biosynthesis in female moths for male attraction (Denlinger, 1985; Zhang *et al*., 2004b). The embryonic diapause of the silkworm, *B. mori*, is induced by the diapause hormone (DH) which is secreted from the neurosecretory cells of subesophageal ganglion (SG) under the control of the other neuroendocrine organs and is a unique process of seasonal polyphenism that is induced transgenerationally in the bivoltine strain. Progeny diapause is determined by the mother's experience during embryonic development (Yamashita and Hasegawa, 1985). The diapause hormone (DH) is a 24-amino-acid peptide amide that is responsible for embryonic diapause and functions by acting on a G-protein-coupled receptor in the developing ovaries during pupal-adult development in females (Homma *et al*., 2006). Cloning of the cDNA for Bm-DH-PBAN resulted in a 800 bp nucleic acid sequence with a 192 amino acid open reading frame that encodes a polypeptide precursor from which DH, PBAN and three

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other neuropeptides (α-, β-, and γ-SG neuropeptide, SGNP) in various insects including *B. mori* (Choi and Vander Meer, 2009). The DH-PBAN gene is composed of 6 exons interrupted by 5 introns and is expressed in 12 neurosecretory cells of the SG. The DH-PBAN-producing neurosecretory cells (DHPCs) are contained in three neuromeres, four mandibular cells (SMd), six maxillary cells (SMx), two labial cells (SLb) located along the ventral midline and four lateral cells (SL), and the locations of the DHPCs are conserved among insect species (Ichikawa *et al*., 1995).

Previous studies the DH-PBAN genes have been characterized and examined its expression in many Lepidoptera. For example, the open reading frame of the cDNA for DH-PBAN in *Helicoverpa armigera* resulted in a 194 amino acid precursor protein that contains a 33-aa PBAN, a 24-aa DH-like peptide, and three other neuropeptides, all of which have a common C-terminal pentapeptide motif FXPR/KL (X ¼ G, T, S). A single message RNA corresponding to the size of Har-DH-PBAN cDNA from pupal SG with significantly higher expression in the SG of nondiapause pupae than diapausing pupae. DH-like peptide expression from SG of both males and females (Zhang *et al*., 2004a). The open reading frame of the DH-PBAN cDNA in *Samia cynthia ricini* encodes a 198 amino acid precursor protein that contains a 33-aa PBAN, a 24-aa DH-like peptide, and three other neuropeptides, all of which share a common C-terminal pentapeptide motif FXPR/KL (X=G, T, S). Northern blots demonstrate the presence of a 0.8-kb transcript in the subesophageal ganglion (SG). The DH-PBAN mRNA was detectable at much lower levels in other neural tissues, such as brain and thoracic ganglia (TG), but not in non-neural tissue, such as the midgut, silk gland, fat body or epidermis. The changes of PBAN-like immunoreactivity in the hemolymph are consistent with PBAN transcripts in the SG during pupal development and increases quickly at adult eclosion (Wei *et al*., 2004). The open reading frame of DH-PBAN cDNA in *Spodoptera exigua* encodes a 197 amino acid precursor protein that contains DH, PBAN, and three other SG neuropeptides, all of which share a conservative C-terminal pentapeptide motif FXPR/KL (X = G, Tor S). The Spe-DH-PBAN mRNA is expressed only in the SG. The expression level is consistently high during larval development. After pupation, the expression sharply decreases to a low level, and then increases again after eclosion (Xu *et al*., 2007).

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1.3.5 Development-dependent and photoperiod-controlled expression of the DH-PBAN gene

Diapause is under photoperiodic control in most insects (Tauber *et al*., 1986). In studies of the effects of photoperiod on the induction of diapause, different groups of experimental insects are usually exposed to a series of day-lengths (all with a 24-h light-dark cycle) and to continuous darkness (DD) and continuous light (LL). The diapause response is most frequently measured in terms of the percentage incidence of diapause in the experimental population. All photoperiodic responses have a feature in common, involving the apparent 'measurement' of day-length (or night-length) (Saunders, 2002). The most important feature of the response is the so-called critical day length (CDL) or critical night length (CNL) that separates the long photophases resulting in non-diapause development from the short photophases that ultimately lead to winter diapause, for example in the zygaenid moth *Pseudopidorus fasciata* enters winter diapause as a fourth instar larva when exposed to short days with a CNL of 10.5 h (Xue and Kallenborn, 1998). Both diapause induction and termination in this moth have been systematically investigated, all results indicate that the larvae are highly sensitive to photoperiod and both diapause induction and termination depend on whether the night-lengths exceed the CNL or not, i.e., 10.5 h for diapause induction; 10 h for diapause termination (Wei *et al*., 2001; Li *et al*., 2003). Thus, this moth is an excellent organism to examine whether photoperiodic time measurement is based on a qualitative or quantitative principle.

The bean bug, *Riptortus clavatus* THUKBERG, exhibits a facultative adult diapause which can be repeatedly induced by a short-day photoperiod and terminated by a long-day photoperiod. A photoperiod with a 13-hr photophase and an 11-hr scotophase (13L-11D) at 25°C, induced diapause but terminated it in some individuals reared under 10L-14D at 25°C. Moreover, the effect of change in photoperiod on diapause termination was examined in adults of *R. clavatus* reared under four diapause-inducing photoperiodic regimes: 8L-16D, 10L-14D, 12L-12D and 13L-11D. Under 12L-12D and 13L-11D, diapause was not terminated regardless of the nymphal photoperiod, but it was terminated in some of those reared as nymphs under 8L-16D or 10L-14D, showing a quantitative difference in the intensity (Numata and Hidaka, 1983).

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However, it is not clear whether such a quantitative difference is found also at long-day photoperiods. It is also unknown whether short-day photoperiods affect the rate of diapause development. Furthermore, it is unclear how the initial intensity of diapause development determines the duration of diapause.

1.3.6 Molecular action of JH on insect molting and metamorphosis

Studies of the action of JH at the molecular level have been hampered due to, in particular, the marked tendency of the hormone to bind to surfaces, but notable advances have been made in the last few years; the most important results have been summarized by Riddiford (2008). In this review, we briefly discuss the most recent findings including the long-awaited discovery of a JH receptor.

Methoprene-tolerant (Met) as a JH receptor

The *methoprene-tolerant* (*Met*) gene, first found in *Drosophila,* is a member of the basic helix-loop-helix (bHLH)-Per-Arnt-Sim (PAS) family of transcriptional regulators (Ashok *et al*., 1998). *Met* null mutants are resistant to the morphogenetic effect of the JH analog methoprene and are viable, although their fecundity is reduced (Wilson and Ashok, 1998). JH is necessary for egg maturation so that yolk protein uptake is controlled (Soller *et al*., 1999); therefore, it has been long thought that the Met protein is involved in the JH signaling pathway. In *Drosophila, germ cell-expressed* (*gce*) is known as a paralog of *Met* (Godlewski *et al*., 2006), but only *Met* has been found in non-*Drosophila* insects (Charles *et al*., 2011; Wang *et al*., 2007). In non-*Drosophila* insects such as *Tribolium*, knockout of *Met* RNA expression by the injection of dsRNA caused precocious metamorphosis, and those individuals are unresponsive to methoprene and JH-III (Konopova and Jindra, 2007; Parthasarathy *et al*., 2008). In *Drosophila*, the removal of CA causes the formation of smaller pupae and death at head eversion (Riddiford *et al*., 2010), and the *Met/gce* double mutant dies during the larval-pupal transition, although both *Met* and *gce* null single mutants are viable because of their redundancy (Abdou *et al*., 2011).

Met protein specifically binds JH-III and other biologically active JH mimics at physiologically relevant concentrations [K_d = 5.3 nM for JH-III in

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Drosophila (Miura *et al*., 2005); K_d = 2.94 nM for JH-III in *Tribolium castaneum* (Charles *et al*., 2011)]. This binding is through the C-terminal PAS-B domain (K_d = 12.3 nM) (Charles *et al*., 2011). Met forms Met/Met homodimers [also Met/GCE heterodimers in *Drosophila* (Godlewski *et al*., 2006)] in the absence of JH, and dimer formation was prevented by JH (Charles *et al*., 2011; Godlewski *et al*., 2006). In the mosquito, *A. aegypti*, a transcriptional coactivator of the ecdysteroid receptor complex FISC (Li *et al*., 2011) and a steroid receptor coactivator SRC (also known as Taiman) (Zhang *et al*., 2011), both of which belong to the member of the bHLH-PAS family, act as functional partners of Met in mediating JH action on target genes such as *Krüppel homolog 1* (Kr-h1). Unlike in the case of the Met/Met formation, Met/FISC and Met/SRC form a complex in the presence of JH (Charles *et al*., 2011; Li *et al*., 2011). Mutations of *Tribolium* Met within the ligand-binding pocket which disrupt JH binding did not affect the formation of the Met/Met dimer complex, but prevented the ligand-dependent dissociation of the Met/Met homodimers and the ligand-dependent interaction of Met with its partner SRC (Taiman) (Charles *et al*., 2011). The evidence of both biological actions and the characteristic nature of Met such as direct and specific binding to JH strongly suggest that Met is a JH receptor with SRC (Taiman) as a partner (Fig. 1.3A).

JH responsive genes and the JH signaling pathway

Kr-h1 is an evolutionarily conserved JH-induced gene that seems to be a crucial factor for molting and metamorphosis in both holometabolous and hemimetabolous insects (Duportets *et al*., 2012; Lozano and Belles, 2011; Minakuchi *et al*., 2008, 2009; Zhu *et al*., 2010). RNAi knockout of the *Kr-h1* gene induces precocious metamorphosis in *Tribolium* (Minakuchi *et al*., 2009) and *Blattella* (Lozano and Belles, 2011), but the JH analog methoprene was unable to rescue this effect so that Kr-h1 is considered to mediate the JH signaling pathway downstream of the hormone's interaction with its receptor. Consistent with this, when *Met* gene expression was suppressed, *Kr-h1* gene expression was also suppressed (Li *et al*., 2011; Minakuchi *et al*., 2009; Zhu *et al*., 2010). Kr-h1 is not only a crucial factor for JH control of larval molting but is also induced by

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ecdysteroid and interacts with other genes such as *broad* (Beck *et al*., 2004), SRC (Zhang *et al*., 2011), and FISC (Li *et al*., 2011).

The induction of *broad* expression by ecdysteroid does not occur until pupal commitment has been performed (Zhou *et al*., 1998b), and its ectopic expression in early second instar *Drosophila* larvae before the rise of ecdysteroid titer prevented molting to the third instar, but caused precocious pupae (Zhou *et al*., 2004). These results show that *broad* is one of the ecdysteroid-induced genes that both specify pupal development and mediate the prevention of the pupal-adult transformation (Zhou and Riddiford, 2002; Zhou *et al*., 2004). RNAi suppression of *Met* or *Kr-h1* expression by dsRNA stimulates broad expression so that precocious metamorphosis results (Konopova and Jindra, 2007; Minakuchi *et al*., 2009); it, therefore, appears that Met bound to JH stimulates Kr-h1 expression, thereby suppressing broad expression so that the larval state is maintained (Fig. 1.3B).

Met/SRC and Met/FISC are required for the expression of JH responsive genes including Kr-h1 (Li *et al*., 2011; Zhang *et al*., 2011), and SRC is required for the expression of ecdysteroid-responsive genes. Therefore, these factors as well as *broad* might mediate cross talk between JH and ecdysteroid to prevent the ecdysteroid-induced switch necessary for metamorphosis.

E75A is also induced by JH in *Drosophila* S2 cells (Dubrovsky *et al*., 2004). This induction of E75A is mediated by an intercellular pathway utilizing GCE/FTZ-F1 and Met/FTZ-F1 that forms transcriptionally active heterodimers, so that the removal of FTZF1 prevents JH activation of E75A (Bernardo and Dubrovsky, 2012; Dubrovsky *et al*., 2011). Although about 20% of E75A mutants undergo precocious metamorphosis in *Drosophila*, this transcription factor does not seem to be involved in the JH signaling pathway; rather the phenotype of the E75A mutant involves a feed-forward pathway that amplifies or maintains the ecdysteroid level, so that there is submaximal ecdysteroid induction of FTZ-F1 expression (Bialecki *et al*., 2002) in turn reducing the latter's ability to act as a competence factor facilitating JH (Dubrovsky *et al*., 2011) and ecdysteroid (Broadus *et al*., 1999) activation of gene expression.

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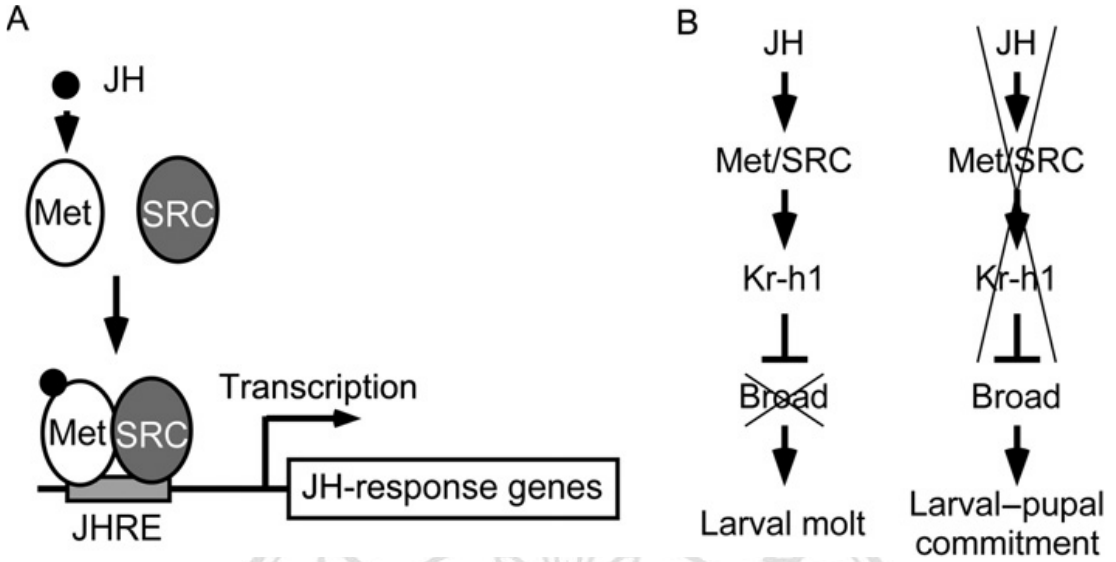

Figure 1.3 Model for Met as a JH receptor in insects (A) and JH signaling pathway during larval-pupal commitment (B) (Hiruma and Kaneko, 2013). JHRE, JH response element. See text for detailed explanations.

1.3.7 Molecular action of ecdysone on insect molting and metamorphosis

The identification of ecdysone as the key molting hormone in the 1950s is a milestone in the history of insect endocrinology. In 1954, Peter Karlson purified 25 mg of ecdysone crystals from 500 kg of silk moth pupae using the *Calliphora* bioassay to track the activity of the hormone (Butenandt and Karlson, 1954). In a series of chemical experiments and the analysis of the crystals, ecdysone was later shown to be a steroid hormone (Huber and Hoppe, 1965; Karlson *et al*., 1965). The first evidence that ecdysone has a direct role in regulating gene expression was based on the puffing of the salivary gland polytene chromosomes. Puffs are enlargements of specific loci on these giant chromosomes and were interpreted as local transcriptional activity. In particular, it was found that some of these puffs were induced rapidly after the addition of ecdysone to cultured salivary glands of the midge *Chironomus* (Clever and Karlson, 1960). Curiously, some of the puffs were responding rapidly to the hormone (early puffs), while others were delayed (late puffs). To test whether puffing was a direct consequence of ecdysone activity, a series of elegant studies by Clever (in *Chironomus*) and later by Ashburner (in *Drosophila*) tested whether protein synthesis was a requirement for the induction of puffs by ecdysone. These studies showed that the early puffs were still induced in the presence of protein synthesis inhibitors, while the late

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puffs were not. Ashburner also found that these early puffs are autoregulated because they failed to regress when protein synthesis was inhibited. Ashburner correctly predicted that the early puffs are direct targets of the ecdysone-bound receptor and that the corresponding early genes encode regulatory proteins that are required for inducing the late puffs (Ashburner, 1974) (Fig. 1.4A).

In *Drosophila*, all major developmental transitions, including the molts and the onset of metamorphosis, are triggered by major pulses of ecdysone (Riddiford, 1993). Each of these pulses has its own characteristics, such as amplitude and duration, which are largely determined by the rate and duration of hormone synthesis, how efficiently the hormone is converted to its biologically active form, and how fast it is degraded. Ecdysone is produced and released from the prothoracic gland cells, which are part of a composite endocrine organ called the ring gland. Once taken up by its target tissues, ecdysone is converted to the biologically active form 20-hydroxyecdysone (hereafter referred to as 20E) (Gilbert *et al*., 2002). Like vertebrate steroid hormones, 20E acts by binding to members of the nuclear receptor superfamily. These are ligand-dependent transcription factors that harbor a highly conserved DNA-binding domain (DBD) as well as less conserved ligand-binding domain (LBD) (King-Jones and Thummel, 2005). The identification of the *Drosophila* ecdysone receptor gene (*EcR*) and the discovery of several early ecdysone response genes established the molecular era of ecdysone biology in the early 1990s (Burtis *et al*., 1990; DiBello *et al*, 1991; Koelle *et al*., 1991; Segraves and Hogness, 1990).

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Ecdysone receptor and ultraspiracle

EcR requires heterodimerization with another nuclear receptor, Ultraspiracle (USP) to form a functional ecdysteroid receptor capable of binding to 20E with high affinity (Thomas *et al*., 1993; Yao *et al*., 1993). *EcR* encodes three protein isoforms, EcR-A, EcR-B1, and EcR-B2, as a result of two promoters and alternative splicing (Talbot *et al*., 1993). All three EcR isoforms are able to interact with USP, and all can bind to 20E with similar affinity. The crystal structure of the EcR LBD suggested that USP is required for forming a ligand-binding conformation, corroborating the observation that EcR alone cannot transcriptionally activate genes (Billas *et al*., 2003; Hu *et al*., 2003). Likewise,

structural studies demonstrated that the LBD of dipteran and lepidopteran USP is locked in an inactive conformation, consistent with the idea that ecdysteroids achieve transcriptional activation through binding to EcR (Billas *et al*., 2001; Clayton *et al*., 2001).

The EcR/USP heterodimer functions at the top of ecdysone regulatory cascade and triggers the transcription of primary and secondary response genes in ecdysone target tissues that play more direct functions during development (Fig. 1.4B). Mutations affecting the region common to all isoforms of *EcR* are embryonic lethal, consistent with the finding that ecdysone signaling plays a critical role during germ-band retraction in the developing *Drosophila* embryo (Bender *et al*., 1997; Kozlova and Thummel, 2003). *EcR-B1* is predominantly expressed in larval tissues that do not contribute to adult structures, and loss of *EcR-B1* function blocks the ecdysone responses in these tissues, resulting in a failure to complete metamorphosis (Bender *et al*., 1997; Schubiger *et al*., 1998). In contrast, the *EcR-A* isoform is expressed in imaginal disks and the ring gland, and animals mutant for *EcR-A* arrest development during late stages of pupal development (Davis *et al*., 2005; Talbot *et al*., 1993), indicating that the different EcR isoforms have distinct functions during development.

The EcR dimerization partner USP is the fly homolog of vertebrate RXR (Henrich *et al*., 1990; Oro *et al*., 1990). Like EcR, USP is required during embryogenesis and metamorphosis, consistent with the idea that USP acts as a key partner for EcR throughout development (Hall and Thummel, 1998; Oro *et al*., 1992; Perrimon *et al*., 1985). USP also dimerizes with the nuclear receptors DHR38 and Seven-up (Baker *et al*., 2003; Zelhof *et al*., 1995), and a recent report found that EcR forms functional dimers with DHR38 as well (Zoglowek *et al*., 2012). In addition, genetic evidence shows that *usp* is not required for the ecdysone-dependent induction of the larval glue genes, raising the possibility that EcR requires a different partner for this response (Costantino *et al*., 2008). The ability of nuclear receptors to form multiple heterodimers adds another layer of regulatory complexity that will be fascinating to unravel in the future.

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Early ecdysone response genes

The molecular characterization of three early ecdysone-inducible genes *BR-C*, *E74*, and *E75* revealed that all of them encode transcription factors, albeit belonging to different DNA-binding protein families (Thummel, 1990). These primary ecdysone response genes are key regulators of the ecdysone genetic hierarchy, which induce the transcription of secondary response genes that in turn execute the appropriate biological effects in response to an ecdysone pulse at the onset of metamorphosis (Fig. 1.4B).

Mutations that disrupt all *BR-C* functions (*npr1* alleles) result in larval lethality, indicating that *BR-C* is an essential gene for entry into metamorphosis (Kiss *et al*., 1988). The broad gene (hereafter referred to as *Broad-Complex* or *BR-C*) maps to the 2B5 early puff and is undoubtedly the most complex of the early genes. FlyBase currently acknowledges 14 transcript isoforms (McQuilton *et al*., 2012), and genetically the locus contains up to four complementation groups (DiBello *et al*., 1991). *BR-C* produces four protein classes, dependent on which zinc finger module, designated Z1 to Z4, is incorporated into a given isoform. The common N-terminal region comprises a BTB/POZ domain, which is a protein–protein interaction domain commonly found in chromatin and transcription factors. The zinc fingers are believed to confer target specificity (DiBello *et al*., 1991; Zollman *et al*., 1994). However, high-affinity DNA binding was never established for BR-C proteins, and existing EMSA (Xiang *et al*., 2010) and footprinting (Von Kalm *et al*., 1994) studies all used uncommonly high BR-C concentrations to achieve DNA binding. Future studies will have to address whether BR-C recognizes its target genes via binding to DNA elements or through interactions with other chromatin-bound proteins, in which case the zinc finger domains may have a less direct role in target gene recognition.

Like *BR-C*, *E74* is directly induced by ecdysone and responsible for the 74EF early puff. Mutations in *E74* confer pupal lethality, indicating that this gene plays essential roles during metamorphosis. *E74* produces two protein isoforms, E74A and E74B, which share a C-terminal ETS DBD (Burtis *et al*., 1990). Both isoforms are precisely controlled by changes in ecdysone titers and display

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complementary profiles. *E74A* is induced when hormone concentrations are high, while *E74B* is abundant when ecdysone concentrations have fallen to intermediate or lower levels. Correspondingly, *E74A* transcript levels fall when ecdysone concentrations start to decline and *E74B* mRNA are repressed by rising hormone titers. This behavioral link between the two isoforms is critical for the proper timing of secondary gene responses (Fletcher *et al*., 1997; Urness and Thummel, 1995).

The *E75* early gene maps to the 75B early puff and encodes a member of the nuclear receptor superfamily. E75 forms at least three protein isoforms (E75A-C) (Segraves and Hogness, 1990). Like all *Drosophila* nuclear receptor genes, alternative splicing tends to produce protein isoforms that differ in their N-terminal sequences but share a common LBD in the C-terminus. This is not any different for E75; however, the E75B isoform represents an unusual nuclear receptor protein: While E75A and E75C both have a complete DBD and LBD domain, splicing of *E75B* removes a part of the DBD domain, which abolishes its ability to bind to DNA. This splice form appears to be a fairly ancient invention, as its closest fly homolog, *E78*, also generates a protein isoform (E78B) with a truncated DBD domain (Stone and Thummel, 1993). Mutations specific for *E75B* are viable, however, molecular data demonstrated that E75B binds to another nuclear receptor, DHR3, in an inhibitory fashion to delay the induction of a third nuclear receptor, βFTZ-F1 (White *et al*., 1997). It should be noted that *E75B* null mutants do not display defects in the timing of *βftz-f1* expression, raising the possibility that E75B and E78B are functionally redundant (Russell *et al*., 1996; Stone and Thummel, 1993).

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In contrast to *E75B*, animals mutant for *E75A* display larval lethality, molting defects, and developmental delays, while *E75C* is required for late pupal development and adult viability (Bialecki *et al*., 2002).

Early-late ecdysone response genes

Early-late genes can be operationally defined as genes that require both the 20E-bound EcR/USP heterodimer and an early gene product for maximal transcriptional induction (Fig. 1.4B). This is typically shown in organ culture assays using protein synthesis inhibitors to block the translation of early gene mRNAs. Two early-late genes with very similar temporal expression profiles are the nuclear receptor genes *DHR3* and *DHR4*. DHR3 is orthologous to the vertebrate retinoid-related orphan receptor (ROR), while DHR4 is represented by the germ-cell nuclear factor (GCNF) in vertebrates. DHR3 and DHR4 expression profiles show a peak at the beginning of prepupal stage, when the expression of early genes such as *BR-C*, *E74A*, and *E75A* is receding, and *βftz-f1* expression is about to be induced. Both DHR3 and DHR4 are sufficient to repress the early genes and are required for maximal *βftz-f1*expression in mid-prepupae (King-Jones *et al*., 2005; Lam *et al*., 1997), strongly suggesting that these two factors act in concert to regulate the early genes and *βftz-f1*. Interestingly, *DHR4* mutants display precocious wandering behavior followed by premature onset of metamorphosis, resulting in a small body size due to a shortened feeding period, a phenotype not observed in any other mutants associated with the ecdysone hierarchy (King-Jones *et al*., 2005). Taken together, the interplay between nuclear receptors E75, DHR3, and DHR4 controls the expression of *βftz-f1* during the prepupal stage, thereby safeguarding the appropriate sequence of programs necessary for the progression of pupal development.

The ecdysone regulatory cascade is best understood at the onset of metamorphosis, and the finding that some components of the hierarchy might also play important roles in the production of ecdysone is not entirely new. One of the first indicators was that *EcR-A* is expressed in the prothoracic gland but not the other two isoforms encoded by *EcR* (Talbot *et al*., 1993). Another study reported that USP modulates PTTH-dependent ecdysone synthesis in Manduca prothoracic glands (Song and Gilbert, 1998). Together, these results suggested that EcR and USP may have roles in ecdysteroidogenesis, possibly through negative feedback regulators in response to rising levels of 20E. Finally, a null mutation in *E75A*

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causes a dramatic decrease in ecdysone levels, indicating that E75A plays a dual role, acting both downstream of ecdysone as an 20E target during the onset of metamorphosis and also upstream of ecdysone as a regulator of ecdysone production in the prothoracic gland (Bialecki *et al*., 2002).

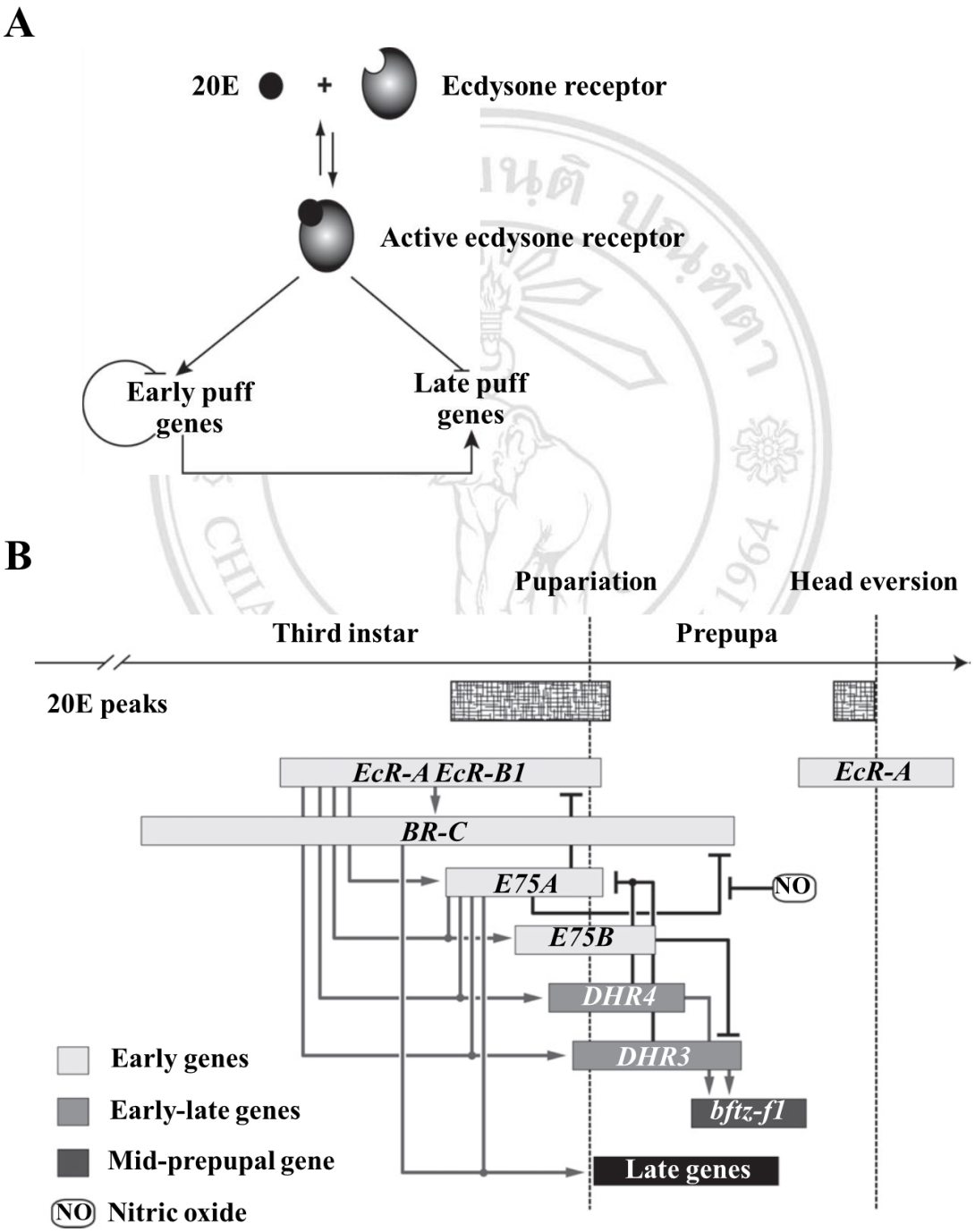

Figure 1.4 Ecdysone hierarchy. (A) Ashburner model. The hormone-bound and thus active ecdysone receptor directly induces the expression of early puff genes and

represses late puff genes. A small set of early puff genes repress their own expression and are required for the induction of a large set of late puff genes (Ashburner, 1974). (B) Overview of *Drosophila* ecdysone hierarchy genes at the onset of metamorphosis. The expression of genes is shown in bars with different shades of gray representing different gene categories (see inset), and the length of the bars indicates the approximate duration of their expression. Positive and inhibitory interactions are shown. Ecdysone peaks are shown in dotted boxes at the top, and approximate timing of puparium formation and head eversion are indicated by dotted lines (Ashburner *et al*., 1974).

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