EVOLUTIONARY CHANGES IN ENDOCRINE REGULATION OF DIAPAUSE TRIGGER DIVERSIFICATION BY A SHIFT IN LIFE CYCLE TIMING

By

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To my parents and Chao, for your love and support

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Many organisms have evolved dormancy as a trait to synchronize life cycle timing with seasonal resource availability. For temperate insect herbivores, diapause plays a critical role in synchronizing their life cycles with permissive conditions, such as newly flushed leaves and fruit ripening of their host plants. Rapid evolution of life cycle timing via shifts in the timing of diapause to ongoing climate change has been reported in several species. While diapause is often regarded as a "resting" stage, it is actually physiologically dynamic and regulated by the neuroendocrine system. However, the endocrine mechanisms as well as the upstream molecular events underlying rapid adaption in diapause timing remain unknown.

Here I conducted endocrine studies in two classic models of ecological speciation via divergence in diapause timing, the apple maggot (*Rhagoletis pomonella*) and the European corn borer (*Ostrinia nubilalis*). Each species has early and late diapausing races or strains that affect the timing of adult activity, thus generating reproductive isolation. They are the early emerging apple and late emerging hawthorn host races in *R. pomonella*, as well as the early emerging BE strain and late emerging UZ strain in *O. nubilalis*. The shifts in diapause timing in both species are associated with release timing of ecdysteroids and the timing of ecdysteroid sensitivity, a key hormone that promotes the start of morphogenesis after pupal diapause in *R. pomonella* and

larval diapause in *O. nubilalis*. Pigment dispersing factor and its receptor were investigated as potential regulators upstream endocrine regulation of diapause in *O. nubilalis*, but the roles in endocrine regulation of diapause were inconclusive.

Further studies characterizing the cellular regulation of larval diapause were conducted in *O. nubilalis*. Larval diapause in *O. nubilalis* is characterized by cell cycle slowdown in both the G0-G1 and G2 phases. Additionally, shallower suppression of the cell cycle during diapause and an earlier resumption of the cell cycle in the BE strain are associated with earlier diapause termination in the BE strain than UZ strain.

CHAPTER 1
INTRODUCTION

Life History Adaptation

For organisms living in seasonal environments, resources are not always available to allow for continuous growth and reproduction all year round. Developing before or after times of resource availability could result in adverse fitness consequence, such as reduced survivorship and fecundity. Therefore, it is important for organisms to synchronize life cycle timing with seasonal environments. Climate change has substantial impacts on seasonal conditions, such as increased overwinter temperatures, a reduction in the duration of winter, and an earlier onset of spring (Williams *et al.*, 2015).

In response to climate change, some organisms will be positively affected, while some may be negatively affected, which can result in "winners" and "losers" respectively (Somero, 2010). For the winners, their population sizes will increase or remain stable after climate change, while losers will show a decrease in population size, geographic ranges, or even become extinct (Clucas *et al.*, 2014). For example, a spring-feeding geometrid moth, *Agriopis aurantiaria*, appears to be a winner because of the increased synchrony between its egg hatching and the budburst of its host plant, sub-Arctic birch, under higher spring temperatures (Jepsen *et al.*, 2011). Because young larvae of *A. aurantiaria* survive better on newly sprouted birch leaves than old leaves, the increased synchrony between the hatch timing of this moth and its host's budburst could lead to increased populations and even an intensive outbreak of this forest pest. Conversely, the caribou, *Rangifer tarandus*, is expected to become a climate change loser because caribous have become seasonally decoupled from the peak availability of their host plants. Specifically, the growing season of host plants has been advancing faster than the migration timing of caribous into the available host-plant ranges, thus causing a mismatch

between peak foliage quality and calf feeding times that results in decreased calf production (Post & Forchhammer, 2008). In the context of climate change responses, "winners" and "losers" are often determined by the degree of synchrony between life history timing of organisms and the availability of their resources. Therefore, further study of adaptation in life history timing is critical to predict and mitigate the effects of climate change on organisms.

Many organisms have evolved dormancy as a trait to synchronize life cycle timing with seasonal resource availability. For temperate insect herbivores, diapause is a form of programmed dormancy that plays a critical role in synchronizing their life cycles with permissive conditions, such as newly flushed leaves and fruit ripening of their host plants. Many examples of shifts in life cycle timing through diapause adaptation in response to climate change can be found in literatures. For example, a shift in life cycle from populations of the fall webworm *Hyphantria cunea* (Drury) is ongoing where populations have shifted from being bivoltine to trivoltine in Fukui, Japan (Gomi *et al.*, 2007). This strategy of life history shift is achieved by using shorter daylength (i.e., longer growing season) to induce larval diapause for *H. cunea* thus allowing another generation each fall. Altered diapause timing can have major fitness consequences for some insect species because shortened or prolonged diapause duration could lead to asynchrony with critical seasonally available host plant resources. Therefore, understanding the endocrine mechanisms under selection for rapid adaptive shifts in diapause timing could help us to predict organisms' ecological responses to climate change and provide insight for conservation efforts. Thus an evolutionary endocrinology perspective may be especially helpful in developing our understanding of the potential for the evolution of lifecycle timing in response to altered seasonality and climate change.

Evolutionary Endocrinology

During the past few decades, the field of evolutionary biology has increasingly focused on the evolutionary genetics of life history traits and development from a micro-evolutionary perspective. Hormones, a class of chemical mediators that act as signals within an organism's body, influence many life history traits and components of development, including body size, wing polymorphisms, and the timing of metamorphosis (Zera *et al.*, 2007; Denver *et al.*, 2009). To better understand life histories and development from a micro-evolutionary perspective, it is important to have a corresponding detailed understanding of the micro-evolutionary endocrine mechanisms underlying various phenotypic expressions (Zera *et al.*, 2007). The need described above gives rise to the synthesis of evolutionary endocrinology.

Evolutionary endocrinology, a subdiscipline of evolutionary physiology, integrates endocrine pathways into evolutionary models and analysis (Zera & Huang, 1999; Nepomnaschy *et al.*, 2009). The broad goal of evolutionary endocrinology is to develop a mechanistic view of organisms' responses (i.e., phenotype) to natural selection and other modes of evolutionary change (Denver *et al.*, 2009). Evolutionary endocrinology becomes increasingly important for not only focusing on understanding how hormonal signaling systems evolve themselves (Denver *et al.*, 2009), but also for exploring the roles of the endocrine system in shaping evolutionary phenomena, such as life history evolution (Cox *et al.*, 2016).

Endocrine Regulation of Evolutionary Changes in Life History Timing

Environmental cues received by organisms are transduced by the neuroendocrine system into corresponding physiological and behavioral responses. Altered endocrine patterns, such as hormone concentrations (titers), the timing of hormone synthesis, and hormone receptivity (e.g., sensitivity) can trigger evolutionary changes in life history traits (Nijhout, 2003). For example, in buckeye butterfly, *Precis coenia*, a light beige fore wing (the *linea* form) under spring and

summer and a dark reddish-brown fore wing (the *rosa* form) under autumn is associated with an earlier rise of ecdysteroid titers after pupation in the *linea* form compared to the *rosa* form (Rountree & Nijhout, 1995). Seasonal polyphenism is also broadly associated with evolutionary changes in hormone titers in Lepidoptera. The African butterfly, *Bicyclus anynana*, shows phenotypic variation in eyespot size as a response to wet and dry seasons, which low or high temperatures that larvae experienced lead to small (dry-season form) or large (wet-season form) adult ventral eyespots, respectively (Brakefield & Mazzotta, 1995; Kooi & Brakefield, 1999). It turns out that adults with larger ventral eyespot wings have higher hormone (ecdysteroids) titers shortly after pupation than smaller eyespot adults do (Koch *et al.*, 1996; Brakefield *et al.*, 1998). The lower hormone titers are triggered somehow by temperature and photoperiod cues that also signal diapause for temperate insect species. Shifts in life history timing via diapause could be also associated with evolutionary changes in endocrine regulation of diapause.

Diapause is a programmed response to environmental stimuli that precede the arrival of unfavorable conditions. While diapause is often regarded as a "resting" stage, it is actually physiologically dynamic with three distinct phases: initiation, maintenance, and termination (Koštál, 2006). In insects, diapause happens at one species-specific life-stage, which can be egg, larva, pupa, or adult. Like other life history traits, diapause is regulated by the neuroendocrine system (Denlinger, 2002). Ecdysteroids are critical endocrine signals of morphogenesis and molting. Larval and pupal diapauses are characterized by failure to molt to the next metamorphic stage, thus, ecdysteroids needed to initiate the next molt can be a critical regulatory step for larval and pupal diapause (Denlinger *et al.*, 2012). Based on previous studies, larval and pupal diapause initiation is hormonally controlled by a suppression of ecdysteroid production throughout the maintenance phase, and then at the end of diapause ecdysteroids are produced and

released thereby triggering the resumption of morphogenesis (Denlinger *et al.*, 2012). To uncover the endocrine mechanisms underlying evolutionary changes in life history timing that are due to changes in larval or pupal diapause timing, it is important to determine the ecdysteroid profiles (e.g., titers, production timing, and sensitivity) of larval and pupal diapause.

Rhagoletis pomonella and *Ostrinia nubilalis*, Evolutionary Models of Shifts in Life Cycle Timing

Specialists can be more susceptible to population declines or even extinctions than generalists can when faced with climate change (Biesmeijer, 2006). Specialists can be more affected when resources are declining in their habitats because they do not have access to alternative resources that generalists may have access to. Because generalists can use a greater diversity of resources, if a resource is impacted by climate change, they are more likely able to shift to another resource that is less influenced by climate change (Clavel *et al.*, 2011). Though specialists can be more sensitive to changing climate than generalists, specialist insects make up the bulk of metazoan biodiversity (Jaenike, 1990; Bush & Butlin, 2004). Understanding specialist insects' responses to ongoing climate change is very important for conservation purposes, and specifically for maintaining biodiversity. The study system in my second chapter is the apple maggot *Rhagoletis pomonella*, a specialist and a textbook example of ecological speciation. *Rhagoletis pomonella*, originally only infested hawthorn, but some shifted to introduced host plant apple. Apple race flies of *R. pomonella* has shifted their life cycle timing one month earlier than hawthorn race flies to match the earlier fruiting of apples. Specialists like *R. pomonella* are closely tied to the phenology of their hosts may be more susceptible to climate change and shifting phenologies.

In my remaining three data chapters I used the European corn borer *Ostrinia nubilalis*, which as a model of divergence in life cycle timing. Similar to *R. pomonella*, *O. nubilalis* has

two strains with an earlier-emerging and later-emerging life cycles. The earlier-emerging BE strain shift their life cycle earlier than the later-emerging UZ strain by terminating larval diapause earlier in spring. *Ostrinia nubilalis* is a generalist but a major pest of cultivated maize in Eurasia and North America (Coates *et al.*, 2018). *Ostrinia nubilalis* can have many generations in the laboratory as long as the conditions (photoperiod and temperature) permit, which allows me to carry out experiments to explore potential molecular mechanisms besides the endocrine basis underlying the adaptation of life history timings.

Research Hypotheses and Aims

This dissertation focuses on investigating the endocrine basis of a shift in diapause timing during the ecological divergence in insects. The general hypothesis of this dissertation is that evolutionary changes in hormonal regulation are associated with rapid evolution of life cycle timing via diapause. In this dissertation, I also test for potential molecular events related to altered patterns of hormones and cellular biology (cell proliferation) during diapause involved in the evolution of life cycle timing. The above hypotheses and objectives were addressed in four research chapters:

Aim 1: Hormonal Regulation of Diapause

- **Chapter 2**: A shift in life cycle timing is associated with changes in hormonal regulation of diapause between the earlier-emerging apple host race versus the later-emerging hawthorn race of *Rhagoletis pomonella*. I predict that a) an earlier peak of ecdysteroid production occurs in apple race pupae during the post-winter stage and/or b) apple race pupae become sensitive to exogenous ecdysteroid earlier than hawthorn race pupae, corresponding to the earlier emergence of apple race flies in summer than hawthorn race flies.

- **Chapter 3:** Similar to *Rhagoletis pomonella*, I predict that an earlier production of ecdysteroids required for diapause termination and earlier sensitive stage to exogenous ecdysteorids in earlier-emerging bivoltine E-strain (BE) than later-emerging univoltine Z-strain (UZ) of *Ostrinia nubilalis* are associated with the divergence in life cycle timing between the BE strain and UZ strain. This chapter will allow me to test the extent to which similar alterations in ecdysteroid concentrations and sensitivity may affect a similar shift in life history timing across deeply divergent insect taxa.

Aim 2: Molecular Mechanisms of Altered Hormone Patterns

- **Chapter 4:** This chapter aims to explore the role of potential upstream elements, pigment-dispersing factor (PDF) and pigment-dispersing factor receptor (PDFR) on the altered hormone patterns of diapause regulation in *O. nubilalis*. PDF and PDFR are well known for their regulation of insect circadian rhythms, and PDF may be involved in regulation of larval diapause by controlling the production of ecdysteorids after receiving photoperiodic cues. The novel role of PDF in stimulating ecdysone biosynthesis has been only reported in *Bombyx mori* to date (Iga *et al.*, 2014), I predict that PDF can also stimulate ecdysone biosynthesis in *O. nubilalis*. In addition, the sensitive stage of prothoracic glands to PDF occurs earlier in earlier-emerging BE strain than later-emerging UZ strain after exposure to diapause-termination conditions.

Aim 3: Cellular Regulation of Diapause

- **Chapter 5:** Cell cycle slowdown is one of hallmarks of insect diapause, and resumption of active cell cycling is indicative of diapause termination. However, we know little about the cellular regulation of diapause in *O. nubilalis*. Also, whether cell cycle resumption coincides with or priors to ecdysteroid production to trigger diapause termination remains unknown. This chapter is directed at characterizing the cell cycle state of larval diapause in European corn borer (*Ostrinia nubilalis*). I predict that a) cells of diapausing larvae in *O. nubilals* arrest in G0/G1 and/or G2 phase of cell cycle, and b) the resumption of cell cycle proliferation occurs earlier in earlier-emerging BE strain than later-emerging UZ strain under diapause-termination conditions.

CHAPTER 2
AN EARLIER PUPAL DIAPAUSE TERMINATION IN APPLE RACE FLIES THAN HAWTHORN RACE FLIES OF *RHAGOLETIS POMONELLA* IS ASSOCIATED WITH AN EARLIER PULSE OF ECDYSTEROIDS AND AN EARLIER ECDYSTEROID SENSITIVITY

Introduction

Organisms living in areas with strong seasonality have a limited period for growth and reproduction each year. Synchrony between an organism's life cycle and the timing of available resources is critical because development before or after times of good resource availability could result in adverse fitness consequences, such as reduced survivorship and fecundity. Anthropogenic climate change can have a big impact on seasonal conditions, especially winter temperatures, winter length, and the frequency of extreme events on the seasonal shoulders in the fall and spring (Kunkel, 2004; Williams *et al.*, 2015). Changes in winter length can lead to shifts in the phenology of some plants and animals. For example, earlier flowering of trees resulting from an earlier ending of winter (Root *et al.*, 2003; Sherry *et al.*, 2007; Colautti *et al.*, 2017) could drive an earlier emergence of caterpillars in spring (Buse and Good 1996), and an earlier migration of birds that exploit caterpillars as food for their nestlings (Walther *et al.*, 2002). Because of this kind of multitrophic relationship, changing the phenology of organisms at one trophic level could cause changes in many aspects of interacting species and even communities (Chapin *et al.*, 1997).

To maximize fitness, insect herbivores must synchronize their active life cycle stages with available host plant resources and dormant stages to escape adverse environmental conditions. The dormant stage, diapause in many insect species, is a critical phase for seasonal life cycle synchronization. Diapause is a state of developmental slowdown with depressed metabolic activity, that is regulated by hormones. Unlike quiescence, a direct response to adverse environmental conditions, diapause is a pre-programmed response to a combination of

environmental stimuli (e.g., photoperiod and temperature) that precedes the arrival of unfavorable conditions. Phenological shifts in response to climate change via shifts in the timing of diapause have been reported in several insect species. For example, pitcher plant mosquitoes, *Wyeomyia smithii*, enter diapause later as a response to longer, warmer growing seasons (Bradshaw & Holzapfel, 2001). Over the last three decades, *W. smithii* has experienced genetic changes in the critical photoperiod for diapause induction that track climate-change induced alterations to the onset of winter in the fall. Similarly, in the winter moth, *Operophtera brumata*, there has been selection for genotypes whose eggs hatch (a product of egg diapause termination) later in the season to match the altered host phenology resulting from climate change (van Asch *et al.*, 2013).

Evolutionary changes in hormonal regulation have been regarded as a potentially critical mechanism underlying life history adaptation (Zera *et al.*, 2007). Numerous studies have shown that interspecific and intraspecific variation in life history traits (e.g., phase, caste, and wing polymorphism) can be due to evolutionary changes in endocrine characteristics, such as hormone concentrations (titers), the timing of hormone synthesis or release, and hormone receptivity (e.g., sensitivity) (Nijhout, 2003). For example, the East African butterfly, *Bicyclus anynana* displays distinct adult life histories. Adults develop fast and have short life spans under warm, wet seasons. In contrast, adults from cold, dry seasons have longer life spans and delayed reproduction compared to wet-season adults. It has been shown that the different life history strategies under these two different environmental conditions are correlated with an earlier ecdysteroid titer peak in wet-season pupae than dry-season pupae of the butterfly *B. anynana* (Oostra *et al.*, 2014). While extensive studies have been done on evolutionary modification of hormonal regulation of polymorphism in life history traits, less attention has been paid to the

endocrine basis of phenological shifts, like those predicted as a response to climate change. While diapause is often regarded as a "resting" stage, it is actually physiologically dynamic and regulated by the neuroendocrine system (Denlinger, 2002). Therefore, we expect that an evolutionary change in endocrine regulation may also be involved in diapause timing adaptation in response to climate change.

Here, I used the apple maggot, *Rhagoletis pomonella* to test the hypothesis that rapid adaptation in seasonal phenology will be associated with alterations in endocrine regulation of diapause. *Rhagoletis pomonella* is a classic model of ecological speciation as a result of temporal reproductive isolation. This fly originally infested fruits of a native host, the downy hawthorn (*Crataegus mollis*). A novel host race of this fly species formed on apples sometime in the mid-1800s after the introduction of domesticated apples (*Malus pumila*) to the United States by European colonists around 400 years ago (Feder *et al.*, 1988). Both of the host races are specialists on their own host plant with only one generation per year (Figure 2-S1). They have a narrow seasonal phenology with adults living only about a month. Each host race must synchronize the timing of adult emergence and egg laying to occur at the right time of the year for ripe host fruits to be available. Because the two host plants flower and fruit at different times in the summer, approximately one month apart in our sympatric field sites in the Midwestern USA, differential timing of host-fruit resources has driven divergence in the timing of adult emergence and reproduction in each host race. After developing in fruits for a few weeks, larvae drop into the soil and enter pupal diapause until the next summer when they will terminate diapause at the appropriate time to emerge as adults and lay eggs in their host fruits. Thus, diapause plays a critical role in life history timing. Because apple trees fruit one month earlier than hawthorn trees and the host fruits are only available for a few weeks, it is critical for apple

flies to terminate diapause and emerge earlier than hawthorn flies to coincide with the availability of apples, contributing to divergence between the two host races due to allochronic reproductive isolation.

Heritable differences in post-winter adult emergence time have been identified between the apple and hawthorn host races of *R. pomonella*. For the first-generation offspring of wild flies, apple flies emerge earlier than hawthorn flies when both are reared under identical conditions (Smith, 1988). Furthermore, laboratory crossing experiments between the apple and hawthorn host races showed that the eclosion time of reciprocal F1 hybrid progeny was intermediate to their parental host races (Smith, 1988). There are also significant differences in allele frequencies at numerous loci spread across three of the six fly chromosomes that are correlated with diapause timing between the two host races (Feder *et al.*, 1993; Michel *et al.*, 2010; Egan *et al.*, 2015; Ragland *et al.*, 2017). These observations demonstrate that there is a genetic basis for the rapid evolution of life cycle timing via diapause timing. Yet, the genetic architecture of lifecycle timing in *Rhagoletis* is complex with many loci associated, and thus we do not know the exact genes or cellular/biochemical processes modified during this adaptive shift. In the current study, I took a targeted physiological approach describing the endocrine correlates of the micro-evolutionary shift in life cycle timing between the earlier emerging apple and later emerging hawthorn host races of *R. pomonella*.

Based on previous studies in other insect species, pupal diapause initiation is hormonally controlled by a suppression of ecdysteroid production throughout the diapause maintenance phase, and then ecdysteroids are produced to trigger the resumption of adult morphogenesis (Denlinger *et al.*, 2012). From previous work in *R. pomonella* and other pupal diapausing insects it is unclear whether: 1) the diapause maintenance phase is ended by a pulse of ecdysteroids that

triggers the resumption of development, or 2) whether diapause is terminated prior to the pulse of ecdysteroids that triggers adult morphogenesis with the pulse of ecdysteroids being a downstream product of diapause termination (Ragland *et al.*, 2011; Chen *et al.*, 2016; Yamada *et al.*, 2016).

One way to understand adaptive shifts in life cycle timing via pupal diapause regulation is to test for evolutionary changes in the ecdysteroid regulation of pupal diapause. For this study, I specifically tested the hypothesis that a shift in ecdysteroid signaling is associated with divergence in lifecycle timing between the apple and hawthorn host races of *R. pomonella*. Within my hypothesis I tested three specific predictions. First, I predicted that the earlier emerging apple host race would show an earlier peak of ecdysteroids after simulated overwintering than the later emerging hawthorn host race. Second, I predicted that the earlier pupal diapause termination in the apple race would be associated with higher concentration of ecdysteroids. Third, I predicted that the earlier pupal diapause termination in the apple host race would be associated with an earlier increased sensitivity to ecdysteroids, especially in the post-winter period. In this set of experiments I quantified ecdysteroid concentrations, the timing of ecdysteroid release, and sensitivity to exogenous 20-hydroxyecdysone (20E) between apple and hawthorn race pupae across the pre-winter, over-winter, and post-winter stage of their lifecycles. In my study, I found that the apple race released ecdysteroids earlier than the hawthorn race after winter, but there was no difference in ecdysteroid concentrations during the peak between two host races. In addition, the apple race was more sensitive to exogenous 20-hydroxyecdysone than the hawthorn race prior to winter and earlier after winter.

Materials and Methods

Insect Collection and Rearing

I collected apple fruits from feral trees infested with *R. pomonella* from Grant, MI, USA (43.34°N, 85.81°W), on 10 August 2016, and hawthorn fruits from Grant, MI and Lansing, MI, USA (42.73°N, 84.56°W), on 13 September 2016. The yield of *R. pomonella* pupae from hawthorn fruits collected from Grant, MI was too low to directly compare ecdysteroid titers and sensitivity between the two host races at Grant, MI. However, I did collect enough hawthorn pupae from the Lansing site to compare both titers and sensitivity to apple pupae at the Grant site. Although apple race individuals from Grant, MI, and hawthorn race individuals from Lansing, MI, do not represent a co-occurring sympatric population pair, these sites are located in the same ecogeographic region at similar latitudes and the ~1 month fruiting time difference mirrors differences observed at sympatric sites throughout MI. Both sites also have similar differences in allele frequencies between the apple and hawthorn host races and have been successfully used for other comparative mechanistic studies (Dambroski & Feder, 2007; Ragland *et al.*, 2012). Furthermore, we did obtain enough hawthorn pupae from the Grant site to assess the degree of similarity in endocrine timing and titers during the post-winter stage between hawthorn pupae collected at Grant and Lansing. I detected no difference in the timing and titers of ecdysteroids between the hawthorn flies from Grant and those from Lansing (Figure 2-S2), further justifying our ability to directly compare Lansing, MI hawthorn race flies with apple race flies from Grant, MI.

Infested fruits were transported to the laboratory located at University of Florida, and kept in a room at 24±1°C and light: dark (L: D) 14:10. Fruits were placed in wire-mesh baskets suspended over plastic trays, so wandering larvae exited the fruits and were collected from trays.

Wandering larvae were collected daily and placed in petri dishes with moist vermiculite for pupariation. Petri dishes were kept at 24±1°C and L: D 14:10. Previous studies have shown that the metabolic rates of diapausing and non-diapausing pupae start to diverge by day seven after pupariation (Ragland *et al.*, 2009), allowing us to clearly distinguish diapausing pupae from pupae that were developing towards adulthood. After 7 days, pupae were removed from vermiculite and placed into individual 5 mL Norm-JectTM syringes (Air-Tite Produts, Virginia Beach, VA, USA) fitted with three-way luer valves (Cole-Parmer, Vernon Hills, IL, USA) for respirometry. *Rhagoletis pomonella* pupae are small enough to fit into the barrel of the leur fitting and thus the syringe plunger could be fully depressed to clear gas from the syringe chamber. After phenotyping with respirometry, diapausing and non-diapausing pupae were placed in clean petri dishes separately in chambers with 85% humidity maintained by a saturated potassium chloride solution (Winston & Bates, 1960). Chambers were kept in the room with 24±1°C and L: D 14:10. A subset of diapausing pupae were used for pre-winter ecdysteroid quantification and sensitivity experiments. Remaining diapausing pupae were transferred to 4±1°C and dark conditions at day 13 after pupariation to simulate overwintering. After 16 weeks in the chilling treatment, pupae were transferred to an incubator at 24±1°C, L: D 14: 10 for post-winter development (Ragland *et al.*, 2011).

Sampling for Ecdysteroid Quantification

I first characterized ecdysteroid concentration in individuals at the following stages before we could distinguish diapause-destined individuals from non-diapause pupae: wandering larvae, pupariation (day 0), as well as days 2, 4, and 6 after pupariation. At day 8 after pupariation, respirometric measurements were conducted to classify pupae as diapausing or non-diapausing where animals that were not in diapause had noticeably higher metabolic rates,

greater than 3 times higher than those in diapause (Ragland *et al.*, 2009). Diapause and non-diapause pupae of both host races were haphazardly sampled every third day from 8 days post-pupariation to 20 days post-pupariation on days 8, 11, 14, 17, and 20. For the over-wintering stage, diapausing pupae from each of the two host races were sampled after simulated overwintering at 4±1°C in a dark refrigerator for 8, 12, or 16 weeks. For the post-winter diapause termination phase, after simulated overwintering at 4±1°C for 16 weeks, pupae were transferred into an incubator at 24±1°C, L: D14: 10. Starting 10 days after transfer, respirometry was conducted daily to track the developmental trajectory of each pupa (Ragland *et al.*, 2009). I used stop-flow respirometry (Lighton, 2008) to physiologically phenotype individuals for diapause termination and post-diapause development in both host races of *R. pomonella*. I followed the developmental trajectory established by Ragland *et al.* (2011). Specifically, I sampled pupae of both host races from early after removal from simulated overwintering that were still in diapause (24h after transfer from 4±1°C to 24±1°C), late after removal from simulated overwintering and still in diapause (two weeks after transfer from 4±1°C to 24±1°C, but prior to the initial metabolic rate increase), 1 and 2 days after the initial metabolic rate increase, and the middle of the plateau of the metabolic trajectory (10 days after the initial metabolic rate increase) for ecdysteroid quantification. I weighed each pupa and pooled six pupae together as one sample. I then snap froze the pooled individuals in liquid nitrogen. Samples were stored at -80°C until further analysis. All assays were performed on five replicate pools, with six pupae in each pool.

Ecdysteroid Extraction and Quantification

Samples were homogenized in 0.5 mL 100% HPLC-grade methanol with Zirconia beads (2.0 mm, Biospec products from Cole-Parmer, Vernon Hills, IL, USA) and then centrifuged at 10,000 × *g* for 10 minutes. A volume of 400 μL of the supernatant was transferred to a 4 mL

glass tube with a PTFE-lined cap (60940D-1 Kimble Chase, Rockwood, TN, USA). Tissue pellets were extracted twice with the same methods and both supernatants were pooled into the same glass tubes. Methanol in the collected extracts was evaporated under a stream of nitrogen gas and the extracts were resuspended in 100 μL 80% methanol.

Ecdysteroids were quantified with high-performance liquid chromatography – mass spectrometry (HPLC-MS) (Westerlund, 2004; Oostra *et al.*, 2011). Specifically, an Agilent 190 infinity HPLC in combination with an Agilent 6550 high resolution Q-TOF mass spectrometer was used for all analysis. The HPLC was equipped with an Agilent 2.1 mm ID x 50 mm Zorbax Extend C18 column that was kept at a temperature of 40°C. The mobile phase consisted of solvent A: H_2O and B: Acetonitrile, both with 0.1% formic acid. The 0.5 mL/min flow was programmed from 95% A, 5% B to 2% A, 98% B over 5 minutes and kept at that concentration for an additional minute. All samples were kept at 10°C and 5 μL was injected per sample. A blank sample (80% methanol) was analyzed in-between each sample to control for and reduce the risk of sample carryover. In addition, the outside of the sampling needle was washed with solvent in-between injections. An extracted ion trace of m/z 447.3000 to 447.3100 and 481.2800 to 481.3400 combined with a retention time of 3.530 min and 3.275 min was used for all ecdysone and 20-hydroxyecdysone quantifications, respectively. Standards of ecdysone (purity≥95% HPLC grade, from ENZO Life Sciences, Farmingdale, NY, USA) and 20-hydroxyecdysone (purity≥93% HPLC grade, from Sigma St. Louis, Missouri, USA) were used for the identification and quantification of detected ecdsyteroids from samples.

Sampling for Sensitivity Analysis to Exogenous 20-Hydroxyecdysone

To compare the sensitivity of the apple and hawthorn host races to ecdysteroids, I injected a series of concentrations of 20E into diapausing pupae. For the pre-winter stage, we

sampled pupae 13 days after pupariation that had low metabolic rates indicative of entry into the diapause developmental trajectory (Ragland *et al.*, 2009). To assess sensitivity in the over-winter stage, pupae that were exposed to simulated overwintering at 4±1°C in dark conditions for 8 weeks were removed from the cold and subjected to injection. Furthermore, for the post-winter stage, after pupae were transferred from 4±1°C to 24±1°C L: D 14: 10, apple race pupae were injected at days 10, 20, and 30 after transfer (average time from transfer to diapause termination is 51.5 days). Hawthorn race pupae were injected at days 10, 20, 30, 40, and 50 after transfer because of their longer post-winter diapause duration (average time from transfer to diapause termination is 64.9 days). For all treatments, pupae were individually kept in the wells of 96-well plates in a room with 24±1°C L: D 14: 10 for observations of diapause termination (Figure 2-1). Across the treatments, sample sizes ranged from 60 to 80 fly pupae each dose.

Hormone Injection

Exogenous 20E for injection was bought as powder (purity≥93%) from Sigma (CAS Number 5289-74-7, St. Louis, Missouri, United States of America) and dissolved in 95% ethanol as a stock solution (5 mg/mL) that was stored at -20°C. The stock solution was diluted on the day of injection into a series of concentrations with 95% ethanol. For the hawthorn race, 10 nL of 0.01, 0.05, 0.5, and 5 mg/mL (0.1, 0.5, 5, and 50 ng) 20E were injected as hormone treatments. For the apple race, 10 nL of 0.01, 0.05, and 0.5 mg/mL (0.1, 0.5, and 5 ng) of 20E were injected. I eliminated the 5 mg/mL dose because we had limited apple race samples. As a control, 10nL of 95% ethanol was injected. Given the possible damage caused by injections, an additional group of pupae that were not injected with anything were used as a further control group. Before injection, the anterior cap of the puparium was removed, because the puparium was too firm to inject through with a pulled glass needle (Drummond Scientific Company, Broomall, PA, USA).

Pulled glass needles were mounted on a microinjector (Nanoliter 2000, World Precision Instruments, Sarasota, FL, USA) used for 20E injection. The 20E was injected into the middle of pupal head (between the two eyes, Figure 2-1). Ragland *et al*. (2009) showed that pharate adult development after pupal diapause termination was characterized by visible morphogenesis. The earliest clearly visible morphogenic landmark is the accumulation of red pigments in the compound eyes. Thus, the appearance of red-eyes was used as a criterion of pupal diapause termination (Figure 2-1).

Statistical Analysis

Ecdysteroid Concentration and Release Timing

Ecdysteroid concentration was shown as pg/mg of flies. We used the concentration of ecdysteroids (pg/μL) detected by HPLC and multiplied it by resuspended volume (100μL) to get the overall ecdysteroid concentrations (pg), then corrected the measure by dividing by the fresh mass of each pooled sample. Wilcoxon signed-rank tests were used to analyze the ecdysteroid concentrations. I tested for differences between the two host races in ecdysteroid concentrations and timing in the pre-winter phase using developmental pathway (diapause or non-diapause), developmental day (wandering larva, pupariation day, 2, 4, 6, 8, 11, 14, 17, and 20 days post-pupariation) and host race (apple and hawthorn races) as fixed factors, with pupal mass as a covariate. I did not detect any ecdysteroids in any of the over-winter samples, and thus performed no statistical analyses of these data. For the post-winter phase, host race (apple or hawthorn) and post-winter timing (early diapause, late diapause, 24 hours, or 48 hours after the initial increase of metabolic rate, and during the metabolic plateau) were treated as fixed factors, and pupal mass was used as a covariate. Analyses were performed in R 3.4.1.

Ecdysteroid Sensitivity

Because the response of *R. pomonella* to 20E changed over the stages of diapause development, different models were fitted for apple and hawthorn races at different time points. For model selection, data were fitted with a linear model, a nonlinear model using generalized least squares, and a Self-Starting nonlinear asymptotic regression model, and the model with the smallest AIC value was selected as the best model and used for dose response comparison between host races. Days taken to terminate diapause were treated as response variables and the log10 (dose+1) transformed dosage and host race were treated as independent variables. Analyses were performed in R 3.4.1.

Results

Ecdysteroid Concentrations

For the pre-winter stage, first, I measured the concentrations of ecdysteroids from the wandering larval stage to day 6, the day before we can separate diapausing pupae from non-diapausing pupae (Ragland *et al.*, 2009). Both 20-hydroxyecdysone and ecdysone content decreased after pupariation in apple and hawthorn race flies, but there was no difference in ecdysteroid content between the host races early after pupariation when diapausing individuals cannot be discriminated from non-diapause individuals (Figure 2-2 and 2-3). Second, I compared the ecdysteroid concentrations between diapausing and non-diapausing pupae of both host races. For the non-diapausing pupae, a peak of ecdysteroids was clearly detected at 17 days after pupariation (Figure 2-2 and 2-3). This peak coincided with pupal-pharate adult metamorphosis in non-diapause pupae, which will complete pharate adult development and emerge as adults 28-30 days after pupariation (Ragland *et al.*, 2009). No difference in ecdysteroid concentrations of non-diapausing pupae was detected between apple and hawthorn races ($P>0.05$). I detected no ecdysteroids in diapausing pupae in monitoring them through 20 days after pupariation (Figure

2-2 and 2-3). These data support my assertion that pupal diapause in *Rhagoletis pomonella* is associated with a suppression of ecdysteroid production and release.

I tested if the degree to which ecdysteroid concentrations or release timing may start to diverge between the two host races during the over-winter stage. However, no ecdysteroids were detected in either apple or hawthorn races flies (Figure 2-4), further justifying that pupal diapause maintenance is associated with a lack of ecdysteroid production and demonstrating that the two host races do not diverge in ecdysteroid concentrations until after over-wintering.

To test whether the timing and concentrations of ecdysteroids differ in the post-winter stage between flies of the two host races, I measured ecdysone and the active hormone 20-hydroxyecdysone at multiple points along the post-winter developmental trajectory from post-winter diapause maintenance to termination and the initiation of pharate adult development (Ragland *et al.*, 2011). As expected, hawthorn race individuals terminated diapause later than apple host race individuals based on accumulated diapause termination (Figure 2-5) and timing of the increase in metabolic rate that is diagnostic of diapause termination. For both host races no 20-hydroxyecdysone was detected before the metabolic uptick that signals diapause termination, but 20-hydroxyecdysone levels were clearly detectable 24h and 48h after the initial metabolic increase in both host races (Figure 2-6B). The concentrations of both 20-hydroxyecdysone and ecdysone were significantly higher during the plateau stage than at sampling points in early diapause (ED), late diapause (LD), or even at 24 hours (24h) and 48 hours (48h) after initial metabolic increase in both host races (Figure 2-6). Compared to the hawthorn race, the plateau stage of the apple race occurred 13 days earlier, suggesting an earlier peak of ecdysteroid concentrations as well as earlier diapause termination in apple race than hawthorn race. There

were no significant differences in the concentrations of 20-hydroxyecdysone and ecdysone between apple and hawthorn races in the post-winter stage (P>0.05).

Ecdysteroid Sensitivity

Exogenous 20-hydroxyecdsone accelerated pupal diapause termination in both apple and hawthorn race flies. The extent of developmental acceleration was directly proportional to the concentration of 20-hydroxyecdysone. There was no detectable difference in the timing of diapause termination between my two control groups, those that were injected with 10nL of 95% EtOH and those that were not injected (P=0.28), so I can rule out the potential effect of ethanol and physical damage on the timing of pupal diapause termination (Figure 2-7). To interpret my dose response, I only included comparisons to the ethanol-injected group.

The dose-response curve was well fit to an exponential decay model with the days taken to terminate diapause decreasing proportionally to the dose applied to pupae. The decay rate was used to compare ecdysteroid sensitivity between host races. For the pre-winter stage, a Self-Starting nonlinear asymptotic regression model was fitted for both apple and hawthorn races (Figure 2-8). Both apple and hawthorn races showed a saturated dose-response. The estimated asymptote was significantly higher in the apple race (18.65 days) than the hawthorn race (17.82 days) (t=17.96, P=0.000) and the intercept was significantly smaller in the apple race (42.78 days) than the hawthorn race (53.99 days) prior to winter (t=11.28, P= 0.000). Most importantly, the decay rate was significantly higher in the apple race (1.35) compared to the hawthorn race (0.45) (t=2.45, P= 0.015), suggesting the apple race was more sensitive to 20E than the hawthorn race at the pre-winter stage.

For the over-winter stage, a nonlinear model using generalized least squares was fitted for both apple and hawthorn races (Figure 2-8). No significant difference in ecdysteroid sensitivity

was detected between the two host races (decay rate: 0.32 and 0.23 for apple and hawthorn race, respectively. t=0.70, P=0.49).

A nonlinear model using generalized least squares was fitted for both host races for the 10-day post-winter stage (Figure 2-8). Similar to the over-winter stage, apple race and hawthorn race did not show a significant difference in sensitivity to 20E at the 10-day post-winter stage (decay rate: 0.31 and 0.3 for apple and hawthorn race, respectively. t=0.23, P=0.82). For the 20-day post-winter stage, the dose responses between host races were compared by a Self-starting nonlinear model (Figure 2-8). The decay rate, which indicates the sensitivity to ecdysteroids, was significantly higher in the apple race (2.0) than the hawthorn race (0.49) (t=2.40, P= 0.017). No difference in decay rate was detected at 30-day post-winter stage between host races (nonlinear model, decay rate: 0.26 and 0.15 for apple and hawthorn race, respectively. t=0.69, P=0.49) (Figure 2-8). However, if I excluded the 5 mg/mL dose that I only injected into hawthorn race pupae, the decay rate was significantly higher in the hawthorn race (0.37) than the apple race (0.26) at the 30-day post-winter stage (t=2.15, P=0.03).

Because different models at different diapause stages could not be used for within host race comparison of sensitivity to 20E, I compromised to use a linear model for within host race sensitivity analysis (linear model had acceptable AIC values, Table 2-1). Both apple race and hawthorn race flies showed essentially flat dose-response to 20E when they were close to their late diapause stage, suggesting a loss of sensitivity to 20E and an increased capacity to terminated diapause over time at post-winter stage. Specifically, a flat dose-response was shown at 30-day post-winter stage in the apple race, but was not shown until 40 days post-winter stage in the hawthorn race.

Discussion

The suppression of ecdysteroid release in pupal diapause is conserved among insect species. In *R. pomonella*, the peaks of both ecdysone and 20-hydroxyecdysone were clearly detectable in non-diapausing pupae during the prewinter period coinciding with the timing of pupa-pharate adult morphogenesis, but there were no ecdysteroids detectable in diapausing pupae in the pre-winter or over-winter periods in either host race. Given that diapause induction and maintenance are associated with the suppression of ecdysteroid synthesis, it is important to test two alternative hypotheses for the role of ecdysteroids in regulating pupal diapause: 1) diapause is terminated by a pulse of ecdysteroids that also triggers pupa-pharate adult metamorphosis, or 2) diapause is terminated by some other factor upstream of ecdysteriods and the peak of ecdysteroids that triggers pupa-pharate adult morphogenesis occurs after the pupa terminates the diapause maintenance phase of diapause development. During the post-winter stage, ecdysteroids were undetectable until after the increase in metabolic rate that marks the termination of pupal diapause in both species. This suggests that ecdysteroids are unnecessary to trigger the initial increase in metabolic rate that signals the termination of pupal diapause. Of course, it is possible that small quantities of ecdysteroids, well below our limits of detection could cause diapause termination, but instead I believe that ecdysteroid production occurs downstream of other events that regulate diapause termination. The substantial peaks of ecdysteroids I observed at the plateau stage well after diapause termination in the metabolic trajectory suggest that large amounts of ecdysteroids are necessary for the pupal-pharate adult molt, corresponding to morphological evidence showed by Ragland *et al.* (2009) (Figure 2-8).

My data are in agreement with a previous *R. pomonella* transcriptomic study showing that transcripts in the ecdysone biosynthesis pathways as well as downstream ecdysone response genes during post-winter stage (Ragland *et al.*, 2011). Halloween genes used for ecdysone

biosynthesis (Huang et al., 2008) showed marked increases between late diapausing individuals and pupae 24–48h after the initial metabolic increase, but not prior to the increase in metabolic rate that indicates diapause termination in *R. pomonella* pupae (Ragland *et al.*, 2011; Meyers *et al.*, 2016). Expression of prothoraciotropic hormone (PTTH), a crucial brain peptide hormone that induces ecdysteroid production (Smith & Rybczynski, 2012), also increased rapidly during the first metabolic rate increase, but not before. The combined effects of changes in expression of Halloween genes and PTTH suggest that an ecdysone pulse occurs after the initial metabolic rate increase that indicates diapause termination. The peak production of ecdysteroids thus happens a few days rather than immediately after the expression of ecdysteroidogenesis genes.

Altered timing of dormancy plays a primary role in shifted phenology in response to environmental changes (Bradshaw & Holzapfel, 2001; Scriber & Ording, 2005; Gomi *et al.*, 2007; Wadsworth *et al.*, 2013). In *R. pomonella,* the timing of the pupal diapause termination is a key life history adaptation to the seasonal differences in host fruiting phenology that reproductively isolates apple race flies and hawthorn race flies (Feder *et al.*, 1993, 1994). Associated with the altered life cycle timing via diapause, I predicted an occurrence of evolutionary changes in hormonal regulation of diapause, including ecdysteroid concentrations, timing in ecdysteroid production and release, and ecdysteroid sensitivity. Consistent with my predictions, the timing of ecdysteroid production and release occurs earlier in apple race flies than hawthorn race flies. The advanced ecdysteroid production and release timing (the difference in plateau stage by 13 days in the apple host race compared to hawthorn race flies) corresponds to the difference in diapause termination timing (the difference in appearance of red-eye: 13.43 days) between apple and hawthorn race flies. However, flies from the two host races showed no difference in the concentrations of either ecdysone or 20-hydroxyecdysone at any parallel

developmental stage. Apple race flies have not evolved higher ecdysteroid concentrations than hawthorn race flies, which may be explained by following reasons. First, the concentrations of ecdysteroids are typically maintained within a narrow range by a series of negative feedback loops and the neurohormone regulators (e.g., PTTH) that regulate the production of ecdysteroids (Zera *et al.*, 2007). Second, the blood titers of hormones are closely related with the abundance of enzymes for biosynthesis and degradation. Ultimately a comparison of abundance of ecdysteroid biosynthesis and degradation enzymes (e.g., P450 enzymes) is needed between apple and hawthorn race flies. Third, the production of hormones can be costly for some traits and the titers might be under strong natural selection. For example, in *Gryllus rubens* and *Gryllus firmus* crickets, the trade-off between flight and reproduction is associated with ecdysteroid titers during late nymphal life. Specifically, an elevated ecdysteroid titer is exhibited in selected lines for the dispersing morph compared with lines selected for the flightless/reproductive morph (Zera, 2004, 2006).

In addition to measuring the timing and concentrations of ecdysteroids across the apple and hawthorn host races, I also tested whether the two host races differed in their sensitivity to exogenous ecdysteroids across several points in the diapause developmental trajectory. My results show that exogenous 20E has an active role in accelerating diapause termination in both host races of *R. pomonella*. Similar effects of injected ecdysone on pupal diapause termination has been reported in *Sarcophaga* flesh flies (Fraenkel & Hsiao, 1968), and several moth species including *Pieris brassicae* (Arpagaus *et al.*, 1986), *Helicoverpa zea* (Zhang & Denlinger, 2012), and *Omphisa fuscidentalis* (Singtripop *et al.*, 2002). For example, in the bamboo borer, *O. fuscidentalis*, after injecting 20-hydroxyecdysone (20E), the mean diapause duration shortened from about 140 days to 30 days (Singtripop *et al.*, 2002; Subta *et al.*, 2017). The responsiveness

to exogenous hormones can differ across the phases of the diapause developmental trajectory. Sensitivity to ecdysteroids is greater when animals are temporally closer to diapause termination compared to early phases in the diapause developmental trajectory (Denlinger *et al.*, 2012). Therefore, sensitivity to exogenous ecdysteroid applications should indicate potentiation to terminate diapause. Specifically, a flat dose-response means that animals are already potentiated and not responsive because they have already made the decision to terminate diapause.

Allochronic reproductive isolation between apple and hawthorn race flies is achieved by two facets of their diapause phenotypes, the initial diapause intensity and the timing of diapause termination (Ragland *et al.*, 2017). Divergent selection on these two phenotypes is driven by differences in the host-related environmental conditions experienced by apple and hawthorn flies. First, apple race flies are exposed to prolonged exposure to warm temperatures before winter cold sets in because of their earlier phenology, thus a greater diapause intensity is favored such that apple race flies should show stronger refractoriness to non-diapause development compared to the hawthorn race flies (Dambroski & Feder, 2007; Egan *et al.*, 2015). Second, colonization of seasonally earlier apple fruits has selected for an earlier adult emergence (pupal diapause termination and resumption of pharate adult development) in apple flies than hawthorn flies (Dambroski & Feder, 2007; Ragland *et al.*, 2017). Therefore, I predicted: 1) with a greater initial diapause intensity, apple race flies would be less sensitive to exogenous 20E to terminate diapause than hawthorn race flies in the pre-winter stage; 2) with an earlier diapause termination, apple race flies would become sensitive to exogenous 20E earlier than hawthorn race flies during the post-winter stage; 3) no difference in sensitivity to ecdysteroids in the over-winter stage because both host races should be refractory to diapause termination at this point. Contrary to my pre-winter prediction, apple race flies were more sensitive to 20-hydroxyecdysone to terminate

diapause than hawthorn race flies just after pupation. Normally, the apple host race shows lower percentage of non-diapausing flies because of their greater initial diapause depth than hawthorn race flies (Feder *et al.*, 1997; Dambroski & Feder, 2007). However, in 2016 the non-diapause incidence of apple race was unusually high with 40.61% of individuals not entering diapause whereas a more normal level of hawthorn flies did not enter diapause in my 2016 samples (5.01%). Temperature is known to affect the propensity of *R. pomonella* to enter diapause (Prokopy, 1968). The relatively high non-diapause incidence in the apple race could be possibly attributed to the rather warm summer in 2016, in Grant, MI, USA (Figure 2-S3). Interestingly, although apple flies were more sensitive to 20-hydroxyecdysone during the pre-winter period, sensitivity to ecdysteroids was reset to the same level between apple and hawthorn race flies during overwintering. The combined observations of sensitivity to ecdysteroids in pre-winter and over-winter stages indicate a genotype by environment interaction. Consistent with my prediction, apple race flies became more sensitive to 20-hydroxyecdysone than hawthorn race flies starting 20 days after artificial overwintering, suggesting that an earlier increase in sensitivity to ecdysteroids is associated with earlier induction of parts of the ecdysteroid response pathways.

The number of studies documenting and predicting the responses of organisms to climate change has increased dramatically over the last three decades, which has driven increased emphasis on the role of phenology in evolutionary ecology. A substantial body of literature has shown that phenological changes are one of the most obvious biological responses to the ongoing climate change (Crick & Sparks, 1999; Fitter & Fitter, 2002; Visser & Both, 2005; Doi, 2008). Some progress has been made in identifying genetic mechanisms of evolutionary changes in phenology, including the genetic basis of advanced flowering time in plants (Wang *et al.*, 2009),

earlier seasonal timing of reproduction in birds (Liedvogel *et al.*, 2009), and diapause induction in insects (Tauber *et al.*, 2009). Our study helps lay a foundation for understanding the complex genetic architecture involved in the process of rapid evolution by understanding the physiological mechanisms underlying rapid adaptive shifts in diapause timing. The next step would be to unravel the mechanisms underlying adaptive variation in endocrine regulation (e.g. upstream events), so we can gain more reliable information in order to predict the ecological responses of organisms to climate change, which will provide insights into biological conservation.

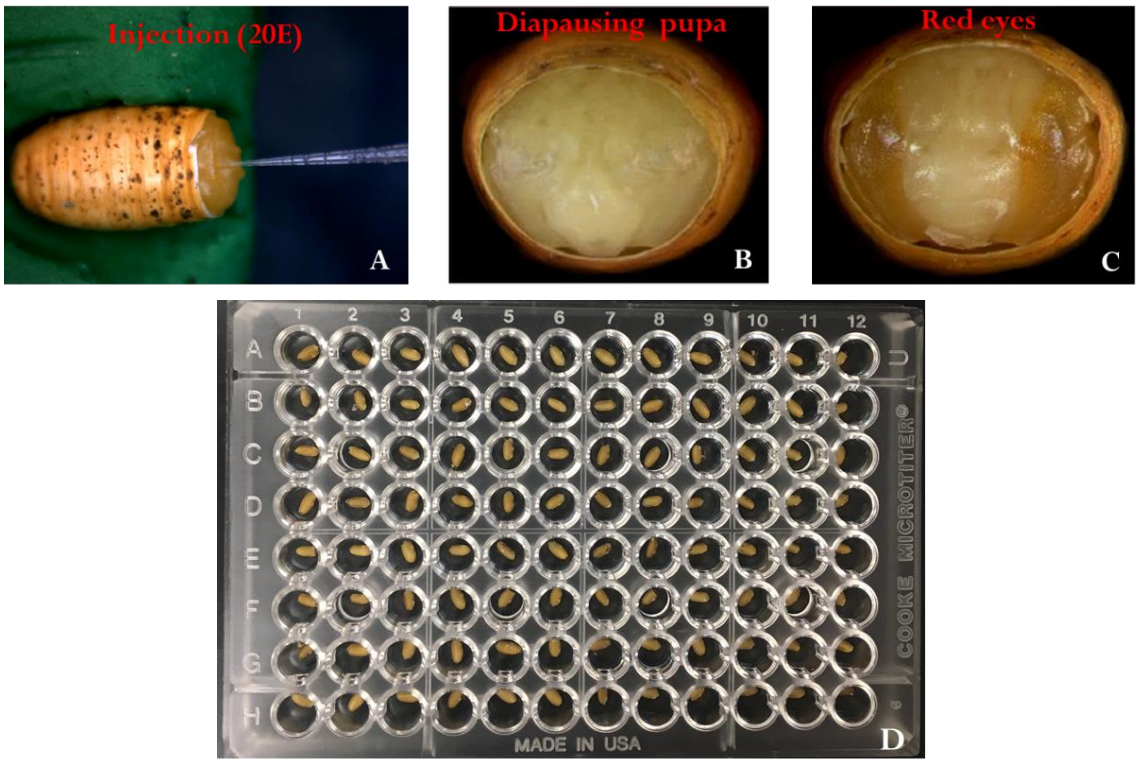

Figure 2-1. Ecdysteroid sensitivity experiment setup of *Rhagoletis pomonella*. A) The injection of 20-hydroxyecdysone into a diapausing pupae of *Rhagoletis pomonella*. B) A diapausing pupae with the anterior cap of the puparium removed. C) Red-eye used as a sign of diapause termination. D) 96-well plate was used for keeping treated pupae and diapause-termination observation. Photo courtesy of Qinwen Xia.

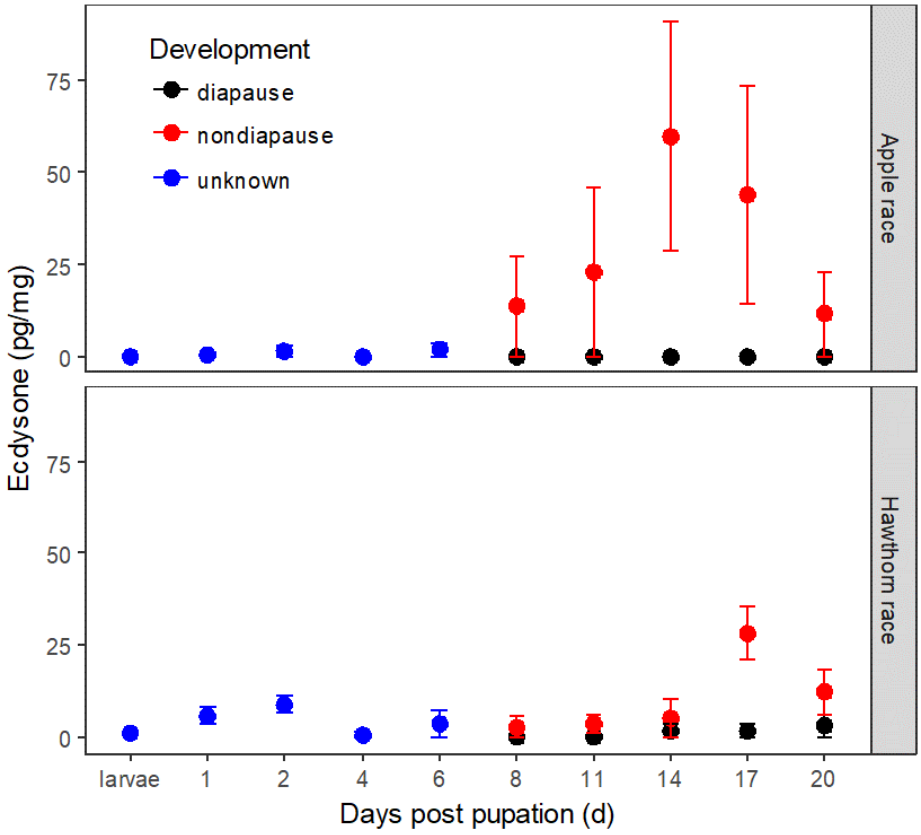

Figure 2-2. Whole-body ecdysone concentrations in diapausing and non-diapausing individuals of apple and hawthorn races of *Rhagoletis pomonella*. Unknown development means the stage when diapause individuals could not be separated from non-diapause individuals by respirometry (blue dots). The symbols represent means and bars represent standard errors.

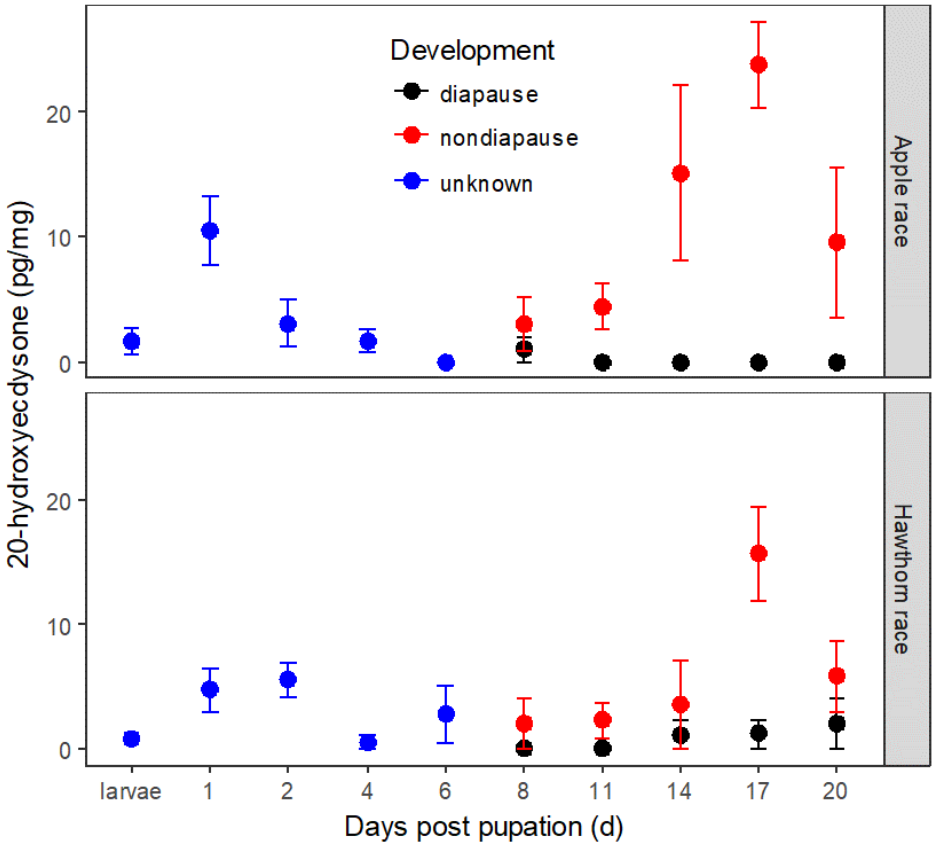

Figure 2-3. Whole-body 20-hydroxyecdysone concentrations in diapausing and non-diapausing individuals of apple and hawthorn races of *Rhagoletis pomonella*. Unknown development means the stage when diapause individuals could not be separated from non-diapause individuals by respirometry (blue dots). The symbols represent means and bars represent standard errors.

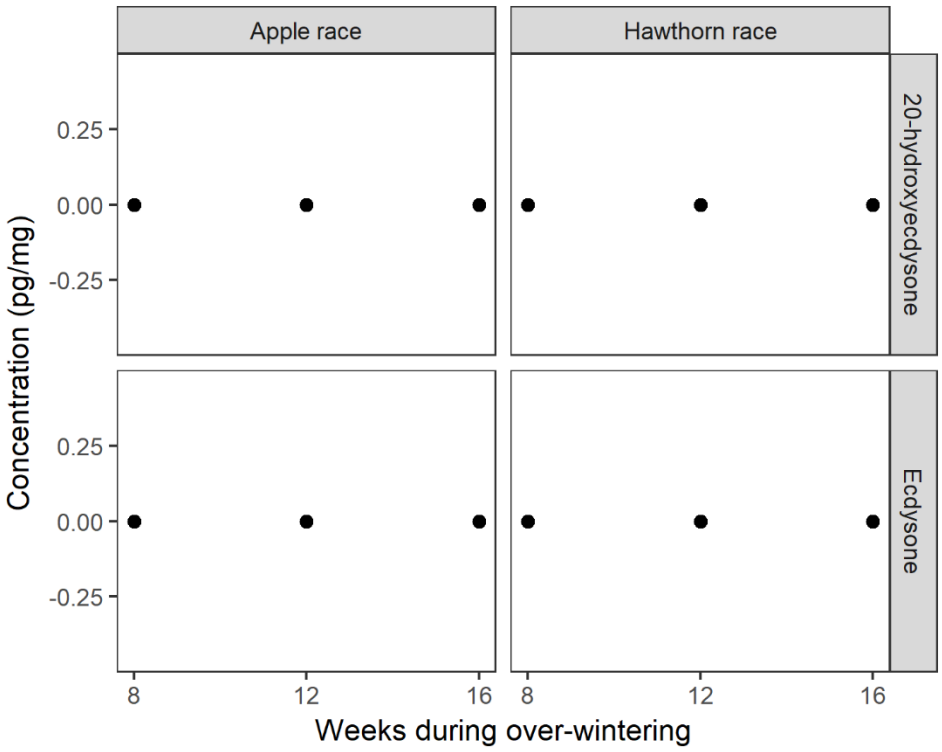

Figure 2-4. Whole-body 20-hydroxyecdysone and ecdysone concentrations in diapausing pupae of apple and hawthorn races of *Rhagoletis pomonella* at weeks 8, 12, and 16 over-wintering.

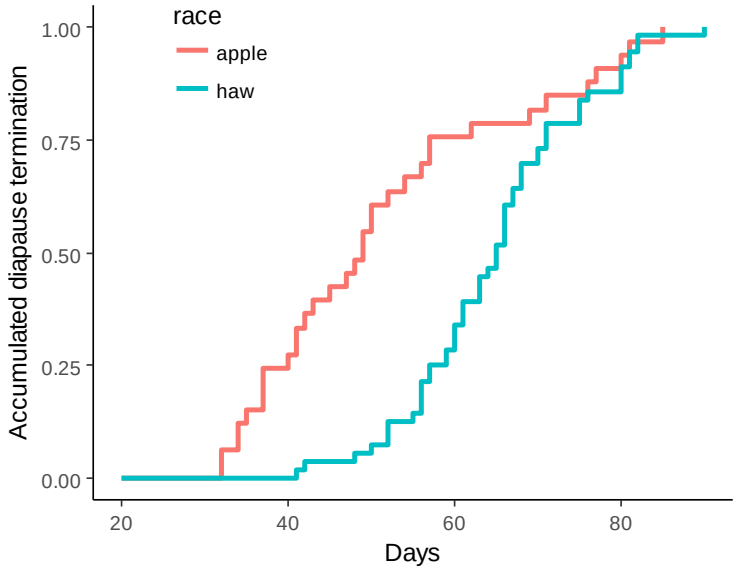

Figure 2-5. Accumulated diapause termination of apple race and hawthorn race flies of *Rhagoletis pomonella* at post-winter stage.

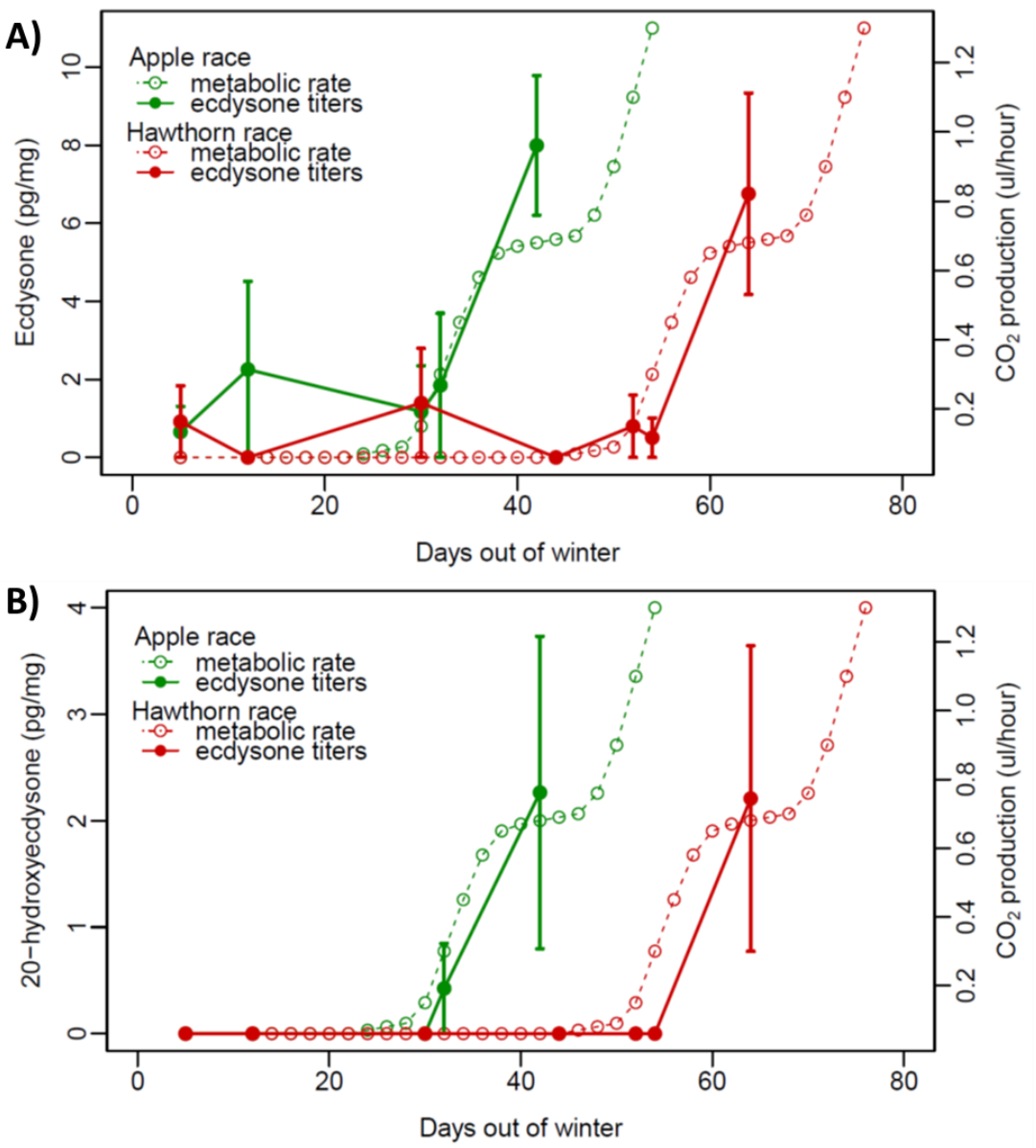

Figure 2-6. The production of ecdysteroids and fitted metabolic rate after the transfer from winter condition in apple and hawthorn races of *Rhagoletis pomonella*. A) ecdysone concentrations. B) 20-hydroxyecdysone concentrations. Solid line represents ecdysteroids, and dash line represents CO_2 production. The symbols represent means and bars represent standard errors.

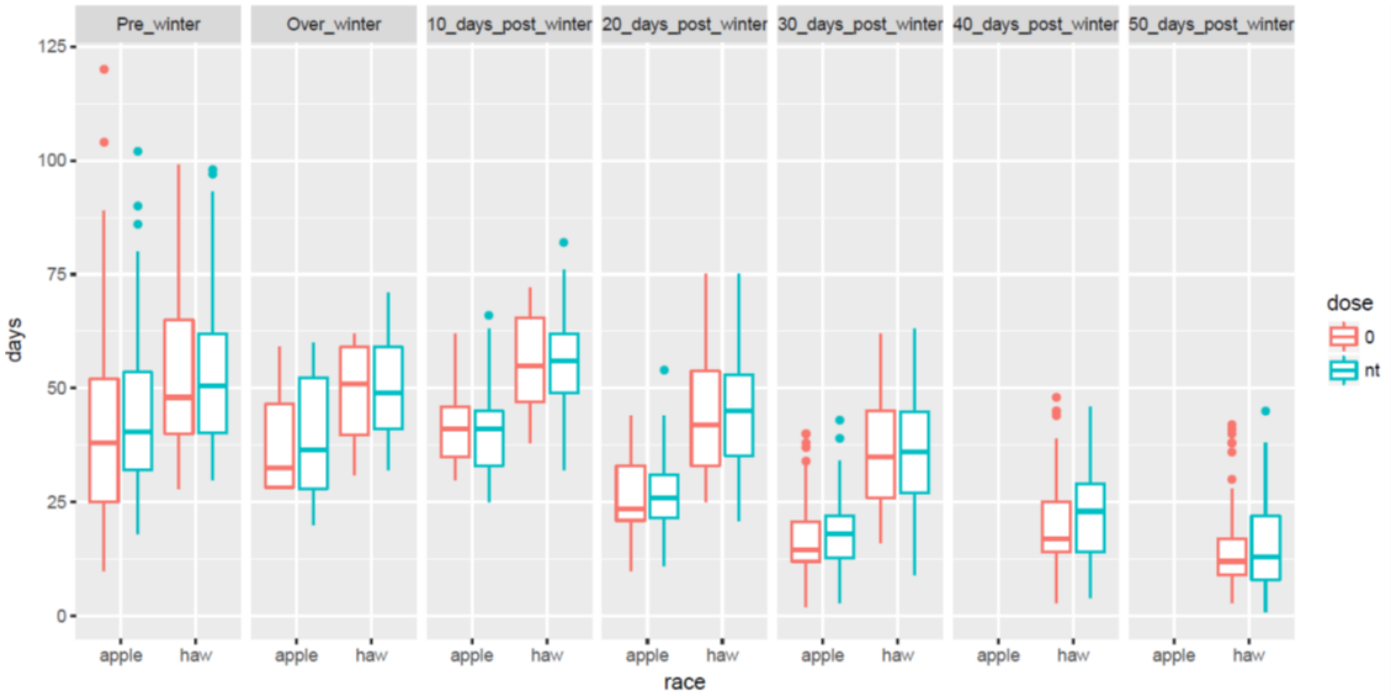

Figure 2-7. Days taken to terminate diapause for two control groups: no-injection and ethanol-injected treatments in ecdysteroid sensitivity experiment of *Rhagoletis pomonella*.

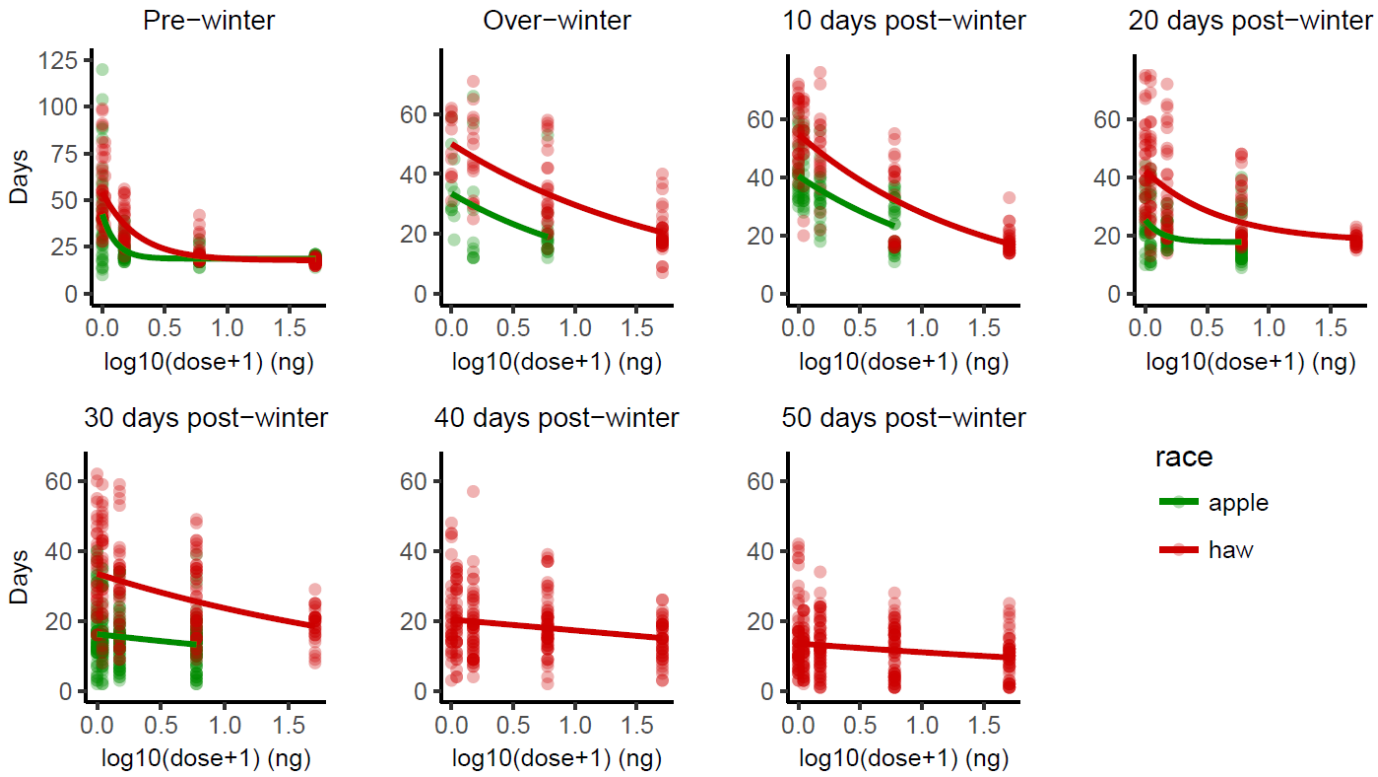

Figure 2-8. Days taken to terminate diapause after treatment with 20-hydroxyecdysone or ethanol at different stage of diapause of *Rhagoletis pomonella*. Pre-winter stage means 13 days after pupariation. Over-winter stage means 8 weeks at 4±1°C. Post-winter stage: 10, 20, 30, 40, and 50 days after transfer from 4±1°C to 24±1°C.

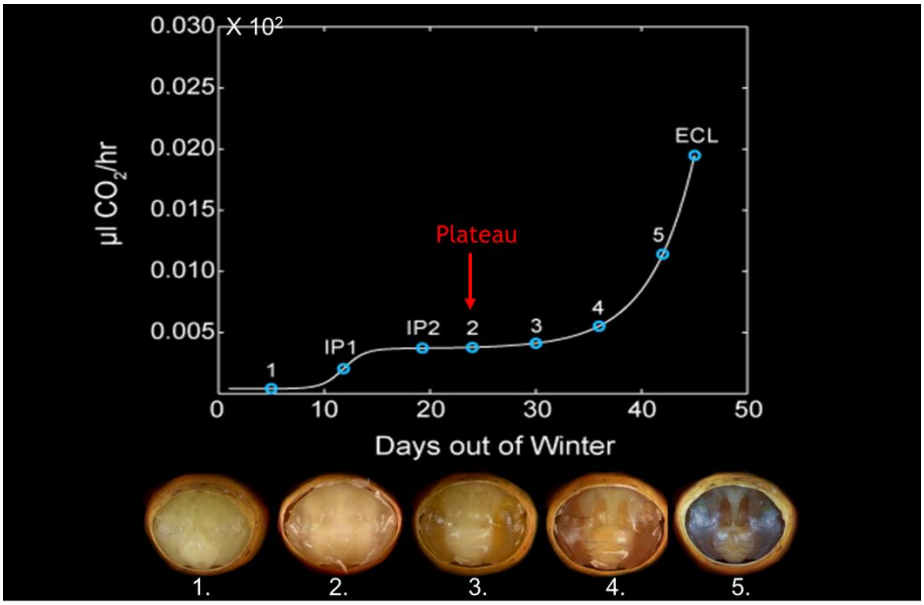

Figure 2-9. Morphological change across the biphasic metabolic trajectory of *Rhagoletis pomonella* (adapted from Ragland *et al*., 2009).

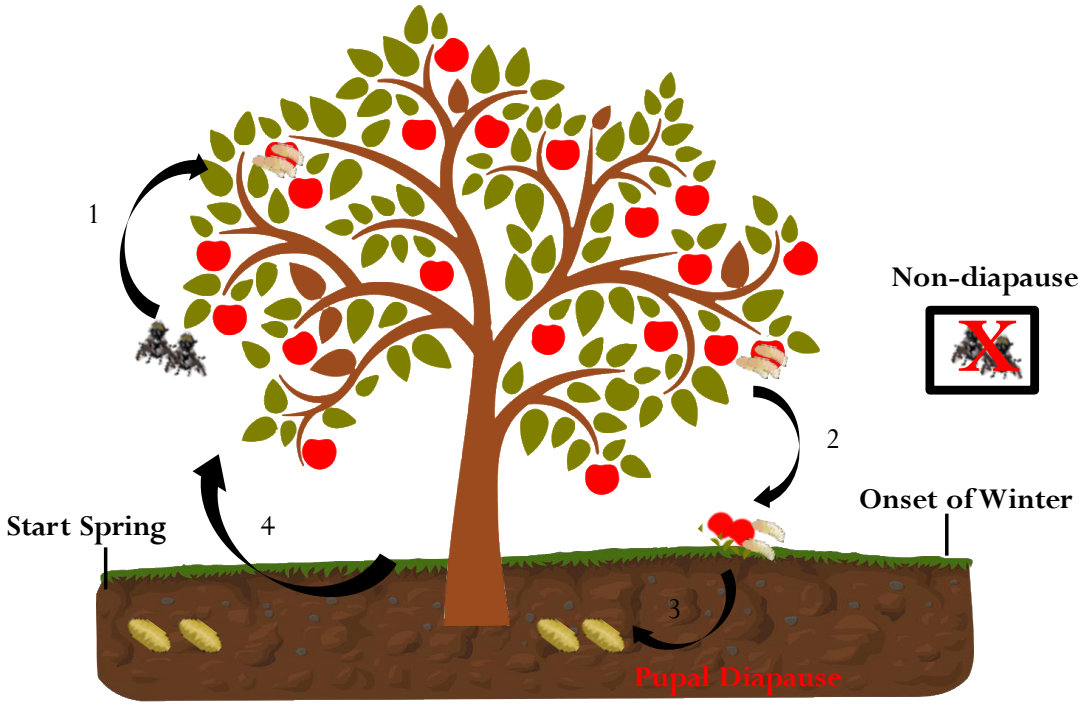

Figure 2-S1. Life history of *Rhagoletis pomonella*. 1. Early summer, adults eclose and mate, and lay eggs into fruit. Eggs hatch into larvae and feed on fruits. 2. Fruits drop from trees and larvae wander out into the soil to pupate. 3. Most pupae enter diapause before the onset of winter, but a small portion of pupae develop directly into adults, those are doomed non-diapause individuals. 4. The diapausing pupae will not terminate diapause until the next spring, when the environment is favorable again to start another generation.

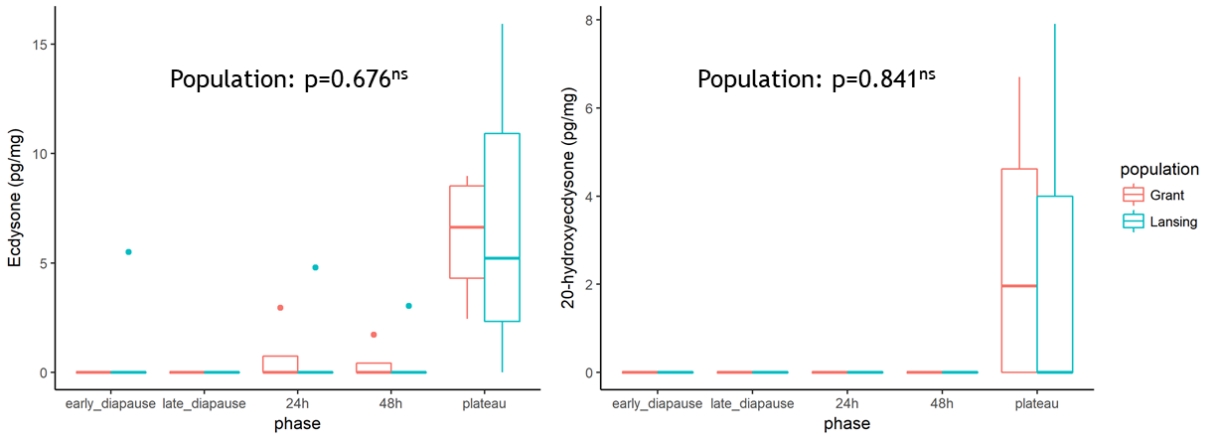

Figure 2-S2. Comparison of ecdysteroid concentrations between Grant and Lansing populations of hawthorn flies at the post-winter stage. ns, non-significant.

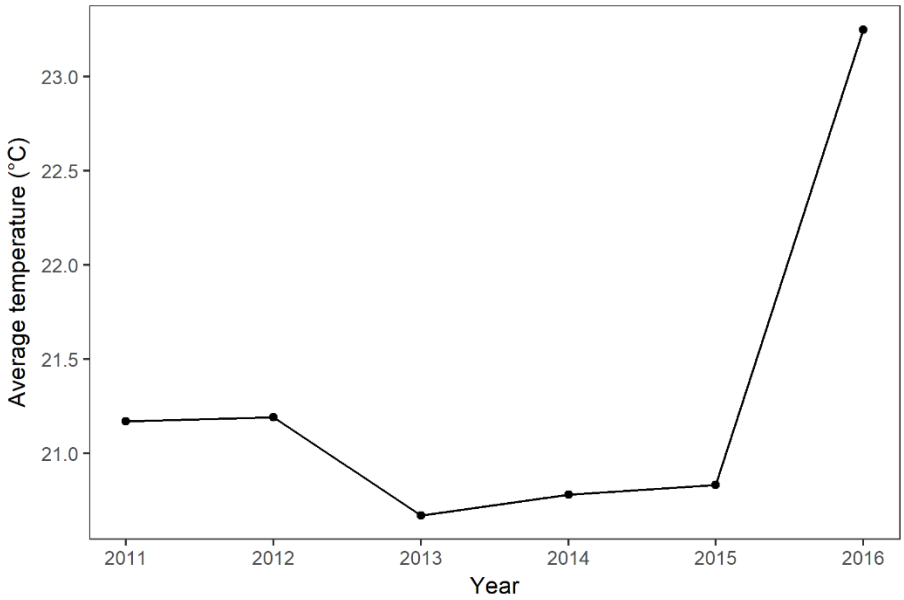

Figure 2-S3. Average temperature of August in Grant, MI, USA from 2011 to 2016. (U.S. climate data)

Table 2-1. AIC value for model selection in ecdysteroid sensitivity analysis.

Phase	Model	AIC value	
		Apple	Hawthorn
Pre-winter	Self-Starting nonlinear asymptotic regression model	1425.723	1250.580
	Linear model	1470.978	1298.507
	Nonlinear model using generalized least squares	1464.318	1275.785
Over-wintering	Self-Starting nonlinear asymptotic regression model	324.1079	713.7617
	Linear model	323.3083	712.0950
	Nonlinear model using generalized least squares	323.0360	712.2010
10-day Post-winter	Self-Starting nonlinear asymptotic regression model	726.1035	926.5628
	Linear model	728.1647	931.7230
	Nonlinear model using generalized least squares	726.8234	924.9934
20-day Post-winter	Self-Starting nonlinear asymptotic regression model	844.0473	1287.778
	Linear model	845.1447	1291.422
	Nonlinear model using generalized least squares	844.8756	1288.046
30-day Post-winter	Self-Starting nonlinear asymptotic regression model	1971.758	1454.752
	Linear model	1970.244	1453.791
	Nonlinear model using generalized least squares	1970.187	1453.042
40-day Post-winter	Self-Starting nonlinear asymptotic regression model		NA
	Linear model		1788.468
	Nonlinear model using generalized least squares		1788.628
50-day Post-winter	Self-Starting nonlinear asymptotic regression model		2048.111
	Linear model		2046.719
	Nonlinear model using generalized least squares		2046.529

CHAPTER 3
AN EXPLORATION OF AN ENDOCRINAL BASIS OF ADAPTION IN LIFE CYCLE TIMING OF EUROPEAN CORN BORER, *OSTRINIA NUBILALIS*

Introduction

The early and late flight strains of the European corn borer (*Ostrinia nubilalis*) are a classic example of allochronic isolation and speciation. The two European corn borer strains are diverging by two major axes: 1) distinct voltinism and adult flight timing driven by divergence in larval diapause timing, and 2) distinct sexual communication systems with each strain favoring a different pheromone blend (Dopman *et al.*, 2010). *Ostrinia nubilalis* enters diapause as a mature 5th instar larva (Beck & Hanec, 1960). The timing of diapause induction, maintenance, and termination are closely related to environmental cues including temperature and photoperiod (Mutchmor & Beckel, 1958; Beck & Hanec, 1960). Latitudinal variation in temperature and photoperiod contributes to distinct diapause timing as well as the number of generations per year. In the United States, the number of generations of *O. nubilalis* shows classical clinal variation from one generation in northern latitudes to three or four generations in southern regions (Sorenson *et al.*, 1992). However, overlaid on this classical clinal variation in voltinism is substructure with the two strains overlapping in some geographic zones. For example, in upstate New York, the one-generation (univoltine) strain and two-generation (bivoltine) strain can both be found in the same location at different times of the year (Glover *et al.*, 1991). Allochronic isolation between the bivoltine and univoltine strains arises from differences in adult breeding seasons. The reproductive timing of the bivoltine strain is early June and late August, whereas the reproductive timing of univoltine strain is in mid-July (Roelofs *et al.*, 1985; Martin *et al.*, 2016). However, above the zone of intermingling of the two strains in upstate New York only the univoltine strain is found and below the zone only the bivoltine strain is found for at least 100 km (Showers, 1981; Levy *et al.*, 2015). Similarly, in a cline across the Midwestern US, the two

strains were found to alternate zones of appearance of one strain, then a zone of co-occurrence, then the other strain with voltinism increasing from north to south in a saw-tooth pattern that is consistent with clinal variation in season length selecting for alternating bands of each strain (Figure 3-1) (Levy *et al.*, 2015). In addition to their differences in seasonal timing, these two strains also differ in the isomeric ratios of pheromone components produced by females and also in male preference for these components. Specifically, the bivoltine E-strain (BE) and univoltine Z-strain (UZ) contain 99:1 vs. 3:97 (E)11- tetradecenyl acetate (E11-14:OAc) and (Z)11-tetradecenyl acetate (Z11-14:OAc), respectively (Roelofs *et al.*, 1985; Martin *et al.*, 2016). Here I focusd on seasonal timing as a major axis of divergence between the two strains.

The one-month shift in adult flight timing between the BE and UZ strains is determined by genetically determined changes in the timing of termination of the overwintering larval diapause in spring (McLeod *et al.*, 1979; Wadsworth *et al.*, 2013). The earlier emerging BE strain has a shorter duration of post-winter diapause development (PDD) than the later emerging UZ strain does. Allele differences in the post-winter diapause development (*Pdd*) region on the Z chromosome have been identified as the major genetic factor that confers the difference in voltinism between BE and UZ populations (Glover *et al.*, 1992; Wadsworth *et al.*, 2013, 2015). Alleles Pdd^{S} and Pdd^{L} correspond to earlier and later emergence in BE and UZ, respectively (Glover *et al.*, 1992). Transcriptome profiling analysis nominated 48 candidate genes for *Pdd* that are potentially involved with divergence in the timing of diapause termination between the two strains (Wadsworth & Dopman, 2015). However, the exact genes that underlie *Pdd* and cause differences in diapause timing remain unclear. An approach targeting the causal downstream endocrine events that regulate diapause termination may provide insights into the molecular mechanisms underlying divergence in diapause timing between these two strains of *O.*

nubilalis. Larval diapause is widely regarded as a result of a failure of the prothoracic glands to secrete the molting hormone ecdysteroids to promote larval-pupal metamorphosis (Denlinger *et al.*, 2012). In a previous study of only one *O. nubilalis* strain, the concentrations of ecdysteroids remained low during the pre-diapausing and diapausing larval stage, but started to increase from day 12 after transferring diapausing larvae into conditions that promote diapause termination (Bean & Beck, 1983; Gelman & Woods, 1983), suggesting the larval diapause of *O. nubilalis* is under the regulation of ecdysteroids.

Here, I proposed that a shift in ecdysteroid signaling is also associated with divergence in life cycle timing between the earlier-emerging BE strain and the later-emerging UZ strain of *O. nubilalis*. Within my hypothesis I tested three specific predictions. First, I predicted that the BE strain would show an earlier peak of ecdysteroids than the UZ strain after exposure to conditions that are conducive to diapause termination. Second, I predicted that the earlier larval diapause termination in the BE strain is associated with higher concentrations of ecdysteroids at the time of diapause termination. Third, I predicted that the earlier larval diapause termination in the BE strain is associated with an earlier increased capacity to terminate diapause and greater ecdysteriod sensitivity. To test those predictions, I measured ecdysteroid concentrations, the timing of ecdysteroid release, and sensitivity to exogenous 20-hydroxyecdysone (20E) in both the BE and UZ strains of *O. nubilalis* across multiple time points in diapause development from diapause induction to diapause termination. In my study, I found that the BE strain released ecdysteroids earlier than the UZ strain after transfer to diapause-terminating conditions, but I found no difference in ecdysteroid concentrations between the two strains. In addition, the BE strain showed an increased capacity to terminate diapause and greater ecdysteriod sensitivity than the UZ strain after exposure to diapause-termination conditions.

Materials and Methods

Insects and Rearing

The BE and UZ strains of *O. nubilalis* were generously donated from colonies maintained by Erik Dopman's lab at Tufts University. Both strains were originally collected from the field in upstate New York and kept under mass-rearing conditions in the laboratory (Glover *et al.,* 1992). Larvae were fed on an artificial diet (LOT# 052418ECB, Southland Products Inc., Lake Village, AR, USA) in a plastic deli-cup (11.75cm top diameter × 9.84cm bottom diameter × 7.62cm height, Bare by Solo, Dart Container Corporation, Mason, MI, USA) at a density of 80 individuals. Non-diapausing individuals were obtained by rearing under long-day conditions including light: dark (L: D) 16:8 at constant 23±1°C. A short-day photoperiod L: D 12:12 at constant 23±1°C was used for larval diapause induction. Some proportion of short-day larvae do not enter diapause, or they have a diapause period too brief to distinguish them from non-diapause larvae. Thus, larvae were determined to be in diapause after they remained as larvae when kept at L: D 12:12 and 23±1°C for 45 days. In contrast, non-diapausing individuals pupated at day ~27 and emerged as moths at day ~36 after egg hatch. Larvae that were clearly in diapause after 45 days were individually transferred from their rearing container into a new chamber that was modified from a 1mL pipette tip containing cotton moistened with water that allowed me to observe each individual for diapause termination (Figure 3-2). No food is needed in this diapause termination chamber because diapausing larvae do not feed during diapause or during larval-pupal metamorphosis. Diapausing larvae are sensitive to long-day and high temperature cues for the termination of diapause and resumption of development from larval diapause into pupal morphogenesis. Thus I shifted both temperature and light cycle to strongly trigger larval diapause termination. Specifically, after 46 days in diapause induction conditions

(L: D 12:12 at 23±1°C), diapausing larvae were transferred to L: D 16:8 at 26±1°C to trigger diapause termination.

Sampling for Ecdysteroid Quantification

To test whether a decline in ecdysteroids was associated with the initiation of larval diapause development of *O. nubilalis*, I measured the concentrations of ecdysone (E) and the active hormone 20-hydroxyecdysone (20E) in diapause-destined and non-diapausing 5th instar larvae. Specifically, non-diapause programed larvae of both strains (L:D 16:8 at 23±1°C) were haphazardly sampled every day from the 4th day after molting into the 5th instar larvae until pupation, specifically I sampled on days 4, 5, 6, 7, 8, 9, 10, 11 (prepupa, Figure 3-3), and also on day 12 once individuals had metamorphosed into pupae. For those diapausing larvae induced under L: D 12:12 at 23±1°C, I also measured the concentrations of E and 20E after exposure to diapause termination conditions (L: D 16:8 at 26±1°C). For the BE strain, I sampled larvae every four days starting from 1 day to 21 days after transfer to diapause-terminating conditions on days 1, 5, 9, 13, 17, and 21 (78.6% of diapausing BE individuals have terminated diapause and initiated larval-pupal metamorphosis by 21 days after transfer to warm, long-day conditions, Figure 3-5). UZ strain larvae were sampled on days 1, 5, 9, 13, 17, 21, 25, 29, 33, 37, 41, 45, 49, 53, 57, 61, and 65 post long-day exposure (80% of diapausing UZ individuals have terminated diapause and initiated larval-pupal metamorphosis by 65 days after transfer to warm, long-day conditions, Figure 3-5). I weighed each larva, and then snap froze it in liquid nitrogen. Samples were stored at -80°C until further analysis. All assays were performed on ten individuals with each individual as a replicate.

Ecdysteroid Extraction and Quantification

Ecdysteroid extraction methods followed those applied to *R. pomonella* in the previous chapter with small modifications. Samples were homogenized in 1mL 100% methanol with Zirconia beads (2.0mm, Biospec products from Cole-Parmer, Vernon Hills, IL, USA) and then centrifuged at 10, 000 × *g* for 10 minutes. A volume of 900 μL of the supernatants was transferred to a 4 mL glass tube with cap (60940D-1 Kimble Chase, Rockwood, TN, USA). Tissue pellets were extracted twice with the same methods and both supernatants were pooled into the same glass tubes. Methanol in the collected extracts was evaporated under a stream of nitrogen gas and the extracts were resuspended in 100 μL 80% methanol.

Ecdysteroids were quantified with high-performance liquid chromatography – mass spectrometry (HPLC-MS) (Westerlund, 2004; Oostra *et al.*, 2011). Specifically, an Agilent 190 infinity HPLC in combination with an Agilent 6550 high resolution Q-TOF mass spectrometer was used for all analysis. The HPLC was equipped with an Agilent 2.1 mm ID x 50 mm Zorbax Extend C18 column that was kept at a temperature of 40°C. The mobile phase consisted of solvent A: H_2O and B: Acetonitrile, both with 0.1% formic acid. The 0.5 mL/min flow was programmed from 95% A, 5% B to 2% A, 98% B over 5 minutes and kept at that concentration for an additional minute. All samples were kept at 10°C and 5μL was injected per sample. A blank sample (80% methanol) was analyzed in-between each sample to control for and reduce the risk of sample carryover. In addition, the outside of the sampling needle was washed with solvent in-between injections. An extracted ion trace of m/z 447.3000 to 447.3100 and 481.2800 to 481.3400 combined with a retention time of 3.530 min and 3.275 min was used for all ecdysone and 20-hydroxyecdysone quantifications, respectively. Standards of ecdysone (purity≥95% HPLC grade, from ENZO Life Sciences, Farmingdale, NY, USA) and 20-

hydroxyecdysone (purity≥93% HPLC grade, from Sigma St. Louis, MO, USA) were used for the identification and quantification of detected ecdysteroids from samples.

Sampling for Sensitivity to Exogenous 20-Hydroxyecdysone

To compare the sensitivity of each strain to ecdysteroids over time between the earlier-emerging BE strain and the later-emerging UZ strain under diapause induction conditions, I injected a series of concentrations of 20E into 46-day old diapausing larvae held in diapause-promoting conditions at 23±1°C, L: D 12: 12, and observed them for diapause termination. Additionally, to compare the sensitivity of the two strains to ecdysteroids under diapause termination conditions, I injected a series of concentrations of 20E into diapausing larvae at different stages after exposure to warm, long-day conditions. Specifically, after *O. nubilalis* were reared at 23±1°C, L: D12: 12 for 46 days, diapausing larvae were transferred to 26±1°C, L: D 16: 8. BE strain larvae were injected at day 1, and weeks 1, and 2 after transfer to warm, long-day conditions. UZ strain larvae were injected at day 1, and weeks 1, 2, 3, 4, 5, 6, and 7 after transfer because of their longer diapause duration. After injection, the larvae were individually kept in a diapause termination chamber at 26±1°C L: D 16: 8 for observations of diapause termination. Across the treatments, three cohorts were conducted, and the total sample sizes ranged from 45 to 60 larvae for each treatment.

20-hydroxyecdysone Injection

Exogenous 20E for injection was bought as powder (purity≥93%) from Sigma (CAS Number 5289-74-7, St. Louis, MO, USA) and dissolved in 95% ethanol as a stock solution (10 mg/mL) that was stored at -20°C. The stock solution was diluted on the day of injection into a series of concentrations with 95% ethanol. Volume of 100 nL 0.01, 0.1, 1, 4, 7, or 10 mg/mL (0.001, 0.01, 0.1, 0.4, 0.7, and 1 μg) 20E were injected. As a control, 100nL of 95% ethanol was

injected. Pulled glass needles (Drummond Scientific Company, Broomall, PA, USA) were mounted on a microinjector (Nanoliter 2000, World Precision Instruments, Sarasota, FL, USA) used for injections. Solvent was injected into the thorax on the ventral side of larva. Pupation was used as a criterion of larval diapause termination. Given the possible damage caused by injections, an additional group of larvae that were not injected with anything were used as a further control group.

Statistical Analysis

Ecdysteroid concentrations were calculated as pg/mg of larva. I used the concentration of ecdysteroids (pg/μL) detected by HPLC and multiplied it by the resuspended volume (100 μL) to get the overall ecdysteroid quantity (pg), then corrected the measure by dividing by the fresh mass of each larva. Wilcoxon signed-rank tests were used to analyze the ecdysteroid concentrations. Ecdysteroid sensitivity was analyzed by a generalized linear model (GLM). The slope of the dose-response relationship to ecdysteroids was used as an indication of the capacity of each strain to terminate diapause. Slopes were compared between the two strains by an ANOVA followed by Tukey's HSD tests corrections for multiple comparisons (R 3.4.1). Strains (BE and UZ), weeks after warm, long-day exposure (day 1, week 1, and week 2), and cohorts were used as fixed factors.

Results

Ecdysteroid Concentrations

To understand the role of ecdysteroids in the regulation of diapause in *O. nubilalis*, I measured the concentrations of E and 20E in diapause-destined and non-diapause 5th instar larvae in both BE and UZ strains. In non-diapause larvae, both E and 20E were under my limit of detection (1 pg/ul) during the early stages of 5th instar larval development, but started to increase when larvae entered the wandering stage (day 8, Figure 3-4). Peaks of E and 20E appeared the

day before individuals became prepupae (day 10) and decreased into pupation (Figure 3-4). However, no ecdysteroids were detected in diapause-destined larvae of *O. nubilalis* (Figure 3-4), supporting the assertion that larval diapause is regulated by a suppression of ecdysteroid production and release (Bean & Beck, 1983; Gelman & Woods, 1983). Throughout this stage, no difference in ecdsyteroid concentrations was detected between non-diapause individuals of the BE and UZ strains ($P>0.05$).

To test whether the release time and concentrations of ecdysteroids differ between the two strains of diapausing *O. nubilalis* individuals that are undergoing diapause termination, I measured E and the active hormone 20E at multiple time points after transferring diapausing individuals into diapause termination conditions (L: D 16: 8, 26±1°C). Corresponding to an earlier larval diapause termination in the BE strain (Figure 3-5), an earlier increase in ecdysteroid titers was detected in the BE strain compared to the UZ strain (Figure 3-6). Specifically, both the peaks of E and 20E appeared at day 13 and 61 post long-day exposure in the BE and UZ strain, respectively (Figure 3-6). I also detected some small peaks before the big peak of E and 20E in the UZ strain (Figure 3-6), reflecting a few relatively early diapause-terminating individuals.

Ecdysteroid Sensitivity

The effect of exogenous 20-hydroxyecdsone on acceleration of larval diapause termination in *O. nubilalis* was strain specific. For statistical analysis, I only included the ethanol-injected group as my control, because I found no detectable difference in timing between control individuals injected with 95% EtOH and control individuals that received no injection. Therefore, I can rule out potential effects of ethanol and physical damage on the timing of larval diapause termination. For the UZ strain, 20E can accelerate larval diapause termination on day 1 ($P<0.000$), week 2 ($P=0.017$), and week 3 ($P=0.002$) after warm, long-day exposure with

detectable response of accelerated diapause termination with higher doses (Figure 3-7). However, after the third week of exposure to diapause-terminating conditions, 20E treatment could not accelerate the process of diapause termination (Figure 3-7). Thus, UZ individuals were initially responsive to exogenous ecdysteroids because they had not yet been capacitated to terminate diapause, but by the fourth week after exposure to diapause-terminating conditions UZ individuals were all capacitated to terminate diapause and exogenous application of ecdysteroids could no longer affect diapause termination timing. This loss of sensitivity to exogenous ecdysteroids suggests that critical regulatory events for diapause termination are occurring between 3-4 weeks after transfer to warm, long-day conditions in the UZ strain.

In contrast, exogenous ecdysteroids were not able to accelerate diapause termination in the BE strain at any time after transfer into diapause-terminating conditions (Figure 3-7). This difference between the strains suggests that the BE strain becomes capacitated to terminate diapause immediately upon transfer to warm, long-day conditions whereas the UZ strain does not become capacitated to terminate diapause for 3-4 weeks after transfer to diapause-terminating conditions. In addition, for those treatments exposed to diapause-terminating conditions after injections, a significant difference in the slope of the dose-response between the UZ and BE strains was only detected in the treatment when injections were done on the first day of transfer to diapause-terminating warm, long-day conditions (day 1, P=0.004) (Figure 3-7). However, for those individuals kept at the diapause-induction conditions after injections, I found no effect of 20E on acceleration of diapause termination in both the BE and UZ strains (Figure 3-8).

In addition to pupation, I also noticed another phenotype resulting from exogenous 20E treatment that is informative of ecdysteroid sensitivity. High doses of 20E stimulated larval-pupal mosaics (Figure 3-9) that will eventually die because of failure to complete pupation.

Following others in the literature (Williams, 1968; Beck & Shane, 1969), I have termed these individuals moribund. I used the proportion of moribund individuals induced by 20E as an indicator of ecdysteroid sensitivity. In the BE strain, 0.4, 0.7, and 1 μg 20E caused some larvae to become moribund as soon as they were exposed to diapause-terminating warm, long-day conditions (week 0), and the proportions of moribund larvae caused by 20E injection were not different for all time points from week 0 to week 2 (Figure 3-10 & Table 3-1). However, in the UZ strain, no moribund larvae were detected until weeks 2 after exposure to diapause-terminating conditions, and the proportions of moribund larvae were very low (Figure 3-10, 4% and 2% induced by 0.4 and 1 μg 20E, respectively). Starting the 3rd week after exposure to diapause-terminating conditions, the proportion of moribund larvae was positively related to the dose of injected 20E (0.4, 0.7, and 1μg), and increased over time for each dose in the UZ strain (Figure 3-10 & Table 3-1). In addition, for the relatively lower dose of 20E (0.1μg), 8% of the BE strain larvae became moribund at week 1 after exposure to diapause-terminating conditions, but I didn't find any moribund individuals induced by 0.1μg 20E in the UZ strain until weeks 7 (6%, Figure 3-10).

Discussion

Pioneering work conducted by Roelofs and colleagues about 40 years ago suggested that ecological differences in voltinism largely contribute to temporal isolation in *O. nubilalis* between the sympatric BE and UZ strains in upstate New York (Roelofs *et al.*, 1985; Dopman *et al.*, 2010). Several studies have shown that the distinct number of generations between the bivoltine BE and univoltine UZ strains can be attributed to a ~30-day shift in timing of diapause termination phase in the spring (Glover *et al.*, 1992; Dopman *et al.*, 2005, 2010; Wadsworth *et al.*, 2013). My study focused on evolutionary changes in endocrine profiles and sensitivity

associated with these differences in diapause timing and thus divergence in life cycle timing between the early-emerging BE and late-emerging UZ strains of the European corn borer. Similar to what I found in *R. pomonella* (chapter 2), my results here clearly showed that changes in the timing of ecdysteroid production and ecdysteroid sensitivity are associated with divergence in timing of diapause termination, and thus adult timing and temporal reproductive isolation between the early emerging and late emerging European corn borer strains.

Ecdysteorid Concentrations

Quantifying the timing and concentrations of ecdysteroids in non-diapause and diapause-destined 5th instar European corn borer larvae supported that larval diapause initiation is characterized by a suppression of synthesis of ecdysteroids (Denlinger, 2002). Previous work by Bean & Beck (1983) had already demonstrated the important regulatory role of ecdysteroids in larval diapause regulation with only one genotype of European corn borer. My work goes beyond this previous effort by investigating the earlier emerging and later emerging strains of European corn borer to test for the role of endocrine profiles in divergence of life cycle timing. Consistent with results of Bean & Beck (1983), in non-diapause individuals, peaks of both ecdysone and 20-hydroxyecdysone were detected the day before forming prepupae, which is associated with physiological and behavioral changes marked transition from wandering larval to prepupal stage. However, I did not detect any ecdysteorids in diapause-destined larvae of both BE and UZ strains, reinforcing that both strains use suppression of ecdysteroid production as a key for suppressing development in diapause. Although both strains suppressed the production of ecdysteroids during diapause, I expected that baselines of ecdysteorid concentrations in diapausing larvae could be higher from the earlier-emerging BE strain than the later-emerging UZ strain. However, I did not detect any difference in ecdysteroid concentrations between diapausing larvae of the BE and UZ strains, because ecdysteroids in both strains were under my

detection limit (1 pg/ul). My data indirectly support the metabolic studies conducted by Wadsworth et al. (2013). Metabolic rate suppression is one of the hallmarks of diapause, and thus metabolic rate can be used to phenotype whether animals are solidly in diapause (Denlinger, 1979). While the baseline metabolic rate during diapause did not differ between the early-emerging BE strain and the later-emerging UZ train, the BE strain increased metabolic rates almost immediately after transfer to diapause-terminating conditions whereas the UZ strain delayed their metabolic uptick by several weeks associated with their later diapause termination and later adult emergence (Wadsworth *et al.*, 2013), which lead to my hypothesis that evolutionary changes in ecdysteroid profiles during the diapause termination phase could contribute to divergence in life history timing between strains of the European corn borer.

Indeed, after exposing diapausing larvae to diapause termination conditions, the BE and UZ strains produced equivalent quantities of ecdysteroids, but the timing of that ecdysteroid peak was ~48 days later in the UZ strain. It should be noted that in the UZ strain, I detected several small peaks before the big peak of ecdysteroids, which could be due to the existence of some individuals that terminated diapause earlier than the bulk of individuals in the UZ strain. The shift in the timing of ecdysteroid release I observed is consistent with previous transcriptomic data from Wadsworth and Dopman (2015). They showed that the transcript abundance of several ecdysteriod-synthesis genes (e.g., *Neverland*, *Spook*, and *Phantom*) increased shortly after exposure to diapause termination conditions in the BE strain, but these same transcripts were not up-regulated for an extended period in the UZ strain (Wadsworth & Dopman, 2015).

Ecdysteroid Sensitivity

The effect of 20E on acceleration of diapause termination in European corn borer is strain specific. In my experimental setting, I found a clear dose response to 20E on weeks 0, 2, and 3 after long-day exposure in the UZ strain, but the effect of 20E disappeared from week 4 to week

7, indicating that individuals of the late-emerging UZ strain had become capacitated to terminate diapause regardless of exogenous 20E as of 4 weeks after transfer to warm, long-day conditions (Figure 3-7). In contrast, the BE strain appeared to be capacitated to terminate diapause as soon as they were transferred to warm, long-day conditions because no dose-response to 20E was detected after exposure to diapause-termination conditions. Interestingly, Beck & Shane (1969) pointed out that large single doses of ecdysone or 20E had no effect on acceleration of diapause termination in their work, but they did not consider the later-emerging UZ strain as I did here.

I also observed that high doses of 20E could cause pathological effects on larval development in both BE and UZ strains of European corn borer, in agreement with work done by Beck & Shane (1969). The ecdysteroid-induced moribund larval-pupal state has also been reported in other Lepidoptera species, such as Cynthia moth (Williams, 1968). A large pulse of either exogenous ecdysteorids alone or the sum of endogenous and exogenous ecdysteroids that exceed normal physiological hormone levels may be toxic and cause these pathological effects of ecdysteroids. Based on my ecdysteroid titer data, endogenous ecdysteroids started to increase soon after larvae were exposed to diapause-termination conditions in the BE strain, but endogenously produced ecdysteroids did not peak for the bulk of individuals in the UZ strain until 61 days after transfer to diapause-termination conditions, although some smaller peaks were detectable at 25 days and 45 days after the transfer to diapause-terminating conditions that are likely indicative of the few UZ individuals that terminated diapause earlier than the bulk of UZ individuals (Figure 3-6). Several low doses of ecdysteorids injected over extend periods may help avoid the pathological effects of single doses of ecdysteroids, a pattern that has been shown in *Manduca sexta* caterpillars (Nijhout, 1976; Bean & Beck, 1983). Therefore, further studies with multiple injections of lower concentrations of ecdysteroids at different time scale could be

helpful in picking apart the effects of ecdysteroids on diapause termination between these two strains of European corn borer.

From my observations, I expect that high levels of ecdysteroids applied before the animal is completely prepared to resume development may disrupt the temporal progression of larval-pupal metamorphosis by forcing the transition from larva to pupa before adequate physiological preparation is completed (Beck & Shane, 1969). Thus, I think that the moribund response is a useful metric for assessing the sensitivity of diapausing larvae to exogenous 20E. I observed that the sensitivity to these pathological effects differed between the two strains with time, suggesting differences in ecdysteroid sensitivity. Specifically, I found that the earlier-emerging BE strain expressed a high proportion of moribund larvae in response to high doses of exogenous 20E as soon as they were transferred to diapause-terminating conditions. In contrast, the later-emerging UZ strain did not respond to exogenous 20E with the production of moribund larvae until 3 weeks after receiving the diapause-terminating cues, and responsiveness of UZ larvae to produce moribund larvae increased from week 3 to week 4 and continued to increase to a consistent level between weeks 5 to 7 (Figure 3-10). Combining the effects of 20E on diapause duration and the moribund response, I can infer that the BE strain became capacitated to terminate diapause by increasing sensitivity to 20E substantially earlier than the UZ strain did, consistent with my predictions.

Here I showed that changes in the endocrine regulation of diapause are associated with shifts in life cycle timing across the early-emerging and late-emerging strains of European corn borer. My long-term goal is to identify the causal genetic mechanisms that may interact with the endocrine machinery to regulate changes in diapause timing. Based on previous studies, genes located in *Pdd* locus on the sex chromosome that are sensitive to photoperiod and temperature

are potential candidates (Wadsworth & Dopman, 2015). Interpreting the molecular and physiological mechanisms underlying this naturally occurring polymorphism in diapause regulation provides insights into our understanding for organisms' potential to respond to global climate change by shifting life-history timing and therefore seasonal phenology.

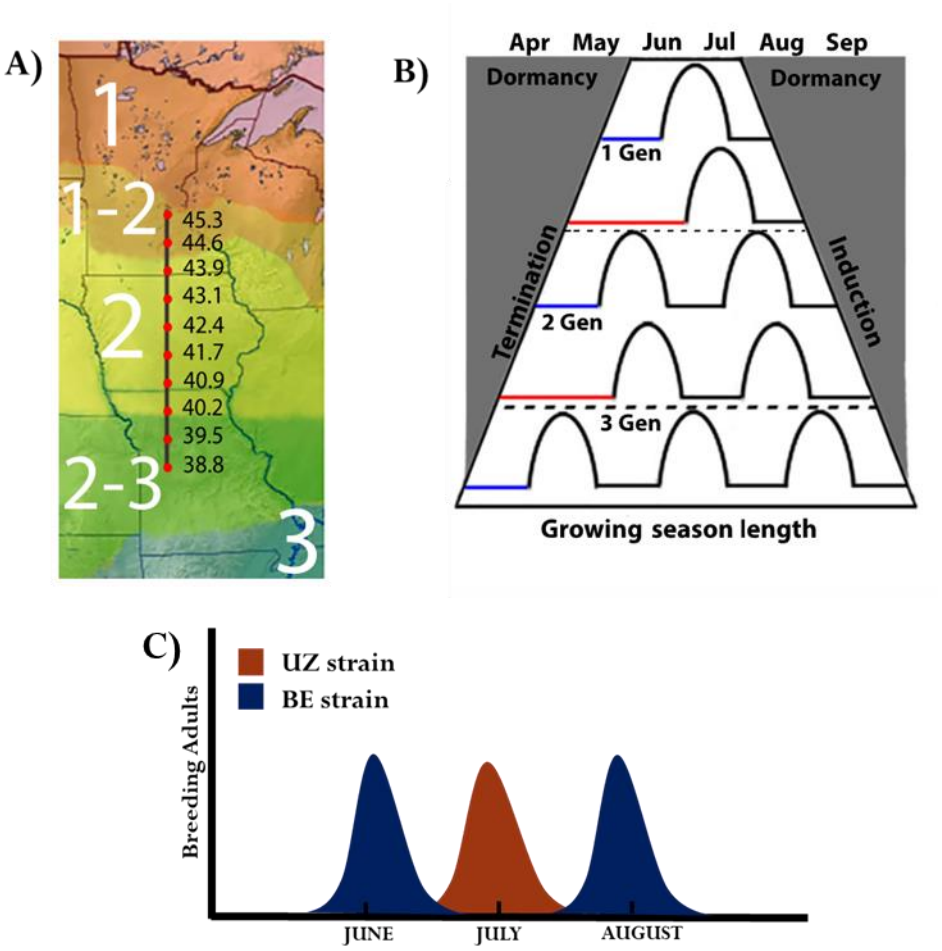

Figure 3-1. Geographical variation in voltinism in European corn borer. A) Clinal variation in voltinism (number of generations per year showed in white) across the Midwestern US (adapted from Showers 1981 and Levy *et al*., 2015). B) Illustration of the relationship between seasonal length and voltinism (dashed lines). Blue and red line represent short and long post-diapause development respectively (adapted from Levy *et al*., 2015). C) Breeding adults of bivoltine E-strain happen in June and August, and in July for univoltine Z-strain.

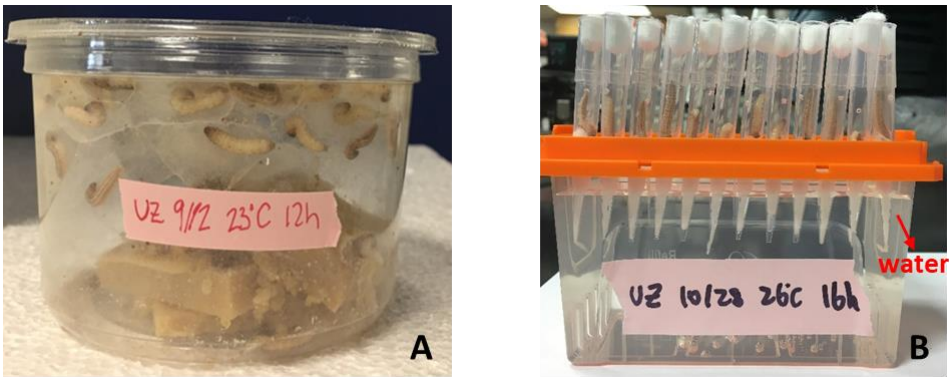

Figure 3-2. Diapause induction and diapause termination of *Ostrinia nubilalis*. A) A container set up for larval diapause induction, which served with artificial diet. B) Diapausing larvae were transferred into modified tips with cotton submerged in water filled 1000 µl pipette-tip box for diapause termination. Photo courtesy of Qinwen Xia.

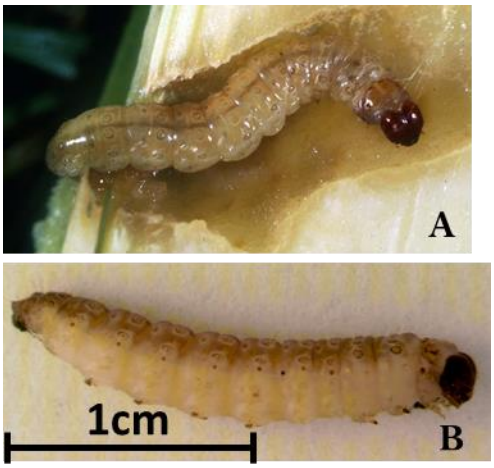

Figure 3-3. Morphological schematic of wandering larva and prepupa of *Ostrinia nubilalis*. A) wandering stage (Photo courtesy of B. Christine). B) prepupa (Photo courtesy of Qinwen Xia)

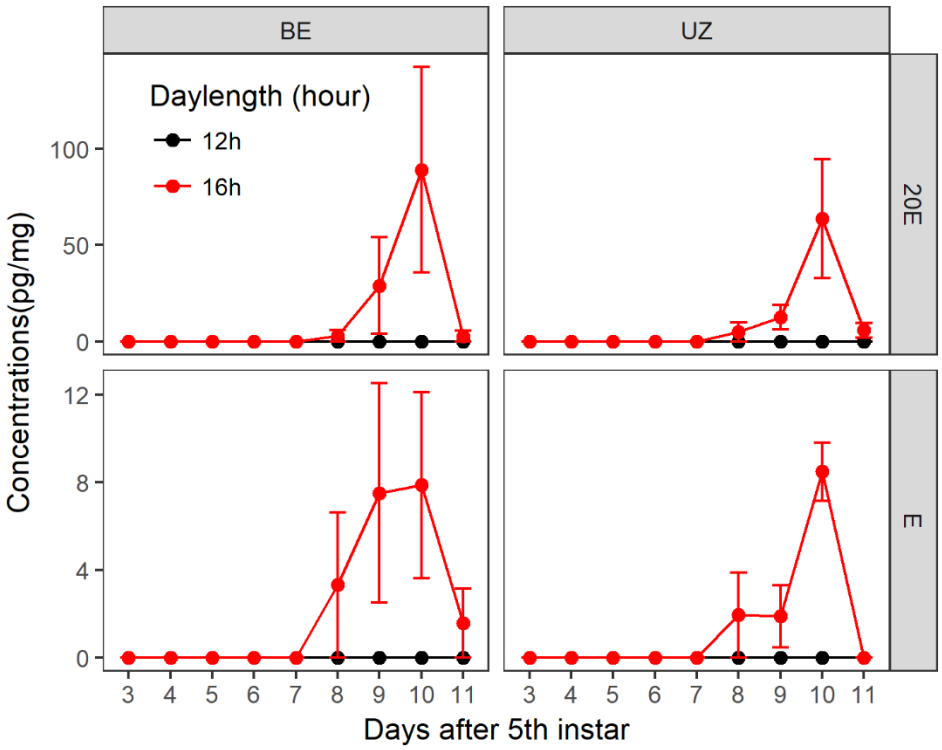

Figure 3-4. Ecdysone (E) and 20-hydroxyecdysone (20E) concentrations of BE and UZ strains of *Ostrinia nubilalis* days after 5th instar molt under the long-day (16h) and short-day (12h) conditions. The symbols represent means and bars represent standard errors.

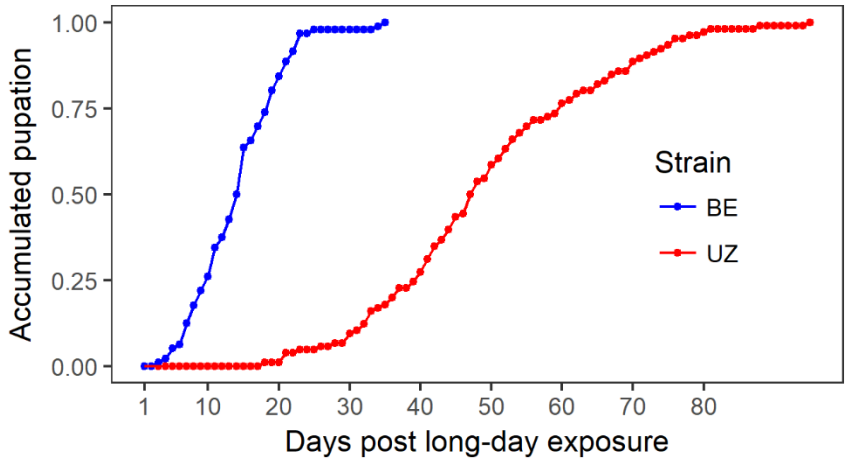

Figure 3-5. Accumulated pupation after warm, long-day exposure in earlier-emerging BE and later-emerging UZ strains of *Ostrinia nubilalis*.

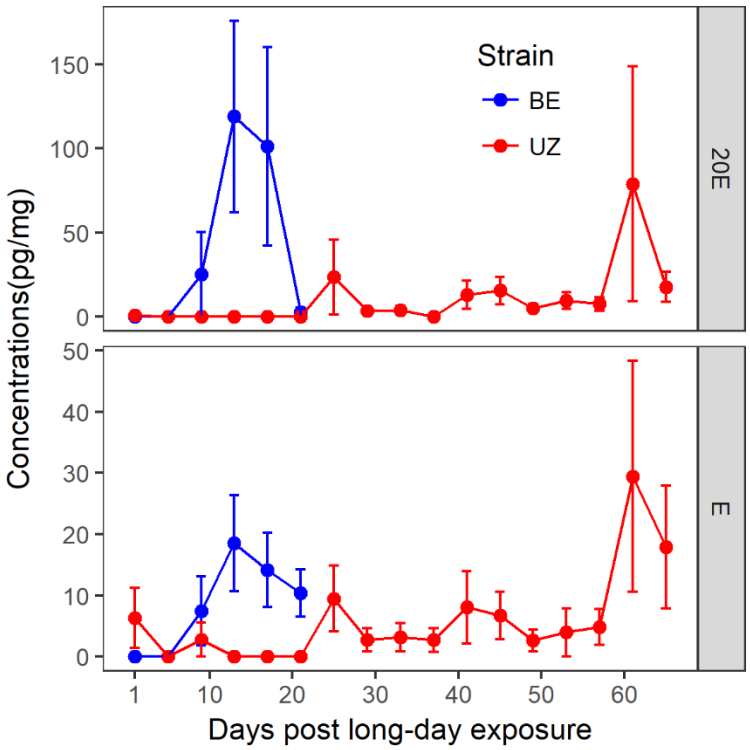

Figure 3-6. Ecdyteroid concentrations after warm, long-day exposure in BE and UZ strains of *Ostrinia nubilalis*. The symbols represent means and bars represent standard errors.

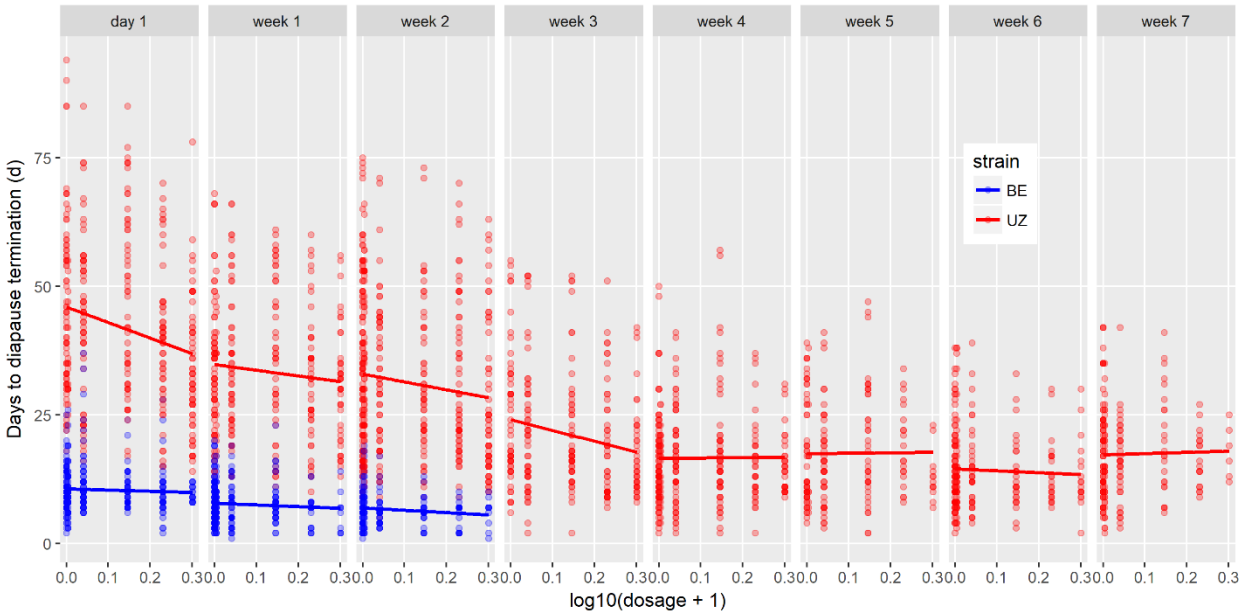

Figure 3-7. Comparison of days taken to terminate diapause between BE and UZ strains of *Ostrinia nubilalis* after treatment of 20-hydroxyecdysone. In the BE strain, injections were applied at day 1, weeks 1, and 2 after transfer to diapause-termination conditions. In the UZ strain, injections were applied at day 1, weeks 1, 2, 3, 4, 5, 6, and 7 after transfer to diapause-termination conditions.

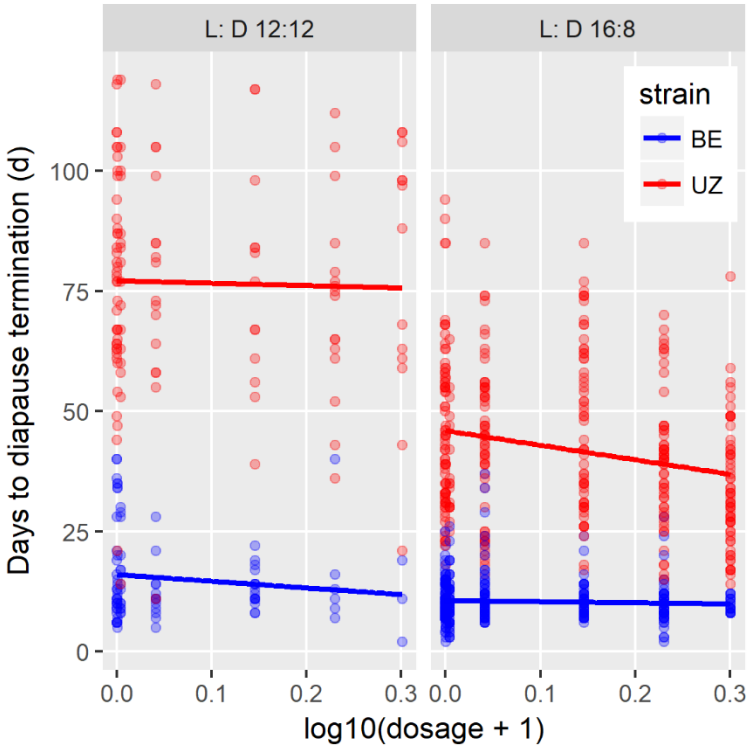

Figure 3-8. The effect of 20-hydroxyecdysone on larval diapause termination of *Ostrinia nubilalis* under diapause induction (L: D 12:12, 23±1°C) and termination (L: D 16:8, 26±1°C) conditions.

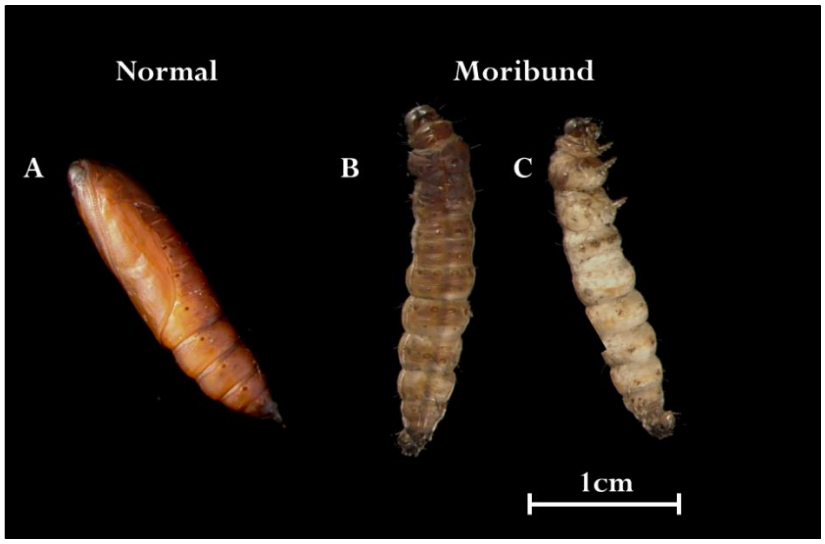

Figure 3-9. Images of a normal pupa and a moribund larval-pupal individual (A: normal pupa, B: dorsal, C: abdomen) of *Ostrinia nubilalis*. Photo courtesy of Qinwen Xia.

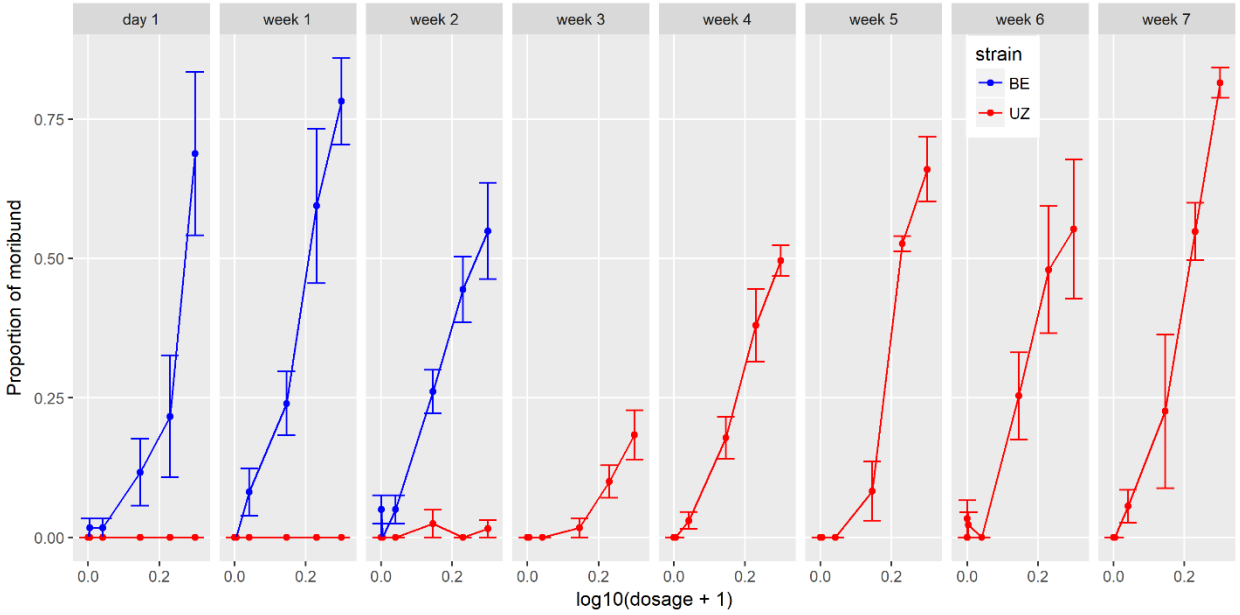

Figure 3-10. Comparisons of proportion of moribund individuals between BE and UZ strains of *Ostrinia nubilalis* after treatments of 20-hydroxyecdysone. In the BE strain, injections were applied at day 1, weeks 1, and 2 after transfer to diapause-termination conditions. In the UZ strain, injections were applied at day 1, weeks 1, 2, 3, 4, 5, 6, and 7 after transfer to diapause-termination conditions.

Table 3-1. ANOVA model fits for the dose-response of moribund to 20-hydroxyecdysone after exposure to diapause-termination conditions.

Model	Df	F	P
BE strain			
Week	2	1.203	0.277[ns]
Dose	1	5.469	<0.000*
Week*Dose	2	4.946	0.946 [ns]
UZ strain			
Week	7	42.59	<0.000*
Dose	1	567.75	<0.000*
Week*Dose	7	66.76	<0.000*

ns, non-significant. Starred terms are significant at $P<0.05$.

CHAPTER 4
EXPLORING THE ROLES OF PIGMENT-DISPERSING FACTOR AND ITS RECEPTOR ON LIFE CYCLE TIMING IN EUROPEAN CORN BORER, *OSTRINIA NUBILALIS*

Introduction

Diapause, a trait that temperate insects use to survive winter stress and to synchronize themselves with favorable periods, has been a target of natural selection under ongoing climate change for decades. Diapause can evolve rapidly in invasive species or species that have been introduced to novel resources (e.g., host-plants) (Feder & Filchak, 1999; Dopman *et al.*, 2010; Ording *et al.*, 2010). Diapause timing can also evolve rapidly in response to the warmer winters and earlier arrival of spring associated with contemporary climate change (Bradshaw & Holzapfel, 2001). A thorough understanding of the mechanisms underlying rapid evolution of life history timing via diapause is urgent for our ability to predict organisms' responses to changing environment. Several transcriptomic studies have been done to reveal candidate genes or pathways involved in evolution of insect seasonality (Ragland *et al.*, 2011; Wadsworth & Dopman, 2015), but the exact genes modified during the evolutionary process remain unknown.

Diapause timing is under the regulation of external environmental factors (e.g., temperature and/or photoperiod) and endogenous factors (e.g., hormones). By carrying out studies on the endocrinology underlying a rapid evolution of life cycle timing via diapause, we can trace back to the upstream molecular events that trigger the diverged endocrine regulation of diapause across host races or strains within insect species and ultimately perhaps gain insights into the diversification of diapause life histories across species. For example, in the European corn borer, *Ostrinia nubilalis*, evolutionary changes in endocrine regulation of larval diapause are associated with diverged life cycle timing between the earlier-emerging BE strain and the later-emerging UZ strain (Chapter 3). Dopman et al. (unpublished data from my collaborator) found that fixed SNPs in the binding site of a hormone receptor for the neuropeptide pigment

dispersing factor (PDF) were associated strongly with early vs. late emergence in European corn borer.

The PDF receptor (PDFR) is a secretin-like G-protein-coupled receptor that is well known for its role in regulating circadian locomotor activity rhythms (Hyun *et al.*, 2005; Lear *et al.*, 2005; Mertens *et al.*, 2005). Pigment dispersing factors (PDFs), the ligands of PDFR, are a family of highly conserved neuropeptides consisting of 18 amino acids in insects (Rao & Riehm, 1989; Park & Hall, 1998; Shafer & Yao, 2014). PDFs were first discovered in crustaceans where they were named after their roles in daily rhythms of pigment dispersion and migration along the dorsal blood vessel (Rao & Riehm, 1989). Insect PDFs were later identified when it was found that they are expressed in two classes of clock neurons: large ventral lateral neurons and small ventral lateral neurons, and ultimately released from the brain to coordinate clocks in peripheral tissues with the central clock neurons in the brain (Helfrich-Förster & Homberg, 1993; Helfrich-Förster, 1997; Renn *et al.*, 1999; Helfrich-Förster *et al.*, 2007). The role of PDFs as important regulators of the insect circadian rhythmic behavior has been well studied in *Drosophila melanogaster*. For example, by knocking out PDF, *Drosophila* flies lost the ability to delay their nighttime peak of activity during long days (Yoshii *et al.*, 2009), showing that PDF may also be important in photoperiodic responses. Besides their best understood role in circadian rhythmicity, PDFs also are involved in regulating other behaviors and physiology, such as flight (Agrawal *et al.*, 2013), copulation (Kim *et al.*, 2013), geotaxis (Toma *et al.*, 2002), and tracheal growth (Linneweber *et al.*, 2014). A novel role of PDFs reported by Iga *et al*. (2014) is stimulation of ecdysone biosynthesis in *Bombyx mori*.

Ecdysone is well known for its regulation in insect growth and development as well as larval and pupal diapause. Ecdysone is synthesized in insect prothoracic glands and its

biosynthesis and release are regulated by several neuropeptides. Prothoracicotropic hormone (PTTH) is the most well-known brain neuropeptide to stimulate the secretion of ecdysone by binding to its receptor tyrosine kinase Torso (Rewitz *et al.*, 2009; Smith & Rybczynski, 2012). In the PTTH signaling pathway, levels of the second messenger cAMP increase in prothoracic glands by stimulation of PTTH, and then cytochrome P450 enzymes (e.g., Phantom, Spook, Disembodied, Shadow, Neverland) that synthesize ecdysteroids are activated in the prothoracic glands. Besides PTTH, a series of other peptide hormones have also been implicated in facilitating the production of ecdysteroids including the lepidoptera insulin-like peptide bombyxin (Ishizaki *et al.*, 1983), diapause hormone (Zhang *et al.*, 2004; Watanabe *et al.*, 2007), and orcokinin (Yamanaka *et al.*, 2011). Recently, PDF was also shown to participate in stimulating ecdysone biosynthesis in the silkworm *Bombyx mori* (Iga *et al.*, 2014).

In *Bombyx mori*, the expression pattern of the PDF receptor (BNGR-B2) corresponded to the pattern of ecdysone titers of continuously developing 5^{th} instar larvae, and applications of PDF can also stimulate ecdysone biosynthesis in the prothoracic glands (Iga *et al.*, 2014). In addition, the PDF signaling pathway partially overlaps with PTTH signaling pathway by increasing the levels of intracellular cAMP in the prothoracic gland (Iga *et al.*, 2014). However, the induction of ecdysone biosynthesis by PDF has only been reported in *Bombyx mori* thus far, and has only been shown in the prothoracic glands of direct developing individuals. Perhaps PDF can also stimulate ecdysone biosynthesis in other insect species or even regulate larval or pupal diapause via its effects upstream of ecdysteroid synthesis. More work on the roles of PDF across different insect species and diapausing individuals is clearly needed.

In this study, I aim to test for a role of PDF and its receptor PDFR as potential candidates for upstream regulation of endocrine divergence between the earlier-emerging E strain (BE) and

later-emerging Z strain (UZ) of European corn borer. In Chapter 3, I showed that earlier production of ecdysteroids as well as an earlier sensitive stage to exogenous 20-hydroxyecdysone (20E) in the BE strain compared to the UZ strain was associated with the divergence in timing of diapause termination. Here, I hypothesized that PDF regulates ecdysteroid biosynthesis in *O. nubilalis*. In addition, PDFR could be the target of selection that drives the diverged sensitive stage to 20E between the BE and UZ strains. Within my hypothesis I tested three specific predictions. First, I predicted that PDF could stimulate prothoracic glands to synthesize ecdysone in *O. nubilalis*. Second, I predicted that exogenous PDF could accelerate diapause termination in *O. nubilalis*. Third, I predicted that an increased transcript abundance of *PDFR* in the prothoracic glands is associated with an increased capacity to terminate diapause after exposure to diapause-termination conditions. My data showed that PDF treated prothoracic glands produced more ecdysone than controls. However, I detected no effect of PDF on accelerating diapause termination. The expression of the PDF receptor did not correspond to the pattern of ecdysone production in the prothoracic glands of non-diapause 5^{th} instar larvae. In addition, the transcript abundance of *PDFR* in the prothoracic glands of BE and UZ strains did not correspond to their patterns of diapause termination under the diapause-termination conditions.

Materials and Methods

Insects and Rearing

Colonies of the BE and UZ strains of European corn borer donated from Erik Dopman's lab at Tufts University were maintained at the University of Florida following the methods of Glover *et al*. (1992). Newly hatched larvae were collected and fed on an artificial diet (LOT# 052418ECB, Southland Products, Lake Village, AR, USA) in a polypropylene deli-cup (11.75 cm × 9.84 cm × 7.62 cm, Bare by Solo, Dart Container Corporation, Mason, MI, USA) at a

density of 80 individuals. Non-diapausing individuals were obtained by rearing under long-day conditions including light: dark (L: D) 16:8 at constant 23±1°C. A short-day photoperiod L: D 12:12 at constant 23±1°C was used for larval diapause induction. Larvae that were clearly in diapause after 45 days in short-day conditions were individually transferred from their rearing container into a new chamber that was modified from a 1mL pipette tip containing cotton moistened with water to track diapause termination. No food is needed in this diapause termination chamber because diapausing larvae do not feed during diapause or during larval-pupal metamorphosis. Diapausing larvae are sensitive to long-day and high temperature cues for the termination of diapause and resumption of development from larvae into pupal morphogenesis, thus I shifted both temperature and light cycle to strongly trigger larval diapause termination. Specifically, after 46 days in diapause-induction conditions (L: D 12:12 at 23±1°C), diapausing larvae were transferred to L: D 16:8 at 26±1°C to trigger diapause termination.

Gene Expression Analysis and Sampling

To test if PDFR expression matches the pattern of ecdysone biosynthesis, I measured the *PDFR* transcript abundance in prothoracic glands from non-diapause 5th instar larvae of the UZ strain. I also measured the transcript abundance of the PTTH receptor *Torso* as a parallel metric of whether the prothoracic gland was ready to receive signals stimulating ecdysteroidogenesis. Prothoracic gland samples were collected at days 3, 4, 5, 6, 7, 8, 9, 10, and 11 after the molt into the 5th instar. I also compared the transcript abundance of *PDFR* between the BE and UZ strains after transfer into diapause-terminating conditions. Prothoracic gland samples of the BE strain were collected at 1 day, and weeks 1 and 2 after transfer to diapause-terminating, long-day conditions and samples from the UZ strain were collected at 1 day, and weeks 1, 2, 3, 4, 5, and 6 after transfer to diapause-terminating long-day conditions because of UZ's longer diapause

duration compared to the BE strain. As a control, *PDFR* transcript abundance was also measured in imaginal wing discs. Prothoracic glands and wing discs were dissected on ice-cold 1x PBS solution and stored at -80°C after snap freezing with liquid nitrogen for future qRT-PCR analysis. Prothoracic glands from 20 to 35 larvae, and wing discs from 4 to 5 larvae were respectively pooled as one replicate. All assays were performed on three or four replicates.

RNA Extraction and qRT-PCR

RNAs from prothoracic glands and wing discs were extracted using the Ambion RNAqueous-Micro Kit (Cat. No. AM 1931, Thermo Fisher Scientific) following the manufacturer's protocol. The extracted RNAs were reverse transcribed with the High Capacity cDNA Reverse Transcription Kits (Cat. No. 4368814, Thermo Fisher Scientific) according to the manufacturer's protocol. qRT-PCR was conducted on a Thermal Cycler CFX96 Real-Time system (Bio-Rad) using SsoAdvanced Universal SYBR Green Supermix (Cat. No. 1725271, Bio-Rad). *Actin* and *Ribosomal protein S03* (*RpS03*) served as internal reference standards. Primers for *PDFR, Torso, Actin,* and *RpS03* were designed from the European corn borer transcriptome (Wadsworth & Dopman, 2015) and can be found in Table 4-1. The PCR product of each transcript was sent to Genewiz (South Plainfield, NJ, USA) for Sanger Sequencing to confirm amplicon identity. The sequences from Sanger Sequencing were blasted against with the European corn borer transcriptomic data showing identity in NCBI Blast.

***Ex Vivo* Tissue Culture**

To evaluate the effect of PDF on ecdysone biosynthesis, prothoracic glands from 8-day old 5^{th} instar direct developing larvae were dissected. Each pair of prothoracic glands dissected from each larva was haphazardly split into two groups. Ten prothoracic glands were pooled together as one sample. One group of 10 dissected prothoracic glands was cultured in 100 µL Grace's Insect Medium (1X, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) with PDF

solution, and the other group of 10 prothoracic glands was cultured in 100 µL Grace's Insect Medium with 1 µL DMSO only. After 4 hours' culture, samples were snap frozen with liquid nitrogen and stored at -80°C for future ecdysone quantification. The PDF from European corn borer is an octadecapeptide with an amino acid sequence of NADLINSLLALPKGMNDA. The protein precursor sequence of PDF was designed from the European corn borer unpublished genome, and the mature protein sequence of PDF was identified by blasting against with *Bombyx mori* PDF sequence (94% identity). PDF used for my experiments was commercially synthesized by GenScript (New Jersey, USA). Concentrations of 0.1, 1, 10, and 100 µM PDF were included in prothoracic gland culture for my dose-response experiments. Five replicates were performed for all assays.

Ecdysone Extraction and Quantification

Ecdysone extraction from *ex vivo* incubations followed the protocol described by Iga et al. (2014) with subtle modifications. After thawing at room temperature, samples were diluted with 100 µL HPLC grade water. Then 400 µL of 1-butanol was added into diluted samples and vortexed for 3 minutes. Samples were centrifuged at 1,000 × *g* for 10 min at 4°C, and the 1-butanol phase (~390 µL) was transferred to a new 4mL glass tube with cap (60940D-1 Kimble Chase, Rockwood, TN, USA). The 1-butanol in the collected extracts was evaporated under a stream of nitrogen gas and the extracts were resuspended in 50 µL 80% methanol. Ecdysone was quantified with high-performance liquid chromatography – mass spectrometry (HPLC-MS) as described in Chapters 2 and 3.

PDF Injection

I tested if PDF could accelerate larval diapause termination in European corn borer. Based on the transcript abundance of *PDFR* after exposure to diapause-termination conditions,

week 1 diapausing UZ larvae were used for PDF injections. A volume of 500 nL containing 1, 5, or 10 mg/ml DMSO-dissolved PDF (0.5, 2.5, and 5 µg) was injected directly into the thorax on the ventral side of larva as my pharmacological treatment. As a control, 500 nL of DMSO was injected. Given the possible damage caused by injections, an additional group of larvae that were not injected with anything were used as a further control group. The pulled glass needle (Drummond Scientific Company, Broomall, PA, USA) was mounted on a microinjector (Nanoliter 2000, World Precision Instruments, Sarasota, FL, USA) used for PDF injection. Pupation was used as a criterion of larval diapause termination. Across the treatments, sample sizes ranged from 30 to 40 larvae in each dose treatment.

Statistical Analysis

For the *ex vivo* ecdysone-titer analysis, one-way ANOVA was used with treatment (PDF or control) as a factor. Pair-wise comparisons of ecdysone-titer differences between each PDF treatment and control were conducted by t-tests. For the qRT-PCR analysis, the abundance of transcripts of *PDFR* and *Torso* were normalized to *Actin* and *RpS03*. The calculation of relative expression followed Pfaffl's methods (Pfaffl, 2001; Pfaffl *et al.*, 2002). An ANOVA model was used to analyze the relative expression, with data log10 transformed to meet model requirements. Day (days 3, 4, 5, 6, 7, 8, 9, 10, and 11 after the 5th instar molt) or week (day 1, weeks 1, and 2 after exposure to diapause-terminating conditions) was treated as a factor for the non-diapause stage and for the diapause termination period, respectively. Tukey's HSD tests were used to correct for multiple comparisons among time points.

Results

Characterization of *PDFR* Transcript Abundance

PDFR transcript abundance in the prothoracic glands of non-diapause larvae peaked on day 11 after the 5th instar molt, the day of prepupa formation (Figure 4-1). A small peak of

PDFR transcript abundance occurred on day 6, the day before gut purge (Figure 4-1). The transcript abundance of *Torso* showed similar pattern with the *PDFR* in non-diapause 5th instar larvae (Figure 4-1).

PDFR transcript abundance increased after transfer into diapause-terminating, long-day conditions in the BE strain (Figure 4-2), which corresponded to the diapause termination pattern. However, the transcript abundance of *PDFR* decreased from week 1 after transfer into warm, long-day conditions in the UZ strain (Figure 4-2). No significant difference in *PDFR* transcript abundance in prothoracic glands was detected between the BE and UZ strains at day 1, weeks 1 and 2 after transfer to diapause-termination conditions (Table 4-2). The transcript abundance of PTTH receptor *Torso* was essentially flat in both the BE and UZ strains after transfer to diapause-termination conditions (Figure 4-2 and Table 4-2). In the wing discs, the transcript abundance of *PDFR* in both the BE and UZ strains increased over time after exposure to diapause-terminating conditions, corresponding to the pattern of diapause termination (Figure 4-2). In addition, the transcript abundance of *PDFR* in the wing discs was significantly higher in the BE strain than the UZ strain ($P<0.001$, Table 4-2).

Effects of PDF on Ecdysone Biosynthesis

The effect of PDF on ecdysone biosynthesis in the prothoracic glands of *O. nubilalis* was evaluated by *ex vivo* culture either with or without PDF. Prothoracic glands cultured with PDF produced more ecdysone relative to controls. Specifically, PDF doses of 0.1, 10, and 100 μM all stimulated significantly higher ecdysone production than controls without PDF (Figure 4-3). Prothoracic glands cultured with 1 μM PDF still produced more ecdysone than controls, but the effect was not statistically detectable (Figure 4-3). Treatments with a low dose of 0.1 μM PDF showed better separation from controls than other higher doses (Figure 4-3). I generated a dose-

response relationship by subtracting the ecdysone in control group from the ecdysone in PDF cultured group for each dose and found no clear pattern (Figure 4-4).

Effects of PDF on Larval Diapause Termination

To test if PDF can stimulate larval diapause termination in European corn borers, I injected different doses of PDF into diapausing UZ larvae and observed them for pupation. Contrary to my prediction, PDF had no effect on acceleration of larval diapause termination in European corn borer when compared to a DMSO control (Figure 4-5). However, all the PDF-treated groups and DMSO control group showed faster diapause termination than the group of larvae that were not damaged by injection (Figure 4-5).

Discussion

GWASS analysis by Dopman et al. (unpublished data) revealed that fixed SNPs in the binding site of PDFR were associated with divergence in life cycle timing between the BE and UZ strains of *O. nubilalis*. Given the effect of PDF on ecdysone biosynthesis reported in *Bombyx mori* (Iga *et al.*, 2014), I inferred that evolutionary changes in PDFR might play a regulatory role in the diversification of diapause timing by endocrine regulation in European corn borer. PDF and PDFR are well known for their regulation of insect circadian rhythms, but their role in ecdysone biosynthesis has only been reported in *B. mori* to date. However, PDF has been associated with diapause initiation in several insects. For example, in the blow fly, *Protophormia terraenovae* bilaterally ablated neurons with PDFs became arrhythmic in locomotor activities, and showed disrupted diapause induction because of being unable to discriminate photoperiod (Hamanaka *et al.*, 2005; Shiga, 2012). Also, surgical removal of neurons with PDF cell bodies led to disruption of photoperiodic regulation of diapause in the bean bug, *Riptortus pedestris* (Ikeno et al., 2014). While in the Northern house mosquito, *Culex pipiens*, adult females entered a diapause-like state even when they were reared under diapause-everting condition by knocking

down PDF genes (Meuti et al., 2015). All three examples mentioned above suggested that PDFs are important for regulation of photoperiodic diapause. However, none of the studies connect PDF or PDFR with endocrine outputs in the context of diapause regulation. In the European corn borer, I expected that PDF may be involved in regulation of larval diapause by controlling the production of ecdysteorids after diapausing larvae received photoperiodic cues. Therefore, in my study, I tested the extent to which PDF could stimulate ecdysone biosynthesis in European corn borers and accelerate larval diapause termination.

Unlike what was observed in *B. mori* (Iga *et al.*, 2014), the peak of ecdysone production occurred one day earlier than the day with most abundant *PDFR* transcript abundance in *O. nubilalis* (Figure 4-1). My observation that *PDFR* transcript abundance peaked just after ecdysteroid titers peaked rather than before could be explained by the idea that the peak of *PDFR* transcript abundance at day 11 after the 5th instar molt (prepupa) might be correlated with the production of more PDFR protein in the prothoracic glands that will ultimately help to stimulate a pulse of ecdysone release to trigger the forthcoming pupal-pharate adult metamorphosis in these non-diapause larvae. Instead, the abundance of the PDFR protein used for the ecdysteroid peak on day 10 after the 5th instar molt may already have been produced from the minor peak of *PDFR* transcript abundance at day 6 after the 5th instar molt. Additionally, PTTH has been shown to stimulate ecdysone biosynthesis in *O. nubilalis* (Gelman *et al.,* 1992) and the transcript abundance pattern of the PTTH receptor Torso also followed a similar pattern to PDFR in non-diapause 5th instar larvae (Figure 4-1). Similarly, in *B. mori* (Mizoguchi *et al.*, 2001; Rewitz *et al.*, 2009) and *Mamestra brassicae* (Yamada *et al.*, 2016), the temporal transcript abundance of *Torso* does not directly correspond to the temporal pattern of ecdysteroids, even though PTTH clearly regulates the production of ecdysteroids in both species. Therefore, effects of PDF and

PDFR on ecdysone biosynthesis in *O. nubilalis* could not be ruled out based on my transcript abundance data. To further examine the roles of PDF and PDFR in stimulation of ecdysone biosynthesis, antibodies are necessary to quantify the protein abundance of PDF and PDFR in non-diapause 5th instar larvae in future studies. I predict a peak of PDFR protein abundance, as well as a peak in circulating PDF, before the pulse of ecdysone (day 10 after 5th instar molt) in non-diapause 5th instar larvae.

With earlier production of ecdysteorids in the earlier-emerging BE strain and later-emerging UZ strain, I expected that the transcript abundance of *PDFR* would correspond to the pattern of ecdysteorid synthesis and larval diapause termination in each strain. The transcript abundance of *PDFR* showed no difference between the BE and UZ strains at day 1, week 1, or week 2 after transfer to diapause-termination conditions. In the BE strain, the transcript abundance of *PDFR* increased over time after transfer into diapause-terminating conditions, which corresponded with my expectations for larval diapause termination. On the contrary, the transcript abundance of *PDFR* decreased over time dropping to its lowest point as the UZ strain began to terminate diapause. The transcript abundance of *Torso* was essentially flat in both the BE and UZ strains after transfer into diapause-termination conditions, suggesting the necessity of antibodies to quantify the protein abundance. Overall my transcriptional data are equivocal with respect to the role of PDFR in diapause termination.

However, the transcript abundance of *PDFR* was consistent with my expectations for a role in diapause termination in the wing discs. Specifically, the transcript abundance of *PDFR* in wing discs of both the BE and UZ strains increased as the larvae became more capacitated to terminate diapause. The transcript abundance of *PDFR* in the BE strain increased earlier than the UZ strain, which may be associated with an earlier diapause termination in the earlier-emerging

BE strain compared to the later-emerging UZ strain. My results with regard to *PDFR* transcript abundance in wing discs suggest that PDFR is associated with diapause termination but may not be involved in ecdysone regulation, because the wing disc is not the tissue to synthesize ecdysone.

I also cultured prothoracic glands of *O. nubilalis ex vivo* with several concentrations of PDF to test for an effect of PDF on ecdysone biosynthesis. Prothoracic glands exposed to PDF *ex vivo* produced more ecdysone than controls treated only with DMSO, suggesting that PDF can stimulate ecdysone biosynthesis in European corn borer. However, there was no clear dose-response, which could be explained by my use of inappropriate doses for the *ex vivo* experiment. One possibility for the unclear dose-response could be that the doses I used are too high, so the response has been saturated. In this case additional lower doses are needed in future studies. Alternatively, the doses I applied are too low, thus my dose-response is still under the lowest effect range. Therefore, higher doses will also be needed in future experiments.

Furthermore, injecting PDF directly into diapausing larvae had no effect on accelerating diapause termination in European corn borer with an essentially flat dose-response. One reason for the failure of acceleration of PDF on diapause termination could be that the doses chosen were not high enough. Perhaps higher doses of PDF are needed in future studies to confirm the role of PDF in stimulating ecdysone biosynthesis. Alternatively, a different sampling time point rather than week 1 after transfer into diapause-termination conditions for injection is needed. For the 20-hydroxyecdysone sensitivity experiment in Chapter 3, a dose-response for diapause termination was only found in day 1, week 2, and week 3 after transfer into diapause-termination conditions. Based on what I found, the potential roles of PDF and PDFR in the evolution of diapause timing remain to be carefully tested.

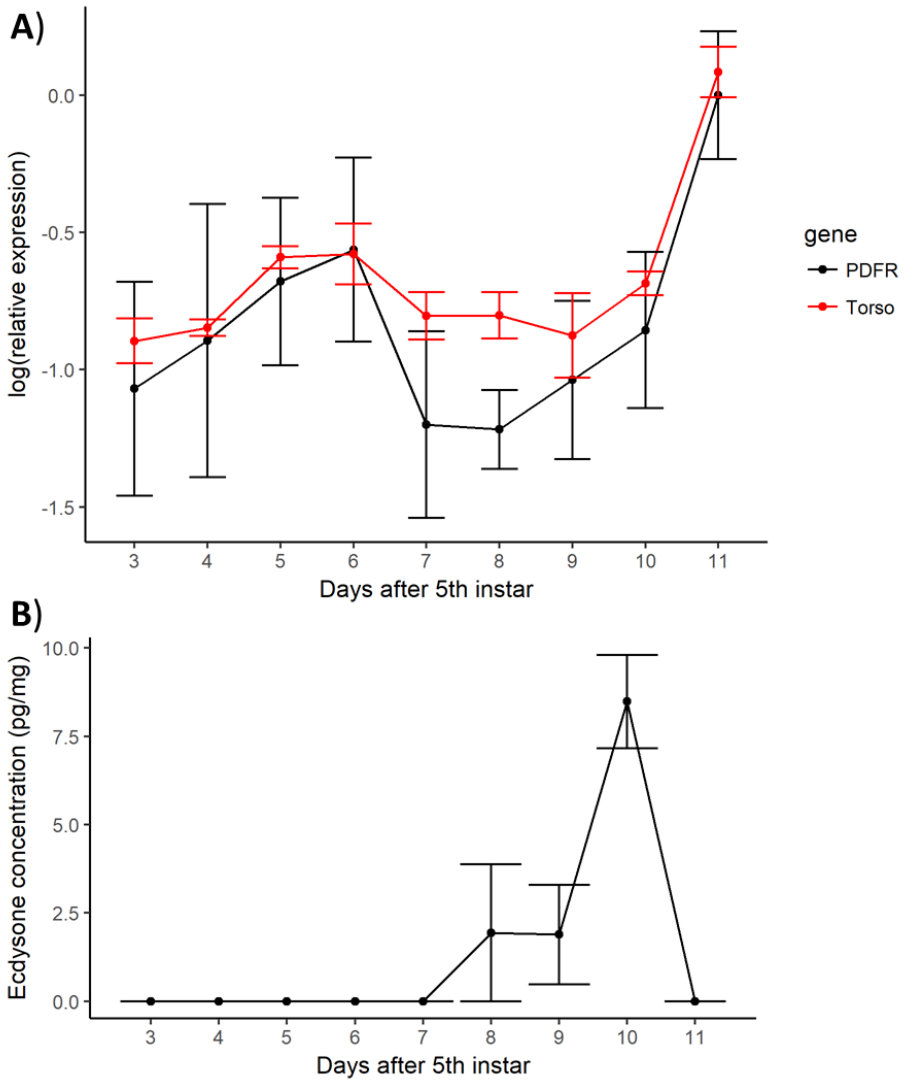

Figure 4-1. Schematic of the relationship between the transcript abundance of *PDFR* and *Torso* and ecdysone synthesis in prothoracic glands of *Ostrinia nubilalis*. A) The transcript abundance of *PDFR* and *Torso* in prothoracic glands of continuously developing 5th instar larvae of *Ostrinia nubilalis*. B), the ecdysone titers of continuously developing 5th instar larvae of *O. nubilalis.* The symbols represent means and bars represent standard errors.

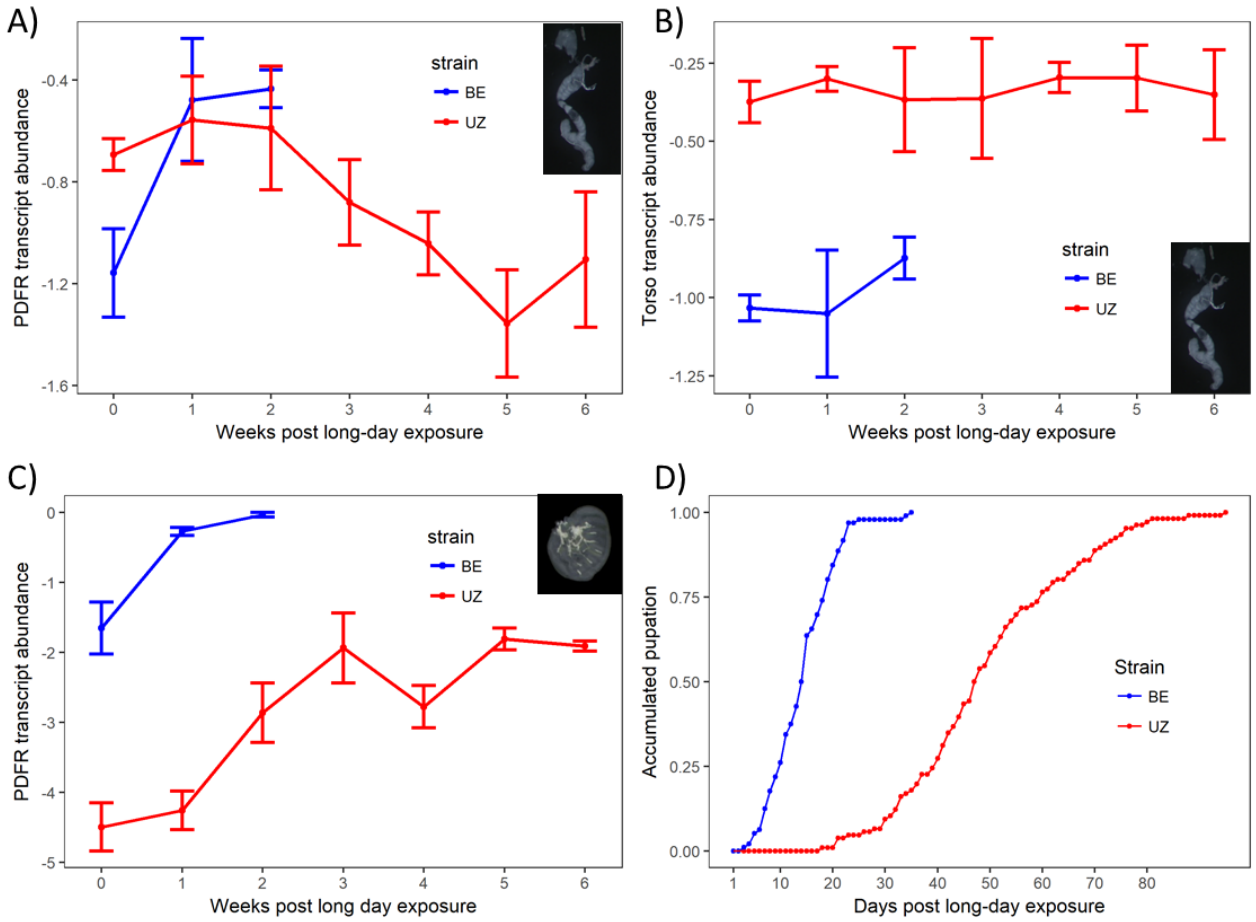

Figure 4-2. The transcript abundance of *PDFR* in the prothoracic glands and wing discs of BE and UZ strains of *Ostrinia nubilalis* after exposure to diapause-termination conditions. A) the *PDFR* transcript abundance in the prothoracic glands. B) the *Torso* transcript abundance in the prothoracic glands. C) the *PDFR* transcript abundance in the wing discs. D) Accumulated pupation of BE and UZ strains after transfer to diapause-termination conditions. The symbols represent means and bars represent standard errors. Photo courtesy of Qinwen Xia.

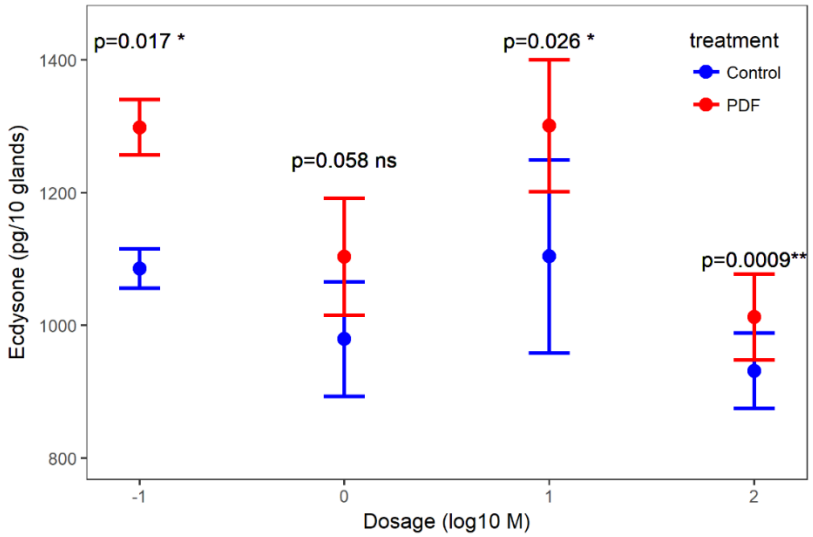

Figure 4-3. Ecdysone production comparison between PDF and DMSO cultured prothoracic glands of 8-day-old 5th instar larvae of *Ostrinia nubilalis*. The symbols represent means and bars represent standard errors.

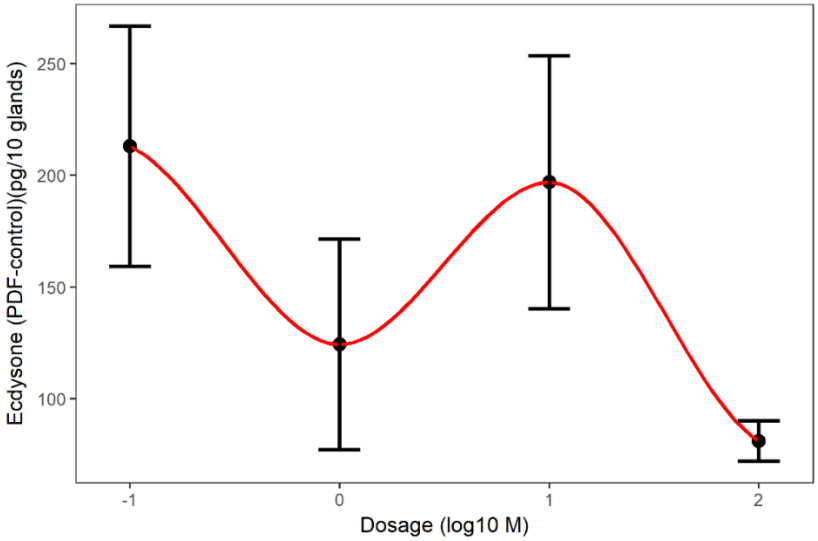

Figure 4-4. Dose-response curve for PDF on ecdysone biosynthesis in the prothoracic glands of 8-day old 5th instar larvae European corn borer. Value of Y axis was generated by subtracting the ecdysone in control group from the ecdysone in PDF group for each dose. The symbols represent means and bars represent standard errors.

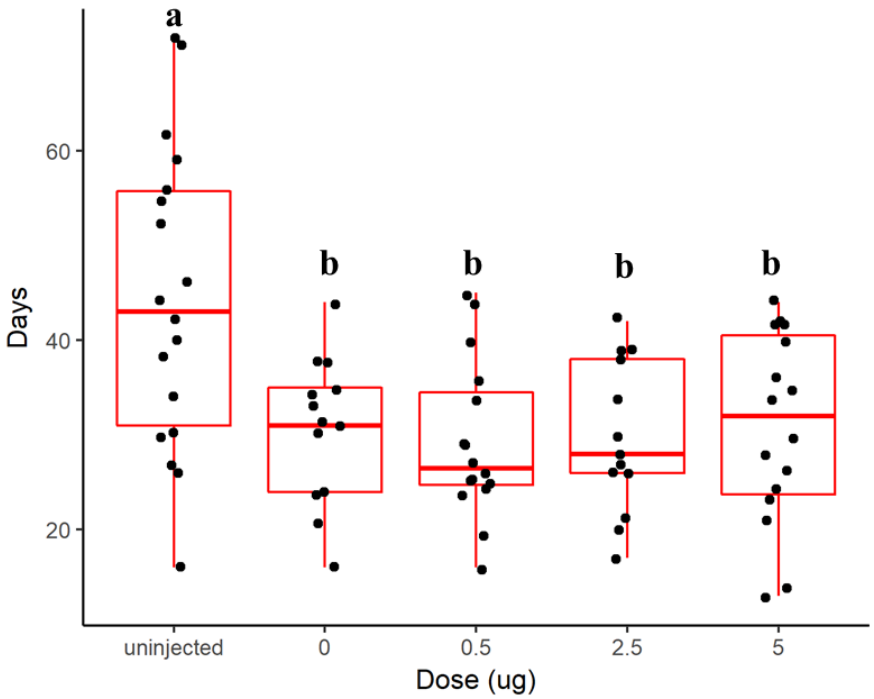

Figure 4-5. The effect of PDF on larval diapause termination in European corn borer. There was no statistical significance between days taken to terminate diapause with injections of 0, 0.5, 2.5, and 5µg PDF. Larvae with injections showed faster diapause termination than the uninjected larvae. Letters denote statistical separation of means using Tukey's HSD.

Table 4-1. Oligonucleotides used in the qRT-PCR.

Gene coding for	Forward (5′–3′)	Reverse (5′–3′)
PDFR	CGGGTGCTGGTGACTAGAAT	GCTATGATCGTCCCTTGGAA
Torso	TCGAATGCTGTCACCCATAA	CGGACTTGGAACCAGAATGT
RpS03 (reference)	ATACAAACTTATCGGAGGTCTCG	ATGATCTTTACCTTGATTCCAAGC
Actin (reference)	ACCTGAGAGGAAATACTCCGTATG	GGCATCATAAATTAGGAACAGTCA

Table 4-2. ANOVA model fits for the transcript abundances of *PDFR* and *Torso* in BE and UZ strains of *Ostrinia nubilalis* after exposure to diapause-termination conditions.

Model	Df	F	P
PDFR (Prothoracic gland)			
Strain	1	0.016	0.901^{ns}
Week	2	2.631	0.116^{ns}
Strain*Week	2	1.620	0.242 ^{ns}
Torso (Prothoracic gland)			
Strain	1	43.797	<0.000*
Week	2	0.329	0.727^{ns}
Strain*Week	2	0.546	0.596^{ns}
PDFR (Wing disc)			
Strain	1	120.760	<0.000*
Week	2	12.098	0.002*
Strain*Week	2	1.769	0.220 ^{ns}

ns, non-significant. Starred terms are significant at *P<0.05*.

CHAPTER 5
A DIVERGENCE IN THE TIMING OF CELL CYCLE PROGRESSION IS ASSOCIATED WITH SHIFTED PHENOLOGIES BETWEEN TWO VOLTINISM STRAINS OF THE EUROPEAN CORN BORER, *OSTRINIA NUBILALIS*

Introduction

Diapause is a developmental strategy that synchronizes life cycles with predictable seasonal changes in the biotic and abiotic environments (Tauber & Tauber, 1981; Danks, 1987). Many insect species enter diapause as a response to a combination of token stimuli (e.g., photoperiod and temperature) prior to the onset of adverse conditions. Specifically, many insects in temperate regions receive the cue that winter is coming when day length falls below a certain duration during the late summer or early fall, termed the critical photoperiod. Reaching this critical photoperiod triggers a switch from continuous growth and reproduction to the diapause developmental trajectory so individuals will enter diapause before the onset of winter. After winter, individuals typically exit diapause and resume development. However, seasonal patterns can be altered by anthropogenic climate change, urbanization, species introductions, host-plant shifts, and an array of other biotic and abiotic factors (Filchak *et al.*, 2000; Bradshaw & Holzapfel, 2001; IPCC, 2014; Williams *et al.*, 2015). In response to altered seasonality, diapause can evolve rapidly by shifting the timing of the onset of diapause initiation or diapause termination (Filchak *et al.*, 2000; Bradshaw & Holzapfel, 2001, 2006; Gomi *et al.*, 2007; van Asch *et al.*, 2013). Subtle shifts in the timing of diapause can substantially alter annual life-history patterns and have big impacts on the synchronization between insect life cycles and environmental conditions (Wadsworth & Dopman, 2015). For example, under the warmer springs resulting from climate change, the hatching of winter moth *Operophtera brumata* eggs showed decreased synchrony with the bud burst of its host oak *Quercus robur* (Visser & Holleman, 2001). After a decade, however, van Asch et al. (2013) showed that the egg hatch of

winter moth become more synchronized with oak bud burst by delaying the timing of termination of their egg diapause. Hence, rapid evolution of diapause timing can be an adaptive response to environmental change (Bradshaw & Holzapfel, 2001; Gomi *et al.*, 2007; van Asch *et al.*, 2013).

To better understand and predict how organisms may respond to novel seasonal environments, numerous studies have focused on exploring the mechanisms underlying shifts in diapause timing (Bradshaw & Holzapfel, 2001; Ragland *et al.*, 2011; van Asch *et al.*, 2013; Wadsworth & Dopman, 2015). Although identification of the specific genes controlling diapause timing has been challenging (Feder *et al.*, 2003; Wadsworth & Dopman, 2015; Wadsworth *et al.*, 2015), some candidate genes involved in diapause regulation have been identified. For example, in *Rhagoletis pomonella*, genes in the Wnt and TOR signaling pathways are very promising candidates for regulating the termination of pupal diapause (Ragland *et al.*, 2011). In *Helicoverpa armigera*, cell cycling and stress resistance genes have been proposed to be important for diapause initiation (Bao & Xu, 2011). However, it is still largely unknown how the regulation of those diapause-related genes are changed by alterations in seasonal information and subsequently transduced into ecologically relevant variation in diapause timing (Sim & Denlinger, 2008).

One of the hallmarks of diapause is a massive slowdown in the cell cycle and the resumption of cell cycle proliferation is a clear indication of diapause termination (Tammariello & Denlinger, 1998; Denlinger, 2002; Koštál *et al.*, 2009; Ragland *et al.*, 2011; Podrabsky & Culpepper, 2012; Shimizu *et al.*, 2016). Therefore, characterizing cell cycle parameters between lineages that differ in diapause life history timing may help us to identify molecular mechanisms underlying alterations of diapause timing as responses to changing seasonality. In this study, I

tested the extent to which the progression of the cell cycle differs across diapause stages between two genetically distinct strains of the European corn borer that differ in their seasonal diapause timing, the BE strain that terminates diapause just after winter and emerges as adults early in the spring season and the later-emerging UZ strain (Eckenrode *et al.*, 1983; Roelofs *et al.*, 1985; Dopman *et al.*, 2010).

Cell division is a critical process in development and morphogenesis where two daughter cells are produced mitotically from one mother cell. The cell cycle consists of four distinct phases: G0/G1, S, G2, and M. The G0 phase is a resting stage where the cell is not actively growing or dividing. G0 is distinguished from the G1 phase because during the G1 phase cells are actively growing, synthesizing RNAs, proteins, and other biomolecules in preparation for the next phase, DNA synthesis (S phase). During the S phase, genomic DNA is copied in preparation for cell division (G2 phase). During the G2 phase, cells evaluate whether there are errors in the duplicated chromosomes and make needed repairs before the genetic material is passed on to a daughter cell. During the subsequent mitotic (M) phase, cells divide into two daughter cells (Canaud & Bonventre, 2015). For phases G1 and G2, cells can either continue the cell cycle and enter the next phase or arrest the cell cycle. The transitions between cell cycle phases are tightly regulated by two key sets of proteins that determine a cell's progress and regulate cell cycling, cyclins and cyclin-dependent kinases (CDKs) that are highly conserved across organisms (Schafer, 1998).

For individuals undergoing diapause, cell division and differentiation are interrupted in target organs such as primordial imaginal structures and the central nervous system (CNS), preventing further development (Tammariello & Denlinger, 1998). Therefore, cell cycle arrest in target tissues is one of the hallmarks of diapause. Cell cycle arrest has been widely studied in

plant dormancy (Velappan *et al.*, 2017), *Caenorhabditis elegans* dauer larvae (van den Heuvel, 2005; Lehmann *et al.*, 2017), and annual killifish embryos (Podrabsky & Culpepper, 2012). However, to my knowledge there are only five reports describing cell cycle parameters in diapausing insects and they are sampled across five different species with substantial differences in their diapause life histories, making a synthesis of cell cycle progression, slowdown, and arrest with respect to the phases of insect diapause a challenge. In the embryonic diapause of the silkworm, *Bombyx mori*, 98% of cells across the whole embryo are in the G2 phase of the cell cycle (Nakagaki *et al.*, 1991). In contrast, 97% of brain cells are halted in the G0/G1 phase in diapausing pupae of the flesh fly, *Sarcophaga crassipalpis* (Tammariello & Denlinger, 1998). During the pupal diapause of the tobacco hornworm, *Manduca sexta*, cells of the optic lobe occur in the G2 phase of the cell cycle (Champlin & Truman, 1998). In the diapausing larvae of the drosophilid fly, *Chymomyza costata*, 86.6% of the CNS cells are found in the G0/G1, and 12.8% in the G2 phase (Koštál *et al.*, 2009). Similarly, in the larval diapause of the jewel wasp, *Nasonia vitripennis*, approximately 80% and 20% of brain cells arrest their cell cycle in the G0/G1 and G2 phases, respectively (Shimizu *et al.*, 2016). From these few studies there is no clear pattern, some species report cellular slowdown predominantly in the G0/G1 phase and some report in the G2 phase during diapause. Some species like flesh flies and silkworms have almost all cells in one phase of the cell cycle, but other species like the jewel wasp and *C. costata* have most cells in the G0/G1 stage, but with a substantial portion also in the G2 stage. What proximate mechanisms and ultimate selective forces may drive these patterns of cell cycle stage during diapause among species is currently unexplored. Furthermore, because the sampling is so sparse and studies have not investigated the same tissues or life stages across species, there is no clear consensus about whether certain tissues within a diapausing insect may arrest in one

cell cycle stage or another. Perhaps tissues differ in their regulatory architectures for cell cycle arrest during diapause, but before one can speculate about the proximate and ultimate mechanisms that may regulate cell cycle arrest across tissues or across species, more work across life stages and tissues within species is clearly needed.

Here, I used the European corn borer, *Ostrinia nubilalis*, to study associations between the cell cycle and shifts in seasonal life-history timing via diapause regulation. Cell cycles status within the brain-SG (subesophageal ganglion) complex and the wing discs were tested because the proliferation of brain-SG and wing discs is synonymous with continuous development, and a stop of proliferation of these key tissues is synonymous with larval diapause (Koštál *et al.*, 2009). I specifically chose two strains of *O. nubilalis* with differences in life cycle timing. In upstate New York, USA where these strains originated, the E strain is bivoltine with one generation that occurs at the beginning of June and another generation at the end of August, while the Z strain is univoltine and has a single generation in the middle of July (Dopman *et al.*, 2010). Wadsworth *et al.* (2013) showed that a one-month shift in the life cycle of the earlier emerging E-strain (BE) from the later-emerging Z-strain (UZ) of *O. nubilalis* is the result of advancing the timing of diapause termination in the spring. I hypothesized that the progression of the cell cycle is associated with divergence in life cycle timing between these two strains of *O. nubilalis*. Within my hypothesis, I tested three specific predictions. First, in diapausing larvae of *O. nubilalis*, the S phase of cell cycle is suppressed to a lower level in the wing discs than the brain-SG complex. I made this prediction because I expect that the brain-SG complex is the main tissue regulating diapause maintenance and termination (Williams, 1946), and thus may be more active in development during diapause than the wing discs. Second, I predicted that the UZ strain suppresses the S phase of cell cycle more than the BE strain during the diapause maintenance

stage of *O. nubilalis*. I predicted this because although diapause is often considered a state of developmental arrest, development can still progress during diapause, but just at a very low rate (Shingleton *et al.*, 2003). Specifically, I expected that the shorter-diapausing BE strain would be experiencing greater levels of cell cycling during diapause than the longer-diapausing UZ strain. Third, I predicted that the BE strain would resume substantial cell cycling earlier than the UZ strain after transfer of both strains to conditions that are favorable for diapause termination. In the present study, I found that cell cycling slowdown in both the G0/G1 and G2 phases during larval diapause, in agreement with observations of larval diapause in *C. costata* and *N. vitripennis*. However, the percentages of cells in the G0/G1 and G2 phases were completely different between the brain-SG complex and wing discs in both strains. During diapause, the proportion of cells in S phase was significantly lower in the wing discs compared to the brain-SG complex. I also found that diapausing BE strain individuals suppressed the cell cycle division less than diapausing UZ strain individuals in both brain-SG complexes and wing discs, which is associated with faster and earlier development of BE strain than UZ strain in spring. Additionally, the resumption of cell cycle proliferation occurs earlier in the earlier-emerging E-strain compared to the later-emerging Z-strain of *O. nubilalis* after exposure to diapause-terminating conditions.

Materials and Methods

Insects and Sampling

The BE and UZ strains of *O. nubilalis* were generously donated from colonies maintained by Erik Dopman's lab at Tufts University. Both strains were originally collected from the field in upstate New York and kept under mass-rearing conditions in the laboratory (Glover et al. 1992). After egg hatch, larvae were fed on an artificial diet (LOT# 052418ECB, Southland Products Inc., Lake Village, AR, USA) in a plastic deli-cup (11.75 cm in top diameter, 9.84 cm in bottom

diameter, and 7.62 cm in height, Bare by Solo, Dart Container Corporation, Mason, MI, USA) for active growth. Non-diapausing individuals were obtained by rearing under long-day conditions including light: dark (L: D) 16:8 at constant 23±1°C. A short-day photoperiod L: D 12:12 at constant 23±1°C was used for larval diapause induction. Some proportion of short-day larvae do not enter diapause, or they have a diapause period too brief to distinguish them from non-diapause larvae. Thus, larvae were determined to be in diapause after they remained as larvae when kept at L: D 12:12 and 23±1°C for 45 days. In contrast, non-diapausing individuals pupated at day ~27 and emerged as moths at day ~36 after egg hatch. Larvae that were clearly in diapause after 45 days were individually transferred from their rearing container into a new chamber that was modified from a 1mL pipette tip containing cotton moistened with water to track diapause termination. No food is needed in this diapause termination chamber because diapausing larvae do not feed during diapause or during larval-pupal metamorphosis. Diapausing larvae are sensitive to long-day and high temperature cues for the termination of diapause and resumption of development from larvae into pupal morphogenesis, thus I shifted both temperature and light cycle to strongly trigger larval diapause termination. Specifically, after 46 days in diapause induction conditions (L: D 12:12 at 23±1°C), diapausing larvae were transferred to L: D 16:8 at 26±1°C to trigger diapause termination.

The brain-SG tissue complex and both pairs of wing discs were dissected from the same individual larva in ice cold 1x PBS buffer and stored separately in 500 µL storage buffer provided with the Cycle TEST Plus DNA Reagent Kit (Becton Dickinson, San Jose, California, USA) at -20°C. Diapausing and non-diapausing tissue samples were collected at days 5, 6, 7, 8, 9, and 10 (the non-diapausing individuals become prepupae at day 10) after the molt into 5^{th} instar. To continue checking the cell cycle of diapausing larvae before transferring them to diapause

termination conditions, samples at days 11, 13, 16, and 23 after the 5th instar molt were also collected for diapausing larvae.

To describe cell cycle parameters during diapause termination, in the shorter-diapausing BE strain, brain-SG complexes and wing discs were collected on the day of transfer into the diapause-terminating long-day conditions, as well as 1, 2, and 3 weeks after being transferred into long-day conditions (78.6% of diapausing BE individuals have terminated diapause and initiated larval-pupal metamorphosis by 3 weeks, Figure 5-1). In the UZ strain, samples were collected at the time of transfer into diapause-terminating conditions as well as 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 weeks after transfer to long-day conditions because of their longer diapause duration (80% of diapausing UZ individuals have terminated larval diapause and pupated by 10 weeks, Figure 5-1).

Flow Cytometry Analysis

The preparation of uniform suspensions of single nuclei was conducted according to the protocol supplied with the Cycle TEST Plus DNA Reagent Kit (Becton Dickinson) with subtle modifications. Specifically, I thawed the sample at room temperature, spun it down at 12,000 x *g* at 4°C for 5 minutes, and removed the storage buffer. Then I added a volume of 125 µL trypsin buffer and gently tapped the sample tube by hand. I allowed the trypsin buffer to react for 10 minutes at room temperature. Then I added a volume of 100 µL trypsin inhibitor and RNase containing buffers into the sample tube and tapped gently by hand. After 10-minute incubation at room temperature, I added 100 µL propidium iodide stain solution and incubated the sample for at least 10 minutes in the dark on ice. Finally, to avoid clogging from cell and tissue fragments, I filtered the sample through 50 µm nylon mesh before flow cytometry analysis. Cellular DNA content was analyzed using an Accuri C6 Flow Cytometer (Becton Dickinson). For each sample

replicate, 10,000 nuclei were analyzed using ModFit LT4.1 software (Verity Software House, Topsham, Maine, USA) and the cells were classified in G0/G1, S, and G2/M phase depending on the intensity of fluorescence peaks (Crissman et al., 1975).

Statistical Analysis

For cell cycle analysis, the percentage of cells in each cell phase was analyzed with an ANOVA followed by post-hoc Tukey's HSD tests for comparing among multiple different groups in R (i386 3.5.0). First, tissue differences in cell cycle progression were analyzed by comparing the percentage of cells in each cell cycle phase between the brain-SG complex and wing discs. The tissue (brain-SG and wing disc) and larval age (days 5, 6, 7, 8, 9, 10, 11, 13, 16, and 23 after the 5^{th} instar molt) were treated as fixed factors. Second, the effect of photoperiod on cell cycle progression was analyzed by comparing the percent of cells in each cell cycle phase between diapause-destined and continuously developing non-diapause 5^{th} instar larvae. The photoperiod (LD vs. SD) and larval age (developmental time) were treated as fixed factors. Only the time points where data were collected for both photoperiodic regimes (L: D 12:12 and L: D 16:8) were included in these analyses (days 5, 6, 7, 8, 9, and 10 after the 5^{th} instar molt). Third, to identify potential strain differences in cell cycle progression under diapause-terminating conditions, the percentage of cells in each cell cycle phase after the switch to long-day conditions was analyzed between the BE and UZ strains. The week (weeks 0, 1, 2, and 3 post long-day exposure) and strain (UZ and BE) were treated as two fixed factors. The results of post-hoc Tukey's HSD tests are shown in the form of letters flanking the mean (different letters when $P<0.05$ among groups). Where the means in one column (day) are flanked with different letters, the difference between them is statistically significant after correction for multiple comparisons.

Results

Photoperiodic Response in the Pre-diapause Preparatory Period

European corn borer larvae either enter diapause at the end of the 5th instar feeding period and prior to pupation in response to short-day and cool temperature conditions, or they develop from larvae to pupae and then reproducing adults under long-day conditions, even in relatively cool temperatures (Mutchmor & Beckel, 1958). In my study, a long-day treatment of L: D 16:8 at 23±1°C induced 100% non-diapausing individuals in both the BE and UZ strains, while a short-day treatment of L: D 12:12 at 23±1°C induced 55.3% and 100% diapausing larvae in the BE and UZ strains respectively (Figure 5-1, P=0.0001).

Photoperiod affected the cell cycle status in both BE and UZ strains. Examples of flow cytometry output of brain-SG and wing discs under short-day and long-day treatments are shown in Figure 5-2. The number of cells in S phase remained consistently high under long-day conditions (9.11% in the brain-SG complexes and 13.62% in the wing discs) and decreased to very low levels (1.48% in the brain-SG complexes and 0.38% in the wing discs) under short-day conditions (Figure 5-3), indicating a substantial slowdown of the cell cycle is a hallmark of diapause in *O. nubilalis*. Generally, cells in diapausing larvae of *O. nubilalis* were in the G0/G1 or the G2/M phase, but the proportion of cells in each phase differed between the brain and wing discs (G0/G1: P<0.000, G2/M: P<0.000). Specifically, in both BE and UZ strains, ~80% of brain-SG cells arrested in the G0/G1 phase and ~20% in the G2/M phase, while ~20% of wing-disc cells arrested in the G0/G1 phase and ~80% in the G2/M phase (Figure 5-3). Although the cell cycle was arrested in both tissues of *O. nubilalis* during diapause, the proportion of cells in S phase, suggesting that these cells were proliferating slowly, was significantly higher in the brain-

SG complexes (1.48%) compared to the wing discs (0.38%) (Figure 5-4, BE strain: P=0.0185, UZ strain: P<0.000).

The morphological development of the wing discs also clearly reflects the effect of photoperiod on cell cycle progression. Under long-day conditions, wing discs developed continuously into the general adult wing shape on the day before prepupa formation (day10 after 5th instar molt) (Figure 5-5). However, under short-day conditions, wing discs remained undeveloped (Figure 5-5), strengthening the idea that most cells in the wing discs of diapausing larvae of *O. nubilalis* are in a state of cell cycle slowdown.

Diapause Initiation and Termination

Numerous studies have shown that the BE strain terminates larval diapause and resumes pupal development sooner after transfer to long-day conditions than the UZ strain of European corn borer (McLeod *et al.*, 1979; Glover *et al.*, 1991, 1992; Dopman *et al.*, 2005; Wadsworth *et al.*, 2013). Thus, I expected that the BE strain would suppress its cell cycle less than the UZ strain during the diapause maintenance stage so that the BE strain would resume the cell cycle earlier and resume larval-pupal metamorphosis faster than the UZ strain under diapause-termination conditions. Indeed, in the brain-SG complexes, the proportion of cells in the S phase was significantly higher in the BE strain (2.51%) compared to the UZ strain (1.43%) during diapause (Figure 5-6, p<0.0001). In addition, after transferring diapausing larvae of both strains into diapause-termination conditions (26±1°C, L: D 16:8), the proportions of cells in the S phase (DNA synthesis) and G2/M phase (cell division and mitosis) increased earlier in the BE strain than the UZ strain in both the brain-SG complexes and wing discs (Figure 5-7 & Table 5-1). Specifically, the increase in the proportions of cells in the S and G2/M phases began week 1 from transfer to diapause-terminating conditions in the BE strain, but not until 7 weeks after transfer in the UZ strain (Figure 5-7). The peak proportion of cells in S phase occurred at week 2

after transfer in the BE strain but not until week 9 for the UZ strain, followed by a decrease at week 3 and week 10 for the BE and UZ strain, respectively (Figure 5-7). In addition, the resumption of cell cycle (BE: day 14, UZ: day 63) corresponded to the pulse of ecdysteroid release (BE: day 13, UZ: day 61) in both the BE and UZ strains after exposure to diapause-termination conditions (Figure 5-7).

Discussion

Insect diapause is characterized by a major slowdown of the cell cycle and resumption of substantial cell proliferation is indicative of diapause termination (Tammariello & Denlinger, 1998; Denlinger, 2002; Koštál *et al.*, 2009; Ragland *et al.*, 2011; Hand *et al.*, 2016; Shimizu *et al.*, 2016). In my study, I compared the progression of the cell cycle between diapause-destined and non-diapause 5th instar larvae of two different strains of the European corn borer, one that emerges earlier in the season due to a shorter diapause and one that emerges later in the season due to a longer post-winter diapause period. My results are consistent with previously published reports of diapausing individuals having a majority of cells in the G0/G1 or G2 phases (Nakagaki *et al.*, 1991; Champlin & Truman, 1998; Tammariello & Denlinger, 1998; Koštál *et al.*, 2009; Shimizu *et al.*, 2016). However, one of the ways my work stands out as novel is that I have studied two different tissues within the same individual, the brain-SG complex and the wing disc. By directly comparing tissues within the same individuals, I have shown that the stage of cell cycle slowdown is not consistent among tissues within a single diapausing individual. Specifically, I found that cells of the brain-SG complex were predominantly in the G0/G1 stage of the cell cycle, as has been reported for the brains of diapausing pupae in the flesh fly, *S. crassipalpis* (Tammariello & Denlinger, 1998) and the brain-SG complexes of diapausing larvae of the drosophilid fly, *C. costata* (Koštál *et al.*, 2009). In contrast, wing disc cells occurred

predominantly in the G2 stage of the cell cycle, consistent with the optic lobes of diapausing pupae in the tobacco hornworm, *M. sexta* (Champlin & Truman, 1998).

The larval wing imaginal disc is an epithelial-derived sheet of undifferentiated cells that develops into the adult wing during metamorphosis (Bryant, 1975; Beira & Paro, 2016). Non-diapausing 5^{th} instar larval wing discs undergo dramatic changes in size and shape (Figure 5-5), supported by active cell division with most cells in the G2/M phase (~80%) ready for mitosis. In contrast to wing discs, the brain-SG complex is mostly developed during late 5th instar larval stage, thus cell division was less active with fewer cells in G2/M phase in the brain-SG complexes (~16%). Although cells of brain-SG complex and wing discs undergo canonical cell cycles (G1→S→G2→M), tissues with different characteristics and functions may be under control of tissue-specific cell cycle regulatory proteins (Boonstra, 2003). One possibility for the distinct differences in the cell cycle phase that brain-SG complex vs. wing discs are arrested in may be due to differences in expression of G1- or G2-phase cyclin/CDK in each tissue. Molecular mechanisms underlying cell cycle arrest have been investigated in only a few diapausing insect species. Most of the studies to date have focused on the cell cycle regulation protein complex (Cyclins and CDKs) and proteins that are known to regulate cyclins and cdks, such as p53, p21, cdc25, etc. (Schafer, 1998). Generally, the expression of proliferating cell nuclear antigen (pcna), a δ DNA polymerase cofactor, is consistently down-regulated the tissues of diapausing individuals (Tammariello & Denlinger, 1998; Koštál *et al.*, 2009; Bao & Xu, 2011; Ragland *et al.*, 2011; Huang *et al.*, 2015; Shimizu *et al.*, 2016). However, the expression patters of transcripts for other regulatory proteins, such as cyclin D, cyclin E, p21, p53, kinases Wee1, Myt1, phosphatase Cdc25 (String), and Dacapo (p27), differ across species during diapause. Taking cyclin D and cyclin E as examples, the relative levels of both the genes were barely

influenced by photoperiodic regime for inducing diapause or direct development in *C. costata* (Koštál *et al.*, 2009), but decreased during diapause in *N. vitripennis* (Shimizu *et al.*, 2016). A detailed study of the expression of cell cycle regulatory proteins is clearly needed in both brain-SG and wing discs of *O. nubilalis* in the future.

Interestingly, instead of completely arresting the cell cycle, both the brain-SG complex (S phase:1.48%) and wing discs (S phase: 0.38%) of diapausing larvae still showed some low levels of cell division. The observation of low levels of S-phase cells reinforces the idea that diapause is actually not a state of complete developmental arrest; instead diapause is a massive programmed slowdown of development. For example, in the pea aphid, *Acythosiphon pisum*, diapausing embryos showed evident cell division and leg growth during the diapause maintenance phase, but at a much reduced rate compared to non-diapause embryos (Shingleton *et al.*, 2003). Continued morphological development during diapause has also been reported in *Austroicetes cruciata* (Andrewartha, 1943), *Cirphus unipunctata* (Saulich, 1975), and *Sesamia nonagriodes* (Gadenne *et al.*, 1997). Thus, the field of diapause regulatory biology may benefit from a shift in thought about whether diapause is really a state of developmental arrest or just a massive, regulated slowdown of development.

Not just slowed proliferation of the cell cycle, the brain-SG complexes and wing discs also suppressed cell division to different levels during diapause. The proportion of cells in the S phase was significantly higher in the brain-SG complexes during diapause than in the wing discs of diapausing larvae. From an energetic perspective, diapausing animals generally shut down or reduce unnecessary costs to save energy reserves (Hahn & Denlinger, 2011). However, diapausing animals also selectively maintain the activity of some tissues, such as the brain, to survive diapause and coordinate their development with diapause-termination cues (Hahn &

Denlinger, 2011). Insect brains are the sensory neural center that receive diverse environmental stimuli and make responses by controlling physiology and behaviors (Wehner, 2003; Srinivasan, 2010; Warrant & Dacke, 2010; Chittka & Skorupski, 2011; Menzel, 2012). Because neural tissue is still metabolically costly to maintain, brains of diapausing insects can suppress the cell cycle partially by selectively shutting down the development of sensory structures such as neuropils related to olfactory rather than light sensing, which might be critical for sensing photoperiod (Lehmann *et al.*, 2017). However, flight ability is not necessary until the adult stage, thus cellular proliferation in wing discs of diapausing larvae of *O. nubilalis* can be largely suppressed during diapause to save energy.

Additionally, the proportion of brain-SG cells in S phase was significantly lower in the later emerging UZ strain than the earlier emerging BE strain during the diapause maintenance stage, suggesting that the faster post-winter diapause termination observed in the BE strain may be facilitated by higher baseline rates of cell division during the larval diapause period. Specifically, with a more active neural system, the BE strain might be more sensitive to diapause-termination cues than the UZ strain. Furthermore, a relatively higher level of cell division of brain-SG in the earlier-emerging E strain might enable a faster diapause development and earlier post-winter diapause termination compared to the later-emerging Z strain. An EdU (5-ethynyl-2'-deoxyuridine) incorporation assay is needed to test this hypothesis by measuring cell proliferation rates in both BE and UZ strains during the diapause maintenance phase and after winter during the diapause-termination phase.

The divergence in life cycle timing between the earlier-emerging E-strain and later-emerging Z-strain of *O. nubilalis* is attributed to a delay in the timing of larval diapause termination wherein the earlier-emerging E strain has a shorter period of post-winter

developmental suppression (Wadsworth *et al.*, 2013). My results also showed an earlier cell cycle resumption in the BE strain compared to the UZ strain. Specifically, the proportion of cells in S phase started to increase rapidly in earlier-emerging E-strain at week 1 and peaked at week 2 after transfer to diapause-termination conditions, whereas the cell proliferation in later-emerging Z-strain remained suppressed until week 7 and peaked at week 9 after transfer to diapause-terminating conditions (Figure 5-7). The proportions of cells in the S phase decreased at week 3 for the BE strain and at week 10 for the UZ strain, suggesting the existence of some deeply diapausing individuals in both strains that had not terminated diapause along with the majority of the population. This difference in the timing of cell cycle resumption corresponds to the average diapause termination timing between BE and UZ strains (~40 days). Earlier transcriptomic studies of diapause development between these two strains of the European corn borer have also shown a rapid increase in the abundance of cell cycle-associated transcripts upon exposure to diapause termination condition in the BE strain but not in the UZ strain (Wadsworth & Dopman, 2015). According to Wadsworth and Dopman (2015), genes involved in active cell cycling (CycA, CycB, Cdk4, Polo, and Pcna) were upregulated within days (day1 and day 7) of long-day exposure in the BE strain, but remained at low abundance in the UZ strain at day1 and day 7 post long-day exposure. Therefore, the earlier resumption of cellular proliferation in the BE strain than the UZ strain is associated with the divergence in life cycle timing between two strains of *O. nubilalis*.

However, the upstream events that might regulate an earlier or later resumption of the cell cycle in response to diapause-terminating temperature and day length cues, and therefore a difference in timing of diapause termination, is still unclear. One potential pathway for cell cycle regulation at diapause termination is Wnt signaling, which promotes proper patterns of growth

and development in insects (metamorphosis) via cell cycle regulation and cellular communication (Logan & Nusse, 2004; Gokhale & Shingleton, 2015; Wadsworth & Dopman, 2015). Many transcripts involved in the Wnt signaling pathway increased in abundance in the BE strain but remained at low abundance in the UZ strain upon exposure to long-day conditions (Wadsworth & Dopman, 2015). In the apple maggot *Rhagoletis pomonella*, the Wnt signaling pathway has also been nominated as a potential upstream candidate for regulation of diapause termination (Ragland *et al.*, 2011). Thus, Wnt signaling, requires further exploration across the early and late-emerging strains of the European corn borer. In addition, because the resumption of cell proliferation (S phase) was mainly due to release from G0/G1 phase, genes involved in G0/G1 checkpoints (e.g. Cyclin D, Cyclin E, CDK2) would be of interest for future relative expression level analysis at diapause termination stage.

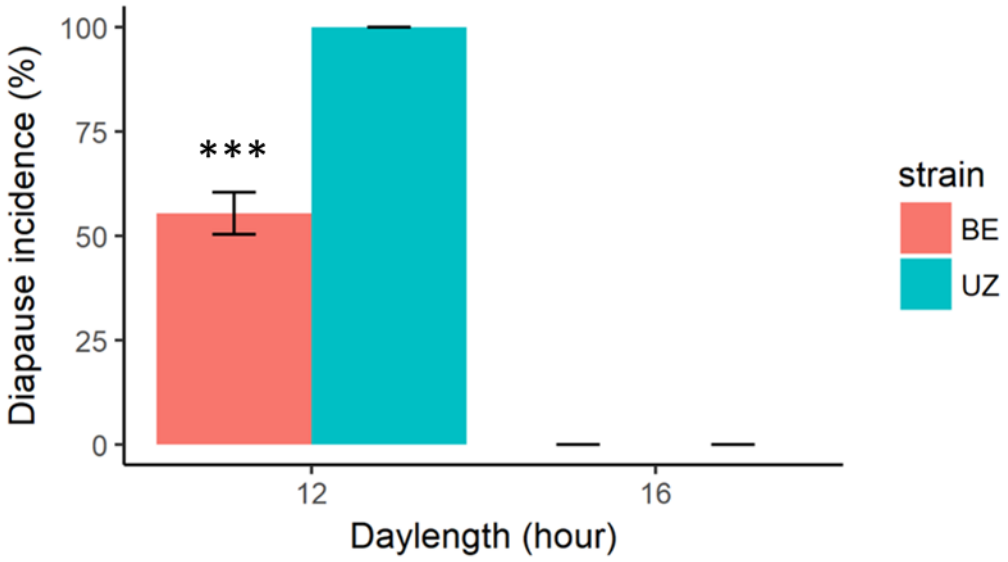

Figure 5-1. Mean ± SE (standard error) of diapause incidence induced under short daylength (12h) and long daylength (16h) in BE and UZ strains of *Ostrinia nubilalis*. Starred terms are significant at $P<0.001$

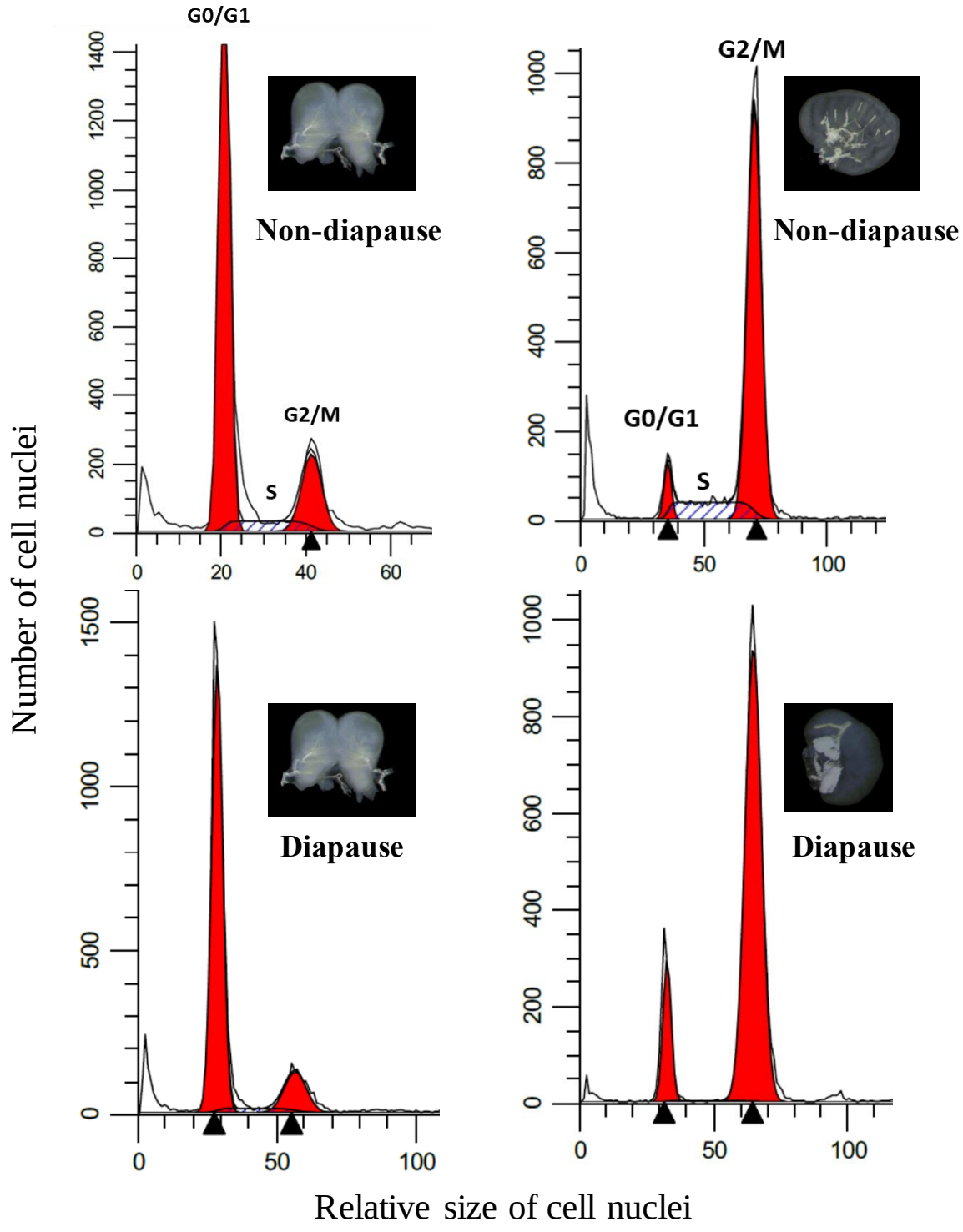

Figure 5-2. The examples of flow cytometry output of brain-SG and wing discs from diapausing and non-diapausing larvae of *Ostrinia nubilalis*. Photo courtesy of Qinwen Xia.

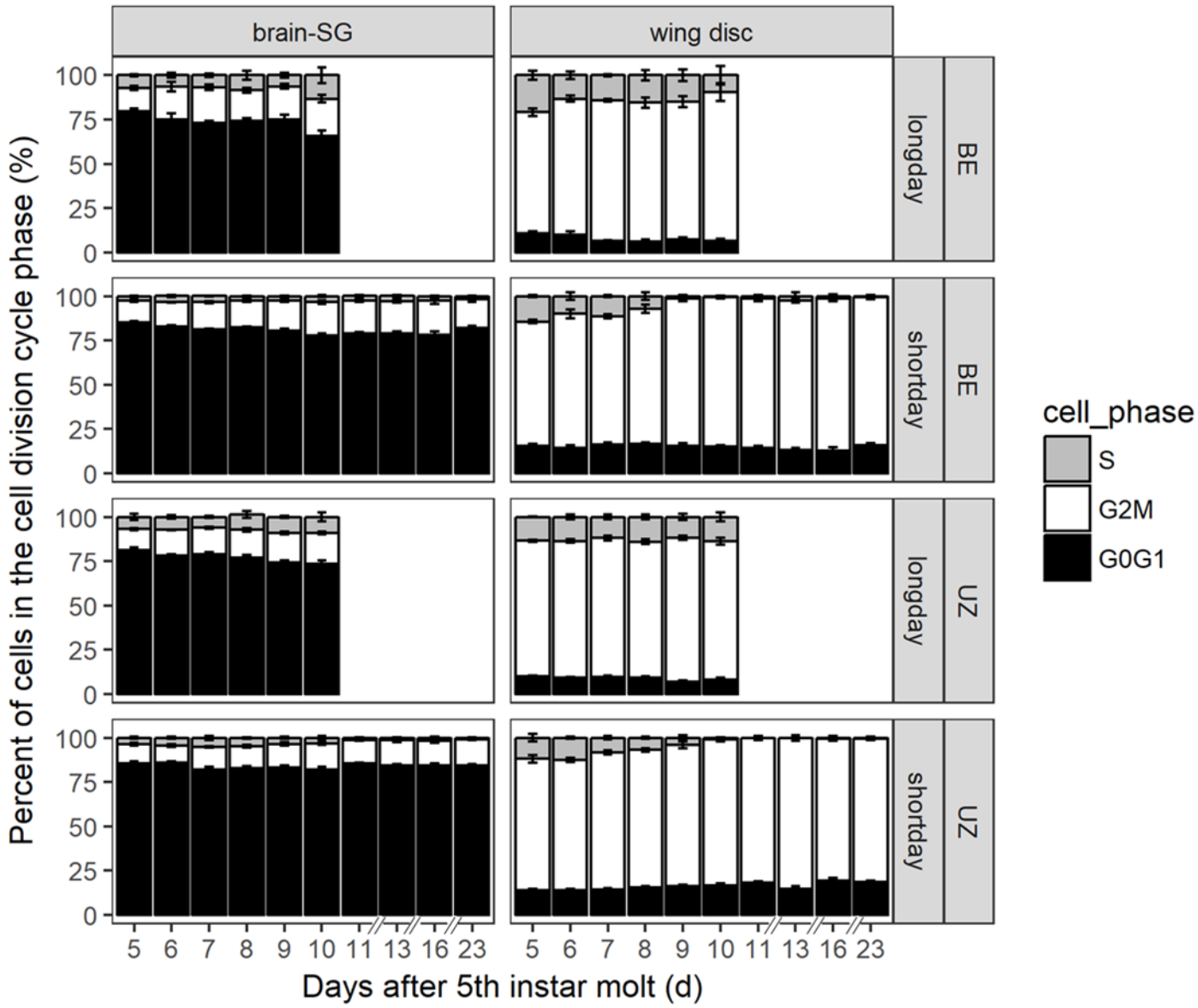

Figure 5-3. Long-day (non-diapausing induction) and short-day (diapause induction) treatment comparison in relative proportions of larval brain-SG and wing disc cells in S, G2/M, and G0/G1phases of cell cycle division in the BE and UZ strains of *Ostrinia nubilalis*. Symbols represent means and bars representing standard error are subsumed within the symbols.

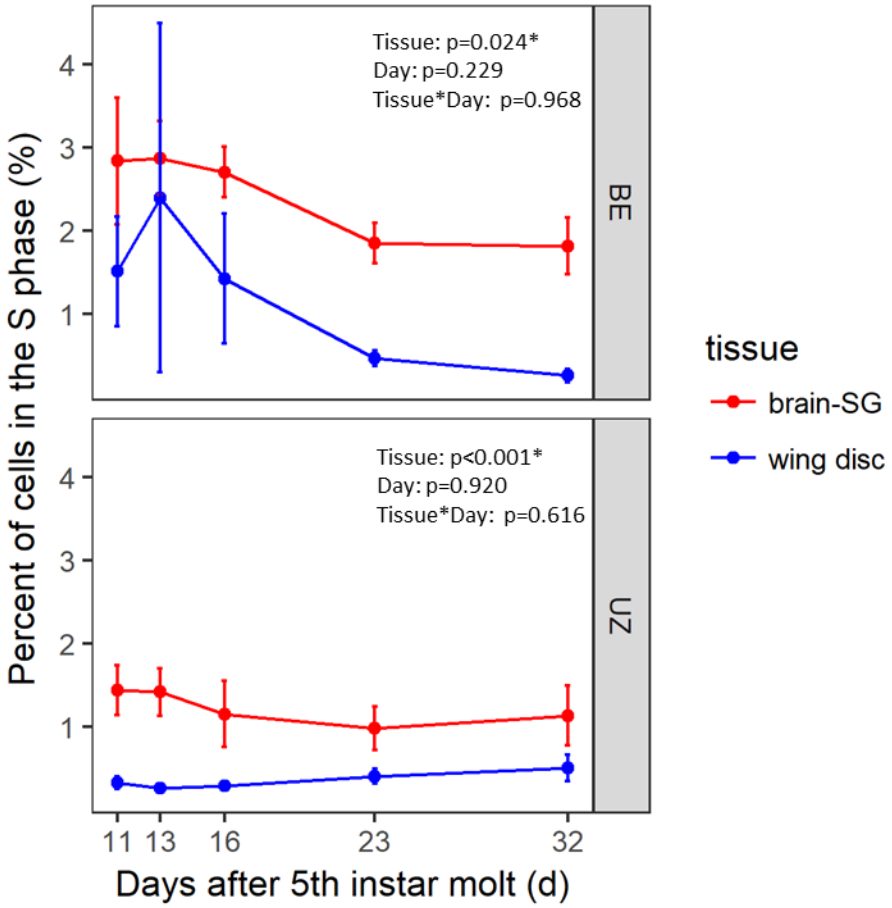

Figure 5-4. Tissue comparisons in relative proportions of cells in S phase of cell cycle in the BE and UZ strains of *Ostrinis nubilalis* during diapause maintenance stage. Symbols represent means and bars representing standard error are subsumed within the symbols. Starred terms are significant at $P<0.05$.

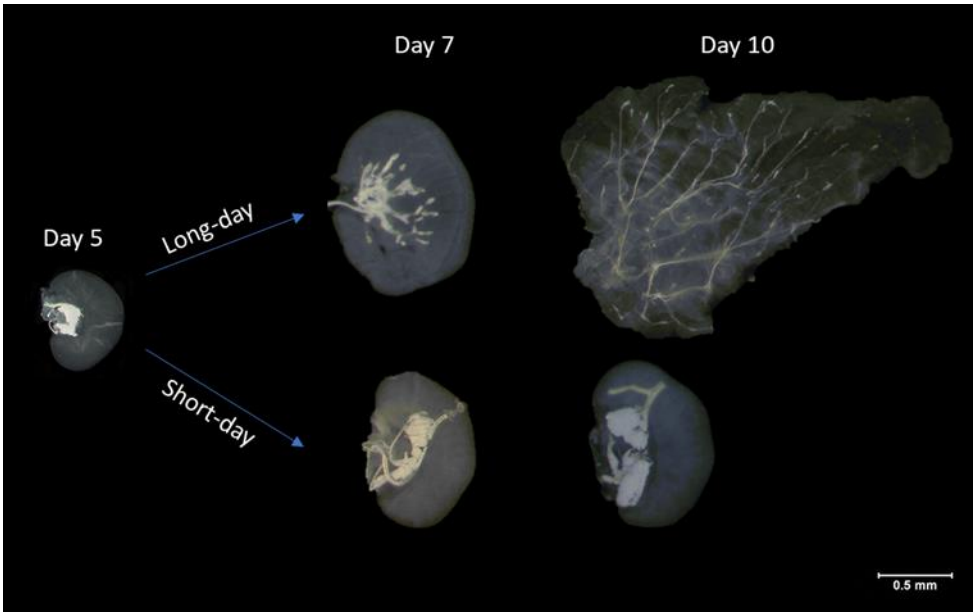

Figure 5-5. Development of wing disc of *Ostrinia nubilalis* under long-day and short-day conditions. Days 5, 7, and 10 represent 5, 7 and 10 days after 5th instar molt. Photo courtesy of Qinwen Xia.

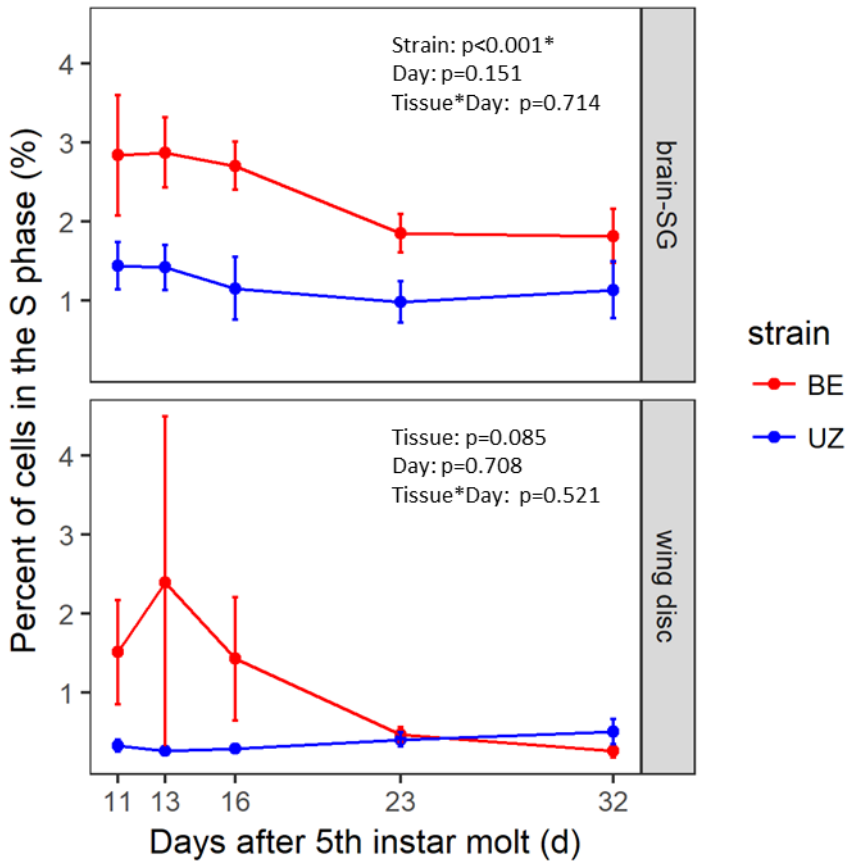

Figure 5-6. Strain comparisons in relative proportions of cells in S phase of cell cycle in the brain-SG and wing disc of *Ostrinis nubilalis* during diapause maintenance stage. Symbols represent means and bars representing standard error are subsumed within the symbols. Starred terms are significant at $P<0.05$.

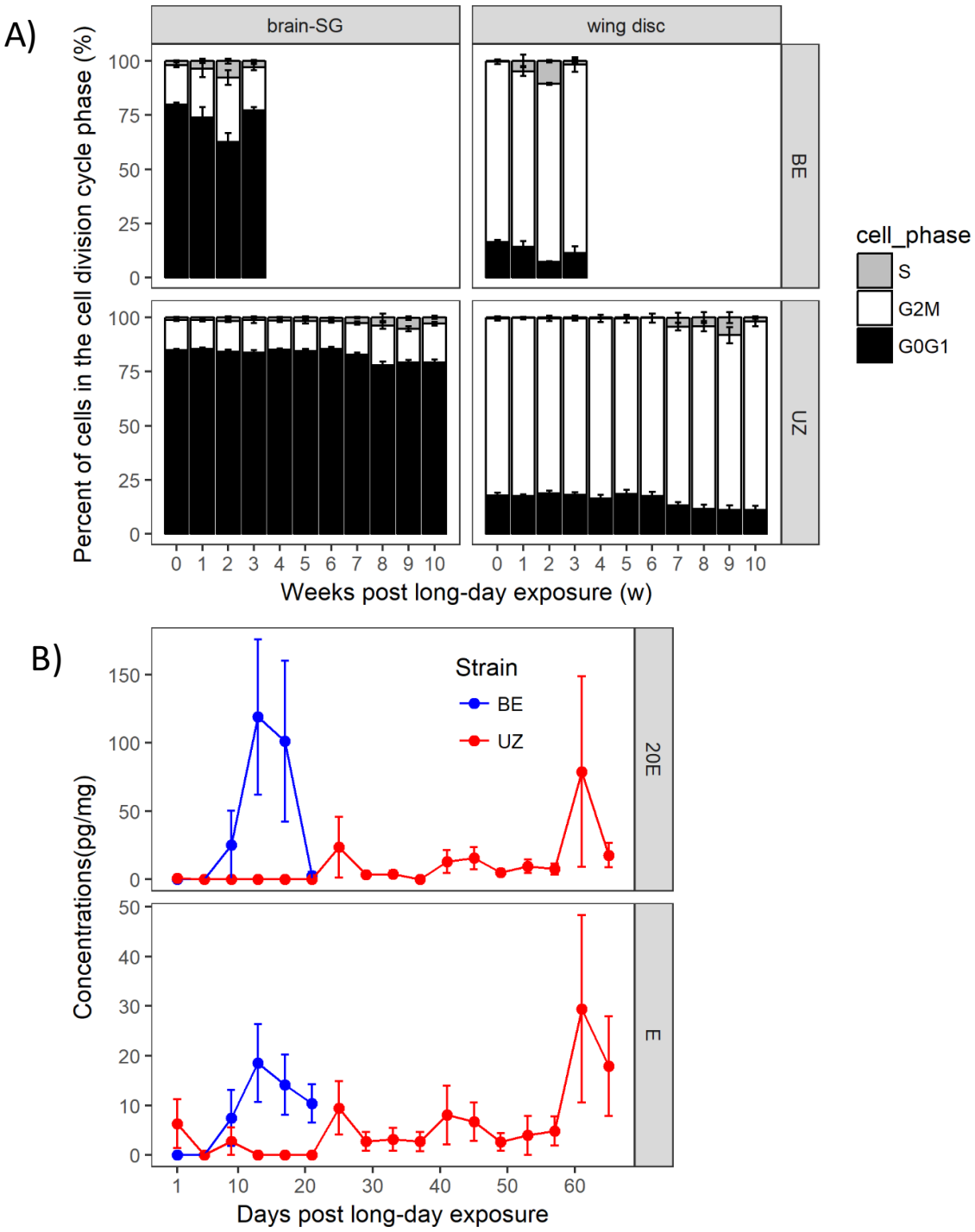

Figure 5-7. A) Strain comparison in relative proportions of cells in S, G2/M, and G0/G1phases of cell cycle division in brain-SG and wing disc of *Ostrinis nubilalis* after exposure to long-day conditions. B) Ecdysteroid concentrations after long-day exposure. Symbols represent means and bars representing standard error are subsumed within the symbols.

Table 5-1. ANOVA model fits for proportions of cells in G0/G1, G2/M, and S phases of cell cycle division after exposure to diapause-termination conditions.

Model	Df	F	P
Brain-SG × G0/G1 phase			
Strain	1	44.812	<0.000*
Week	3	5.469	0.003*
Strain*Week	3	4.946	0.005*
Brain-SG × G2/M phase			
Strain	1	23.589	<0.000*
Week	3	3.130	0.0361*
Strain*Week	3	2.754	0.550^{ns}
Brain-SG × S phase			
Strain	1	38.133	<0.000*
Week	3	9.646	<0.000*
Strain*Week	3	7.253	<0.000*
Wing disc × G0/G1 phase			
Strain	1	24.063	<0.000*
Week	3	2.193	0.104^{ns}
Strain*Week	3	3.443	0.026*
Wing disc × G2/M phase			
Strain	1	2.803	0.102^{ns}
Week	3	1.189	0.326^{ns}
Strain*Week	3	1.266	0.299^{ns}
Wing disc × S phase			
Strain	1	16.182	<0.000*
Week	3	4.826	<0.000*
Strain*Week	3	4.984	<0.000*

ns, non-significant. Starred terms are significant at $P<0.05$.

CHAPTER 6
CONCLUSIONS

With accelerated global climate change, predicting organisms' biological responses becomes increasingly urgent to protect biodiversity. Establishment of predictive models is a promising tool to foresee responses to climate change. However, most current models are still inefficient to use for accurate prediction with incomplete biological parameters or mechanisms. Nowadays, scientists start to focus on improving the current predictive models by including six key biological mechanisms: 1) species interactions, 2) evolutionary potential, 3) dispersal and range dynamics, 4) phenotypic plasticity in the context of the environment, 5) physiology, and 6) demography and phenology (Urban *et al.*, 2016). One of the obstacles to build a model with six biological mechanisms is to get all the missing information and data required for more realistic models. My dissertation helps fill this gap by providing information about the physiological mechanisms underlying a rapid shift in phenology in two important insects.

Phenology enables organisms to cope with seasonally variable environments by adjusting the timing of their life cycles. Phenotypic plasticity or genetic differences could result in changes in phenology (Danks, 2006; Forrest & Miller-Rushing, 2010). Changes in phenology could further lead to disruption in synchrony of active developmental or reproductive stages with suitable conditions. For example, a migratory butterfly, the red admiral *Vanessa atalanta*, has advanced its return date to Britain from France while its host plant flowering time has not advanced over the past two decades (Sparks *et al.*, 2005). Such decrease in matches of phenology may have a significant impact on decline in biodiversity. Alternatively, shifts in phenology could create opportunities for new resources or habitats. For instance, those temporally diverged individuals could be exposed to reduced intra- or interspecies competitions and/or natural enemies (Feder *et al.*, 1995). Ultimately, allochronic separation and speciation may be a possible

result of selection on individuals with shifted phenology (Ritchie, 2001; Scriber & Ording, 2005; Savolainen *et al.*, 2006; Branco *et al.*, 2017).

My central hypothesis was that evolutionary changes in the endocrine regulation of diapause timing are associated with a rapid shift in phenology (life cycle timing). To test my hypothesis, I analyzed the concentrations and release timing of ecdysteroids (the hormone regulating the shift from larval and pupal diapause to resuming morphogenesis) as well as the sensitivity to exogenous 20-hydroxyecdysone at different time points across diapause stage in two host races of *Rhagoletis pomonella* (Chapter 2) and two strains of *Ostrinia nubilalis* (Chapter 3) that differ in their life cycle timings. Both *R. pomonella* and *O. nubilalis* are classic models of incipient speciation by shifts in life cycle timing via diapause regulation. Although *R. pomonella* and *O. nubilalis* enter diapause as pupae and larvae, respectively, I found changes in the timing of ecdysteroid release and sensitive stage to exogenous 20E are associated with the divergence in the timing of diapause termination between two host races or strains in both study systems. By revealing the downstream endocrine basis of the evolutionary changes in diapause timing, we can gain insight into the potential upstream genes involved in the process of shifts in life cycle timing. Because of their univoltine lifecycle and difficulty in establishing lab colony of *R. pomonella*, using *O. nubilalis* enabled me to explore molecular mechanisms that are potentially upstream of ecdysteroid production (PDF and PDFR study in Chapter 4). In addition, we know little about the cellular regulation of diapause in *O. nubilalis*. In Chapter 5, I carried out a flow cytometry study to investigate the cell cycle pattern at diapause initiation, maintenance, and termination phases. As a summary of my whole dissertation, I list what I found as follows:

- **Chapter 2**: The production of ecdysteroids occurred earlier in apple race pupae compared to hawthorn race pupae of *Rhagoletis pomonella* at post-winter stage (Figure 2-5). No differences in ecdysteroid titers and release timing were detected between the two host races at pre-winter and over-winter stages (Figure 2-2 and 2-3). Apple race pupae

were more sensitive than hawthorn race pupae at pre-winter phase, but was at the same level of sensitivity to 20-hydroxyecdsyone (20E) at over-winter phase (Figure 2-4). During post-winter phase, the apple race pupae became sensitive to 20E earlier than hawthorn race pupae (Figure 2-7). Therefore, an earlier release of ecdysteroids and sensitive stage to 20E in apple race compared to hawthorn race at post-winter phase is associated with the divergence in life cycle timing via diapause regulation.

- **Chapter 3**: Similar to *R. pomonella*, earlier-emerging E strain (BE) showed an earlier production of ecdysteroids compared to later-emerging Z strain (UZ) of *Ostrinia nubilalis* after transfer into diapause-termination conditions (Figure 3-6). No difference in ecdysteroid concentrations was detected between two strains. BE strain became capacitated to terminate diapause earlier than UZ strain (Figure 3-7). BE strain showed an earlier sensitive stage compared to UZ strain based on moribund response to injected 20E (Figure 3-10).

- **Chapter 4**: Based on the fact that fixed SNPs were found in the binding site of pigment dispersing factor receptor (PDFR) and its possible regulation on endocrine outputs, I tested for the role of PDFR in evolutionary changes in diapause timing of *O. nubilalis*. The pigment dispersing factor (PDF), a ligand of PDFR, can stimulate the production of ecdysone in the prothoracic glands of non-diapause 5^{th} instar larvae of *O. nubilalis* (Figure 4-3), with no clear dose-response was detected (Figure 4-4). However, the transcript abundance of *PDFR* in the prothoracic glands did not show a pattern corresponding to the ecdysone concentrations of non-diapause larvae (Figure 4-1), suggesting the effects of PDFR might not be mediated by transcript but translational or post-translational regulation. Therefore, protein analysis of PDFR is clearly needed in future studies. The relative abundance of PDFR transcript in earlier-emerging BE strain and later-emerging UZ strain did not match the pattern of diapause termination of these two strains (Figure 4-2). My results also showed that exogenous PDF had no effect on accelerating diapause termination in *O. nubilalis* (Figure 4-5). In conclusion, the role of PDF and PDFR on adaptation of life cycle timing in *O. nubillalis* remains to be confirmed.

- **Chapter 5**: Larval diapause of *O. nubilalis* was characterized by cell cycle slowdown at G0/G1 and G2 phases, and the percentage of cells in each phase was tissue specific. In brain-SG complex, ~80% cells were in the G0/G1 phase and ~20% in the G2 phase; while ~20% of wing-disc cells occurred in the G0/G1 phase and ~80% in the G2 phase (Figure 5-3). The proportion of brain-SG cells in the S phase was significantly lower in the later-emerging UZ strain than the earlier-emerging BE strain during the diapause maintenance stage (Figure 5-6), suggesting that the faster post-winter diapause termination observed in the BE strain may be facilitated by higher baseline rates of cell division during the larval diapause period. Additionally, the BE strain resumed cell cycle proliferation earlier than the UZ strain after transfer into diapause-termination conditions (Figure 5-7), suggesting divergence in cellular regulation of diapause is also involved in evolutionary changes in life cycle timing in *O. nubilalis*.

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BIOGRAPHICAL SKETCH

Qinwen Xia was born in 1989 in Jiangxi, China. She entered Jiangxi Agricultural University and studied agronomy in 2006. During this four-year program, she learned basic knowledge of agriculture, including cultivation, plant breeding, plant pathology, and entomology, etc. At the last semester of her undergraduate program, she interned in Dr. Fangsen Xue's lab to work with insect diapause and life history adaptation. She was inspired by Dr. Xue's passion and persistence on science since then. After Qinwen received her Bachelor of Science degree in agronomy from Jiangxi Agricultural University in 2010, she continued her study as a master student at the same institution with Dr. Xue from 2010 to 2013. Her master's project focused on the study of geographical variation in diapause and inheritance of the photoperiodic larval diapause induction of Asian corn borer. She received her Master of Science degree in zoology in the summer of 2013.

Qinwen started her doctoral program at the University of Florida in August 2013. She worked on the endocrine basis of divergence in life cycle timing under the guidance of Dr. Dan Hahn. After receiving her Doctor of Philosophy degree in entomology and nematology from the University of Florida in Fall 2018, Qinwen intends to return to China to start her new career.