THE MECHANISMS AND CONSEQUENCES OF PHENOLOGICAL EVOLUTION

A dissertation submitted by

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ABSTRACT

Phenological shifts are clearly biologically important as they contribute to both ecological speciation and persistence during global warming. However, the genetic and physiological mechanisms behind shifts remain unclear. This is due to complicated genetic and genomic bases, as well as the cryptic nature of the phenotype. The consequences of phenological shifts also remain largely unexplored. We expect that a novel temporal habitat will have a different thermal profile than the ancestral habitat due to seasonal thermal variation. Therefore, for successful phenological shifts we predict correlated evolution in thermal tolerance. My dissertation investigates both the mechanisms and evolutionary consequences of phenological shifts, and adds to a growing body of literature on these topics.

I first investigate the physiological mechanism underlying evolution in life-history timing of corn borers. Using metabolic trajectories, I find that variation in the length of the diapause termination phase underlies an ~30-day shift observed in seasonal timing between E and Z-strains. While the E-strain exits diapause within 7 days, the Z-strain remains in diapause termination for ~30 more days.

These metabolic trajectories are further used to target days for whole transcriptome profiling to nominate molecular mechanisms for the shift in timing. I nominate 48 candidate genes within the quantitative trait locus (QTL) for seasonal timing, and find that shorter E-strain termination is defined by a burst of

genome-wide transcriptional activity involved in cell cycling, the stress response, and hormone production.

I next interrogate the genomic architecture surrounding the QTL for differences in seasonal timing. I investigate if a chromosomal inversion could be present near the QTL for timing as previous studies found evidence of a large region of suppressed recombination in this region. Our results confirm that there is evidence for a large-scale rearrangement covering at least 20% of the Z (sex) chromosome that harbors the basis for seasonal timing differences.

A final study looks at the consequences for evolved differences in seasonal timing between corn borer strains. As shifts in phenology displace populations across a season, they also expose populations to novel thermal environments. I find that earlier-emergence from winter diapause is correlated with enhanced cold-tolerance.

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CHAPTER 1

INTRODUCTION

Why study the evolution of phenology?

Global climate change due to anthropogenic effects has been one of the largest drivers of a reduction in our planet's biodiversity over the past four decades, with the rate of decline showing no signs of decreasing (Butchart et al., 2010). Ongoing climate change will have dramatic impacts on the local abiotic environment including changes in mean daily temperatures, an increase in the duration and severity of extreme temperature events, and an impact on the length of the growing season (Easterling et al., 2000; Parmesan, 2006). These rapid abiotic changes present a challenge to the persistence of species (Thomas et al., 2004; Møller et al., 2008; Pereira et al., 2010; Maclean & Wilson, 2011).

There is clear evidence that global climate change is restructuring the geographic and temporal distribution of species (Walther et al., 2002, 2005; Root et al., 2003; Parmesan & Yohe, 2003; Parmesan, 2006). A rich literature has documented northward range shifts associated with climate warming in insects (Ford, 1945; Kaisila, 1962; Parmesan, 1996; Parmesan et al., 1999; Crozier 2003, 2004; Hickling et al., 2005; Breed et al., 2012), amphibians (Pounds et al., 1999, 2006), and birds (Gudmundsson, 1951; Harris, 1964; Kalela, 1949, 1952; Salomonsen, 1948; Thomas & Lennon, 1999). Consistent with the latitudinal pattern of species seeking cooler climates as the environment warms around them, documentations of elevation shifts have been reported for both plants and animals (Grabherr et al., 1994; Pauli et al., 1996; Moiseev & Shiyatov, 2003; Pounds et

al., 2005; Beckage et al., 2008). However, while tracking favorable climate bands is important for population persistence, this strategy is limiting for organisms with low dispersal ability or co-evolved specialists that depend on them to survive (e.g., predator-prey, herbivore-plant, parasite-host interactions.).

Shifts to phenology, or seasonal timing, due to climate change have also been extensively documented. Phenological shifts have been reported in plants (Cayan et al., 2001; Menzel, 2005; Menzel & Dose, 2005), amphibians (Beebee, 1995), birds (Brown et al., 1999; Both et al., 2004; Gordo et al., 2005), insects (Roy & Sparks, 2000; Forister & Shapiro, 2003; Stefanescu et al., 2003), and phytoplankton (Winder & Schindler, 2004). The majority of these species show advancement of seasonal events from their historical occurrence dates (e.g., earlier spring-time flowering in plants, earlier egg-laying or migrations in birds, earlier adult flights in insects, etc.) (Walther et al., 2002; Parmesan, 2006). This pattern is most likely due organisms taking advantage of lengthened growing seasons due to climate warming (Menzel & Fabian, 1999). In an advanced season, earlier timing of seasonal events may lead to an inter- or intraspecific competitive edge in the battle for resources, in which an earlier bird does in fact have a better chance of getting the worm. These phenological shifts may initially be due to phenotypic plasticity, but genetic adaptive evolution of phenology may be the only way for species to persist in the long-term (Hoffman & Sgro, 2011).

So why study the evolution of phenology? There is no question that our climate is changing on an unprecedented scale. Shifts to seasonal timing have been documented in many organisms, and therefore may be important to

persistence in a changing environment. While critically important, the physiological and genetic mechanisms behind evolution in phenology remain largely unknown in many organisms. Furthermore, the consequences of evolution in phenology remain unexplored, as shifts will expose populations to novel abiotic and biotic environmental conditions. Therefore, we may expect correlated evolution of phenology and other traits involved in survival in novel environments. In this dissertation, I study the genetic and physiological mechanisms associated with differences in phenology, and ask about the consequences of these phenological differences in the European corn borer moth. I will describe the physiological mechanism for shifts in phenology in Chapter 2, use whole transcriptome sequencing to query candidate genes for the evolution of phenology in Chapter 3, describe the presence of a large structural rearrangement harboring the genetic basis for seasonal timing in Chapter 4, and describe correlated evolution between phenology and thermotolerance in Chapter 5.

Dissertation research goals

Understanding the Mechanisms of Phenological Evolution

The timing of life-history events throughout a season is important for organisms to be able to survive reoccurring adverse abiotic conditions (e.g., winter) as well as to be able to synchronize life-stages with the appropriate biotic resources (e.g., host-plants, prey, etc.) (Tauber et al., 1986). As the growing season length changes, and thermal profiles and biotic resources shift across the year due to climate warming, organisms must be able to shift their life-history

timing to cope. While clearly important for persistence, both the physiological and genetic mechanisms behind evolution in seasonality remain unclear.

Insects are an excellent model system to begin to understand evolution in seasonal timing. In temperate environments, insects invoke a seasonal diapause overwinter, with the entrance and exit from diapause commonly synchronizing insect populations throughout the year (Tauber et al., 1986). Diapause is a dynamic alternative developmental pathway, and is composed of pre-diapause, diapause, and post-diapause stages (Koštál, 2006). Diapause itself is further composed of an initiation phase in which direct development ceases, a maintenance phase in which development is arrested through adverse conditions, and a termination phase in which the potential for direct development returns (Tauber et al., 1986; Koštál, 2006). Studies have shown that evolution in the timing of the onset or termination of diapause is common, with many shifts believed to be caused by environmental perturbations such as changing climates, anthropogenic impacts, and species introductions (Pickett & Neary, 1940; Tauber & Tauber, 1976; Bradshaw & Lounibos, 1977; Glover et al., 1992; Bradshaw & Holzapfel, 2001, 2006, 2008; Bradshaw et al., 2004; Filchak et al., 2000; Mathias et al., 2005; Schmidt et al., 2005; Gomi et al., 2007).

While these shifts in diapause timing are commonly reported in the literature between populations of insects, the mechanisms behind these shifts are still obscure. This is due to the genetic bases often being encased within complex genomic architectures (e.g., chromosomal rearrangements) or having a complicated genetic basis (e.g., polygenic, epistasis) (Tauber et al., 1977; Feder et

al., 2002; Bradshaw, 2005; Mathias et al., 2007; Emerson et al., 2010; Wadsworth et al., 2015). This is also due to the cryptic nature of the phenotype, as insects will often have no visible outward signs that are indicative of the transitions between the distinct phases of diapause (Tauber *et al*. 1986).

While there are tantalizing clues for the genetic and physiological bases for ecologically relevant shifts in seasonal timing in the literature, more work needs to be done in this area to diversify the taxonomic groups studied, clarify how to define the relatively cryptic transitions between diapause stages, and further characterize the mechanisms involved. My dissertation research adds a moth system to a literature dominated by studies in flies, illuminates a useful method for classifying the cryptic phenotype of diapause transitions, and nominates some candidate physiological and genetic mechanisms for variation in seasonal timing.

Understanding the Evolutionary Consequences of Phenological Shifts

One of the founders of modern evolutionary biology, Charles Darwin, described life as a tree (Darwin, 1859). From the sturdy trunk of the tree springing upwards, life continuously divides into smaller branches, ever diversifying and stretching outwards from a common root. Each bud at the tip of each branch, blossoms into a unique species. This imagery aptly describes life, each species splitting under selective forces and connected by common ancestry. There are millions of species on Earth today and there have been billions of species that have lived and died, and countless species have yet to emerge on our planet. Each

of these species is a spectacular assortment of modifications that have given rise to distinct, or not so distinct, forms. This understanding of life leads us to a fundamental question in biology which is, how do new species form?

Speciation is the evolutionary process by which new species form, and is instrumental to creating biological diversity (Coyne & Orr, 2004). Speciation ultimately results from barriers to reproduction. Recently, there has been resurgence in interest on ecological barriers to reproduction, and how divergent habitat or resource usage is key to focus on because they can complete speciation in the face of gene flow (Harrison, 2012). While in geographically isolated populations genetic drift is a large force involved in speciation, in scenarios with ongoing gene flow divergent selection must be strong in order to overcome gene flow and complete speciation. Despite the clear importance of classifying ecological barriers and their underlying genetic bases, few genes responsible for these barriers have been characterized. Some of the difficulties in understanding ecological isolating barriers are that they, 1) can be hard to identify, 2) can be composed of many loci, 3) can be hard to quantify, and 4) can be hard to measure in a laboratory setting. This is one of the reasons that ~70% of studies on speciation have focused on post-zygotic barriers, mainly in model organisms (Coyne & Orr, 2004).

Variation in seasonal timing is a strong ecological barrier that isolates populations in time. Rapid fixation of alternate alleles for timing between populations has the potential to eliminate gene flow almost instantaneously, so could be an important mechanism for isolation during speciation with gene flow

(Coyne & Orr, 2004). Indeed, many models for speciation research of sympatric populations vary in seasonal timing (e.g., *Rhagoletis* (Pickett & Neary, 1940), *Enchenopa* (Wood & Keese 1990; Wood et al., 1999), *Magicicada* (Lloyd & Dybas, 1966), and *Ostrinia* (Roelofs et al., 1985)).

While seasonal timing shifts have been commonly documented, the complex evolutionary consequences of these shifts are less clear. Phenological shifts displace populations in time and expose organisms to novel environmental conditions. Therefore, as a consequence of phenology evolution we expect, 1) reproductive isolation due to the phenological shift, and 2) correlated evolution in traits involved in increasing fitness within the new temporal environment. For example, if the new temporal environment differs in temperature or host plant availability, we would expect evolution of traits to adapt to these novel conditions. The fixation of these correlated traits may in fact enhance genetic divergence and speciation, where when alleles from the ancestrally timed population migrate into the derived population in the novel temporal niche, they may be at a selective disadvantage. My dissertation research not only adds to the speciation literature by providing some insight into the mechanisms behind an important ecological isolating barrier (phenology), but also provides an initial look at correlated evolution in thermotolerance, which is a predicted trait that should evolve in populations that are transplanted into novel temporal habitats across a season. This work provides the basis for future work on the combined effects of diapause timing and thermotolerance on overall reproductive isolation.

Study System

The European corn borer moth (ECB, *Ostrinia nubilalis*) is an invasive pest species in North America, and is one of the most impactful lepidopteron pests of corn (*Zea mays*) in both its native and invasive ranges. Native to Europe, Asia, and northern Africa, multiple populations of corn borers were first introduced to the United States of America in the early 1900's unintentionally from Italy and Hungary in shipments of broomcorn (*Sorghum vulgare*) (Caffrey and Worthley, 1927). The first invasion occurred in the area surrounding Boston, MA around 1910, but the insects were not officially identified until 1917 after populations became abundant and the destruction to corn crops became a large problem for farmers in the area (Vinal and Caffrey, 1919; Palmer et al., 1985). In 1919, a second infestation was discovered in Scotia, NY and it was soon clear that this infestation was widespread in the Schenectady area (Caffrey and Worthley, 1927). In 1919-20, infestations were further discovered around lake Erie, both in the United States and Canada, probably due to ships transporting goods up the Erie Canal (Smith, 1920; Caffrey and Worthley, 1927; Wressell, 1961).

Since these and possibly other undocumented introductions, corn borers have spread to all corn-producing regions of the United States and Canada (Palmer et al., 1985). Although the primary concern of this pest species in North America are commercial sweet corn crops, larvae also feed or shelter in over 200 additional plant species, including several other economically valuable plants (Caffrey and Worthley, 1927). Damage to corn plants is primarily caused by the

larval stages tunneling through the tassels, stalks, ear stems, and ears of plant which causes large yield losses (Caffrey and Worthley, 1927). Estimated losses to corn crops annually from larval corn borer damage have been estimated to exceed $1 billion USD annually (Ostlie *et al*. 1997).

Distinct strains of corn borers exist in the native range, and through the multiple introductions of the pest were brought to North America (Caffrey and Worthley, 1927; Palmer et al., 1985). As there are distinct preferences for different host plants in Europe which are not present in the introduced North American populations (Malusa et al., 2007; Dopman et al., 2010), strains are most likely in a period of extensive secondary contact. North American corn borer strains fall into distinct populations due to evolved differences in seasonal temporal isolation, circadian temporal isolation, male orientation, female discrimination, oviposition, F_1-male behavioral dysfunction, and F_1-oviposition, that all act as barriers to reproduction; although the strength of these barriers depends on geographic location and the combination of barriers present (Dopman et al., 2010).

The largest component of reproductive isolation between corn borer strains is the pheromone communication system (Dopman et al., 2010). Corn borers are characterized by being of either the 'E' or 'Z'-strain, where strains differ in the pheromone blend that females produce and the response of males to that blend. Z-strain females produce a 3:97 blend of (E) and (Z)-11 tetradecenyl acetates and Z-strain males preferentially fly towards this blend, while E-strain females produce a 99:1 blend of (E) and (Z)-11 tetradecenyl acetates and E-strain

males preferentially fly towards this blend (Klun et al., 1973; Klun & Maini, 1979; Kochansky et al., 1975; Roelofs et al., 1987; Zhu et al., 1996). The genetic basis of differences in the biochemical pathway that produces different ratios of female pheromones has been mapped to the autosomal locus, fatty-acyl reducatase (*pgFAR)* (Lassance et al., 2010). The genetic basis of male response has been mapped to the Z (sex) chromosome, although the gene responsible is still unknown (Roelofs et al., 1985; Glover et al., 1992; Dopman et al., 2004).

The second largest component of reproductive isolation between strains is seasonal temporal isolation (Dopman et al., 2010). Seasonal temporal isolation is caused by differences in the time it takes for an overwintering larvae in diapause to become a pupa in the spring; where the short developing type takes on average 14 days to pupate and the long developing type takes on average 44 days to pupate (Glover et al., 1992; Dopman et al., 2005). This difference in seasonal timing separates adult flights by ~30 days in the field, limiting the potential for these populations to mate (Dopman et al., 2010). Z-strain moths are polymorphic for voltinism type, with some borers being of the long type and others being of the short type, whereas E-strain moths seem to be exclusively short in their development time (Roelofs *et al*. 1985). The genetic basis of this trait (*Pdd*) is known to map to the Z (sex) chromosome, but the gene responsible and exact location remain unknown (Roelofs *et al*. 1985; Glover et al., 1992; Dopman et al., 2005). Interestingly, genetic markers that are closely linked to *Pdd* have been shown to oscillate in allele frequency along a latitudinal cline which correlates with shifts in generation number (voltinism) (Levy et al., 2015). This suggests

that *Pdd* is intimately connected to voltinism phenotype. While clearly important for both reproductive isolation and the geographic distribution of strains, we still do not entirely understand the physiological or genetic mechanisms behind this trait.

Overview and Major Research Findings

One phase of the dormancy developmental pathway is critical for the evolution of insect seasonality: In order to elucidate the physiological mechanism behind evolutionary shifts in the diapause developmental pathway, despite the cryptic nature of diapause, I use metabolism as a biomarker. Using metabolic trajectories through a time-course I interrogate three distinct hypotheses. My results unequivocally demonstrate that evolution in seasonal timing is driven by a shift in the length of the dormancy termination phase, where E-strain corn borers rapidly exit the suppressed metabolic state of diapause within days of experiencing a photoperiodic cue, and Z-strain borers remain in this suppressed state for ~30 days longer.

Transcriptome profiling reveals mechanisms for the evolution of insect seasonality:

In order to illuminate the molecular mechanisms behind shifts in seasonality, I use whole transcriptome sequencing of a time-course through dormancy of both strains. I (1) illuminate putative upstream casual factors for shifts in seasonal dormancy timing, and (2) describe downstream molecular pathways involved in

resumption of active development. I nominate 48 candidate genes within a known quantitative trait locus (QTL) for a shift in the length of the dormancy termination phase; several of which fall within the insulin signaling or circadian rhythm pathways. I also find that genome-wide transcriptional activity is negligible during the extended Z-strain diapause termination phase, whereas the shorter E-strain diapause termination phase is characterized by a rapid burst of regulatory changes involved in resumption of the cell cycle, hormone production, and stress response. This suggests that the evolution of insect seasonality is driven by a genetic switch that once activated, results in widespread restructuring of gene regulatory networks.

A recombination suppressor contributes to ecological speciation in *Ostrinia* moths: Chromosomal rearrangements can act to enhance speciation via the suppression of gene flow between populations. In this study, I set out to test for the presence of a chromosomal rearrangement and its contribution to speciation between the E and Z-strains of the European corn borer moth. In order to do this I use a combination of genetic crosses, phenotyping of a trait underlying ecological isolation, and population genetic analysis of wild populations. The results suggest the presence of a large chromosomal rearrangement between corn borer strains encompassing ~20% of the Z (sex) chromosome. The observation of 50% lower genetic variation inside the rearrangement vs. outside is congruent with a selective sweep on the region. Loci within this region also show elevated genetic differentiation between strains, supporting a role for rearrangements in

recombination suppression and the maintenance of genetic divergence between populations. Finally, I find that the rearrangement harbors the genetic basis of variation in the length of the diapause termination phase.

Genetic differences in basal cold-tolerance correlated with phenological shifts in Ostrinia moths: Organisms live in heterogeneous thermal environments that vary spatially, and also temporally throughout a season. Therefore, a consequence of phenological shifts is exposure to novel thermal environments as populations are displaced in time through a season. Using archived data, I find a significant difference in the thermal habitats that E and Z-strain borers inhabit in upstate NY. In this location earlier-emerging E-strain pupae experience subzero temperatures, while the later-emerging Z-strain pupae never experience subzero temperatures. In parallel, I find evidence of enhanced basal thermotolerance in the E-strain in the two direct developing life-stages investigated. This strongly suggests that evolution in phenology and exposure to novel temporal habitats is driving correlated evolution in thermotolerance.

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CHAPTER 2

ONE PHASE OF THE DORMANCY DEVELOPMENTAL PATHWAY IS CRITICAL FOR THE EVOLUTION OF INSECT SESONALITY *

ABSTRACT

Evolutionary change in the timing of dormancy enables animals and plants to adapt to changing seasonal environments and can result in ecological speciation. Despite its clear biological importance, the mechanisms underlying the evolution of dormancy timing in animals remain poorly understood because of a lack of anatomical landmarks to discern which phase of dormancy an individual is experiencing. Taking advantage of the nearly universal characteristic of metabolic suppression during insect dormancy (diapause), we use patterns of respiratory metabolism to document physiological landmarks of dormancy and test which of the distinct phases of the dormancy developmental pathway contribute to a month-long shift in diapause timing between a pair of incipient moth species. Here, we show that divergence in life cycle between the earlier-emerging E-strain and the later-emerging Z-strain of European corn borer (ECB) is clearly explained by a delay in the timing of the developmental transition from the diapause maintenance phase to the termination phase[1]. Along with recent findings indicating that life-cycle differences between ECB strains stem from allelic variation at a single sex-linked locus, our results demonstrate how dramatic shifts

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* Wadsworth, CB, Woods, WA, Hahn, DA, & Dopman, EB (2013). One phase of the dormancy developmental pathway is critical for the evolution of insect seasonality. *Journal of Evolutionary Biology*, **26(11)**: 2359-2368.

[1] Our interpretation of this pattern has been updated in all other chapters to follow Koštál (2006), whereby the shift in developmental timing between E and Z-strains is explained by variation in the length of the diapause termination phase: Koštál, V (2006). Eco-physiological phases of insect diapause. *Journal of Insect Physiology*, **52(2)**: 113-127.

in animal seasonality can result from simple developmental and genetic changes. Although characterizing the multiple phases of the diapause developmental programme in other locally adapted populations and species will undoubtedly yield surprises about the nature of animal dormancy, results in the ECB moth suggest that focusing on genetic variation in the timing of the dormancy termination phase may help explain how (or whether) organisms rapidly respond to global climate change, expand their ranges after accidental or managed introductions, undergo seasonal adaptation, or evolve into distinct species through allochronic isolation.

INTRODUCTION

An essential component of long-term persistence for animals and plants is the ability to synchronize organismal life cycle with seasonal environments. Organisms face continuous challenges to the maintenance of their seasonal synchrony due to a range of environmental perturbations including changing climates, anthropogenic impacts and species introductions. Such perturbations might often increase the risk of local extinction, but they can also open novel temporal seasonal niches that provide opportunities for ecological adaptation, allochronic or temporal reproductive isolation, and even population divergence and ecological speciation. Examples of evolutionary responses to changing seasonal environments are now common in the literature, with many involving rapid life-cycle evolution via shifts in the timing of dormant life stages (Tauber et al., 1986; Bradshaw & Holzapfel, 2001; Bradshaw et al., 2004; Schmidt et al., 2005; Bradshaw & Holzapfel, 2006; Gomi et al., 2007; Bradshaw & Holzapfel,

2008). Thus, key insight into several fundamental issues in biology – from the origin of species to climatic adaptation – can be found by understanding how dormancy timing evolves.

Defined as a state of environmentally induced developmental arrest, dormancy is a physiologically dynamic developmental trajectory characterized by distinct phases and is a nearly ubiquitous life-history strategy for animals and plants living in temperate and polar environments. In recent years, plant biologists have made substantial strides towards identifying key genes and physiological mechanisms that matter for seasonality in *Arabidopsis* and corn (Caicedo et al., 2004; Stinchcombe et al., 2004; Chiang et al., 2009, 2011; Coles et al., 2010; Wilczek et al., 2010; Hung et al., 2012). Progress in animals remains limited in comparison and has often focused on insects. Extensive work shows that insect dormancy (diapause) is often environmentally induced, usually by temperature and/or photoperiod (Tauber et al., 1986; Danks, 1987) and is typically regulated by the alteration of a critical developmental hormone, such as juvenile hormone or ecdysteroids (Denlinger, 2002; Denlinger etal., 2005). A series of companion studies to the endocrine work have successfully induced diapause-like states or caused diapause to end prematurely using pharmacological or RNAi approaches, nominating physiological pathways important for diapause (Denlinger et al., 2005; Sim & Denlinger, 2008). However, it remains unclear whether these pathways harbour natural genetic variation that can provide the raw material for the evolution of insect seasonality.

The fly *Drosophila melanogaster* is an exception among insects, with

multiple studies identifying naturally occurring allelic polymorphisms associated with diapause adaptation along latitudinal clines. Variation in clock genes (Tauber et al., 2007), insulin signaling (Williams et al., 2006), *couch potato* (Schmidt et al., 2008) and other loci provide needed clarity on life-history evolution. However, most *Drosophila* studies focus on the ability to enter diapause as a binary response and do not address whether the identified loci are responsible for change in diapause timing and synchronization with seasonal environments. In contrast, the evolution of diapause timing has been emphasised in moths, butterflies, and pitcher plant mosquitoes, leading to clearly identified quantitative trait loci (QTL) (Dopman et al., 2005; Mathias et al., 2007; Kunte et al., 2011). Yet, the identity of the causal loci in these genomic regions and how allelic variation leads to altered physiological pathways and shifts in seasonal timing in nature all remain as outstanding questions.

One complication for understanding the role of dormancy timing in the evolution of animal seasonality is that dormancy is neither an inactive state nor a binary characteristic, but rather a physiologically dynamic alternative developmental pathway with several distinct phases including induction, maintenance and termination (Denlinger et al., 2005; Koštál, 2006). It is now clear that genetic and physiological variation must be partitioned across these developmental phases to understand the basis for seasonal adaptation, allochronic isolation, and other aspects of organismal and population biology influenced by seasonal timing. However, a critical challenge to this goal and a long-term limitation in diapause research has been a lack of anatomical landmarks to discern

which phase of diapause an individual is experiencing (Hodek, 2002; Denlinger et al., 2005; Koštál, 2006). Fortunately, over a half-century of physiological research and hundreds of studies have uncovered a nearly universal feature of diapause – suppressed metabolic rate associated with morphogenic and developmental arrest (Hahn & Denlinger, 2007).

Suppression of respiratory metabolism in diapausing insects has two profound implications for dissecting the basis of shifts in seasonal timing. First, respiratory gas exchange within species can be tracked in individuals through time to probe morphologically covert physiological transitions within the dynamic diapause development trajectory (Wipking et al., 1995; Hahn & Denlinger, 2007; Singtripop et al., 2007; Ragland et al., 2009). Specifically, metabolic rates can be used to document physiological landmarks and identify the induction phase – wherein metabolic rates drop precipitously, the maintenance phase – wherein metabolic rates remain depressed, and the termination phase – wherein metabolic rates increase precipitously (Denlinger, 2002; Koštál, 2006; Singtripop et al., 2007; Ragland et al., 2009). Second, metabolic profiles of individuals from different locally adapted populations or species provide a means to identify the variable developmental phase(s) of diapause relevant to evolved shifts in diapause timing. Thus, the characterization of the multiple phases of the dormancy developmental programme by respiratory metabolism creates a framework in which to identify and interrogate the mechanistic functions of candidate physiological pathways and genetic polymorphisms that underlie the evolution of animal seasonality. We apply this comparative approach to dissect the phases of

the diapause developmental programme underlying seasonal life-history adaption and allochronic reproductive isolation between incipient species of *Ostrinia nubilalis*, the European corn borer moth.

Introduced to North America in 1909–1914 from multiple European regions (Caffrey & Worthley, 1927), the European corn borer (ECB) moth is a textbook example of speciation (Coyne & Orr, 2004), in which one species splits into two through the evolution of multiple forms of reproductive isolation. Asynchrony in seasonal flight timing of adults, and thus their mating period, contributes disproportionately to speciation between incipient lineages of ECB that are commonly referred to as 'Z' and 'E' strains (Dopman et al., 2010). Allochronic isolation stems from differences in the number of generations per season (voltinism), in which bivoltine E-strain populations have one generation at the beginning of the season (June) and a second generation at the end of the season (August), whereas univoltine Z-strain insects have single generation in the middle of the summer (July) (Eckenrode et al., 1983; Roelofs et al., 1985; Dopman et al., 2010) (Fig. 2.1a). Life cycles are determined primarily by genetic changes in the timing of emergence from diapause in the spring (McLeod et al., 1979). An uncharacterized Mendelian locus on the Z (sex) chromosome named post-diapause development (*Pdd*) influences the time needed for caterpillars in diapause to reinitiate development and become pupae after winter (Glover et al., 1992; Dopman et al., 2005), when temperatures once again become warm enough to permit development. Two major co-dominant alleles occur at *Pdd*. One allele confers earlier emergence from larval diapause and thus earlier adult flight times

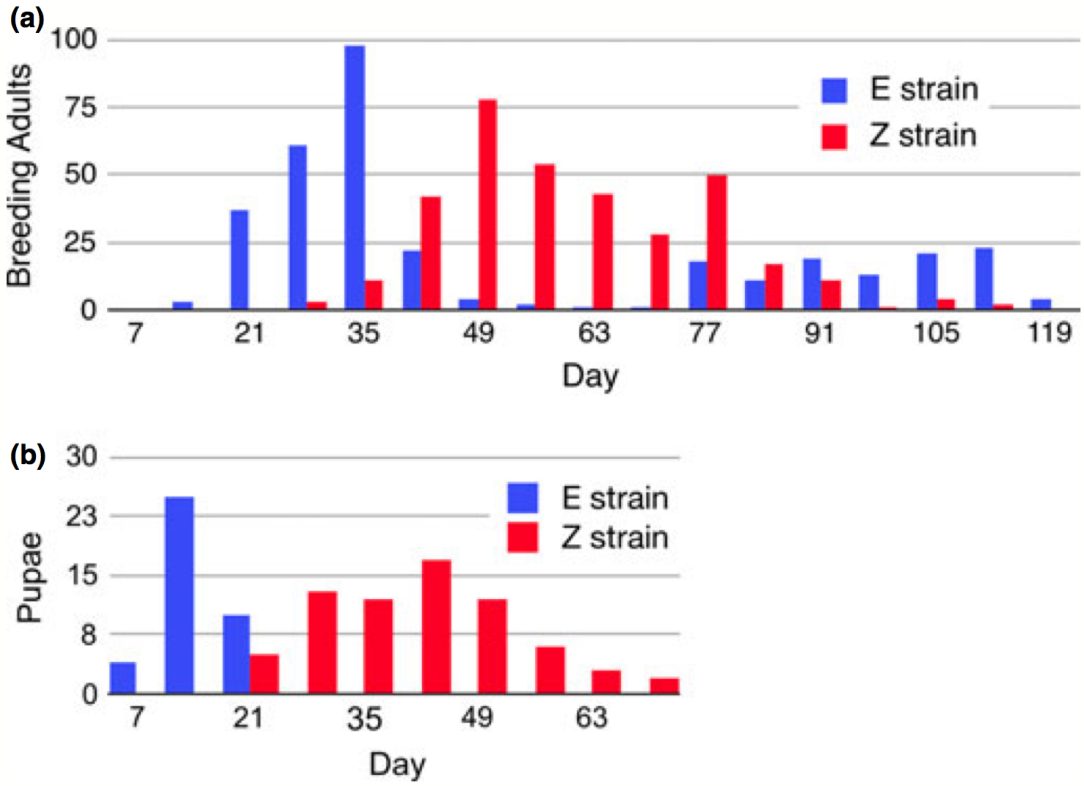

Figure 2.1 Allochronic isolation and diapause timing in the European corn borer. (a) Breeding cycle differences between bivoltine E-strain ECB (blue) and univoltine Z-strain ECB (red) in the wild (Farmington, NY) (adapted with permission from Dopman et al., 2010). (b) Post-diapause development time in earlier-emerging E-strain/Pdd^{S} (blue) and later-emerging Z-strain/Pdd^{L} (red) moths reared in the laboratory (adapted with permission from Glover et al., 1992).

(*Pdd*^{S}), whereas the other confers later emergence and later flight times (*Pdd*^{L}). Upon experiencing identical post-winter cues, univoltine Z/*Pdd*^{L} moths transition from larval diapause and resume active pupal development 20–30 days later than early emerging bivoltine E/*Pdd*^{S} moths under both laboratory and field settings (Glover et al., 1991, 1992; Dopman et al., 2005) (Fig. 2.1b). Because the duration of pupal and adult morphogenesis is similar between the earlier-emerging E-strain and the later-emerging Z- strain (Liebherr & Roelofs, 1975), the month-long shift in the time to pupation (Fig. 2.1b) has the potential to fully account for genetic differences in adult voltinism among field populations (Fig. 2.1a). Hence, the timing of the transition between the end of larval diapause and the initiation of pupal development is the trait that sets adult mating timing and thus allochronic isolation between ECB strains.

The earlier end of larval diapause and the initiation of pupal development in the bivoltine E-strain could be the product of changes in several phases within the diapause developmental programme. First, the degree of metabolic suppression during the diapause maintenance phase could differ (Fig. 2.2a). In this case, diapausing larvae of the earlier-emerging E-strain should be less metabolically depressed than the later-emerging Z-strain, allowing them to more quickly move from the diapause maintenance phase into the diapause termination phase. Second, the earlier-emerging E-strain could enter the diapause termination phase earlier than the later-emerging Z-strain (Fig. 2.2b). The beginning of the diapause termination phase can be precisely identified by a persistent increase in metabolism compared with the metabolically depressed diapause maintenance

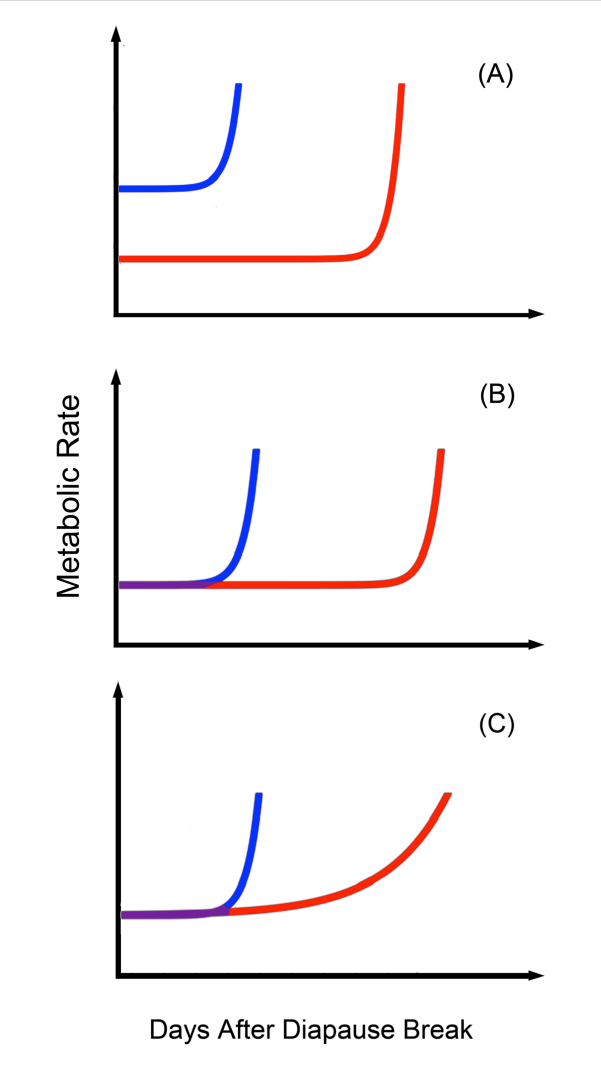

Figure 2.2 Hypothetical metabolic trajectories for ECB strains differing in diapause timing. (a) Bivoltine E-strain ECB (blue) and univoltine Z-strain ECB (red) differ in the severity of metabolic suppression. (b) Physiological response to diapause-breaking cues is delayed in the Z-strain compared to the E-strain. (c) Strains respond to diapause-breaking cues at similar times, but differ in the rate of release from metabolic arrest.

phase and the termination phase will eventually lead to the end of larval diapause and pupal formation. Third, the earlier-emerging E-strain may end larval diapause and initiate pupal development earlier than the later-emerging Z-strain because the diapause termination phase progresses more rapidly from the initial increase in metabolism to pupation in the earlier-emerging E-strain (Fig. 2.2c). We use changes in CO_2 production as a metric for metabolic rate to test which of the three components of the diapause programme, or combinations of components, have diverged to produce the clear differences in life-cycle timing between the earlier-emerging bivoltine E-strain and the later-emerging Z-strain of ECB.

MATERIALS AND METHODS

Inducing and terminating diapause: Diapausing ECB larvae do not feed during or after diapause, but during the active growth phase all larvae were fed a standard artificial diet for ECB (Southland Products, Lake Village, AR, USA). Photoperiod and temperature are two critical cues that influence both entry into larval diapause and the end of larval diapause and resumption of pupal development in ECB. In the laboratory, diapause is efficiently initiated under short-day photoperiod, even under a variety of temperatures that would normally be permissive for development (Takeda & Skopik, 1985; Skopik & Takeda, 1986; Skopik et al., 1986; Glover et al., 1991, 1992). Previous studies of ECB diapause have shown that populations react similarly in both the laboratory and the field, including the degree of metabolic depression observed in diapausing larvae (Beck & Hanec, 1960; McLeod & Beck, 1963). Therefore, after hatching, a 12:12 light/dark (L:D) photoperiod at constant 23°C was used to induce diapause

(Glover et al., 1992) in two ECB strains that were generously donated from colonies maintained by Charles Linn at the New York State Agricultural Experiment Station in Geneva, NY. The earlier-emerging bivoltine E-strain/Pdd^{S} and later-emerging univoltine Z-strain/Pdd^{L} lines were originally derived from field collections in central NY and are maintained in culture under mass-rearing conditions (Glover et al., 1992). After 35 days of exposure to 12:12 L/D, diapausing borers are at the 5th instar larval stage, whereas non-diapausing insects will have already pupated or enclosed as adults.

Diapausing larvae are sensitive to photoperiod and exposure to long-day photoperiodic conditions can effectively end larval diapause and promote active pupal development in both laboratory and field-collected insects (McLeod & Beck, 1963; Beck, 1964; Skopik & Bowen, 1976). Therefore, 36 days after diapause was induced, diapausing larvae were transferred from short-day conditions that maintain diapause (12:12 L:D and 23 °C) to conditions that will precipitate the end of diapause at 16:8 L:D and 26 °C (Glover et al., 1992).

Measuring metabolic rates: Metabolic rates were quantified every other day starting at mid-morning and at the same time (±1 h), beginning on the day before transfer to long-day conditions permissive to ending diapause. Measurements were continued until pupal development. ECB larvae were weighed and individually transferred into a constant-volume respirometry chamber, which consisted of a 60-mL syringe fitted with a three-way luer valve. Syringes were purged, filled with medical grade air (<1 ppm CO_2; Airgas, Cambridge, MA, USA) and then returned to incubators. After 3 ± 0.5 h, a syringe

pump was used to inject 50 mL into the sample air column of a Li-Cor 6262 CO_2/H_2O analyzer operating in differential mode (Li-Cor Biosciences, Lincoln, NE, USA), with sample and reference airflow maintained at 500 mL/min by Sierra Side-Trak 840 mass flow valves (Sierra Instruments, Monterey, CA, USA) operated by a Sable Systems mass flow controller (Sable Systems, Las Vegas, NV, USA). Correction was made for water vapour loss by the Li-Cor unit. Following injection of the gas bolus and CO_2 quantification, ECB larvae were removed from the syringes, weighed, placed in 30 mL portion cups with moistened dental wicking and returned to their incubators. Preliminary studies indicated that humidification of the air in respirometry chambers was unnecessary because ECB showed no change in mass before and after measurements, and larval mortality was low despite repeated exposures to dry air during the brief measurement period.

All metabolic measurements were recorded and results transformed and integrated using Sable Systems Datacan V Software (Sable Systems), in which CO_2 free air served as a baseline. Empty syringes were used as a control for leakage of CO_2 into the chambers. The manual bolus integration method was used to calculate the rate of CO_2 production per individual per day (Lighton, 2008; Ragland et al., 2009):

$$R = \frac{\int_{ti}^{tf} CF\, dt}{e},$$

in which R is respiration rate (cc CO_2/h), C is instantaneous CO_2 concentration (cc/L), F is flow rate (cc/h), e is elapsed time from purge to injection (h), and t_i -

tf is the time interval in which CO_2 was integrated (h). Data were corrected for the volume injected *vs*. the total syringe volume and for mass (cc CO_2/mg/h). After correcting for larval mass, there were no significant differences in CO_2 production between sexes and data were pooled across sexes.

A model for metabolic rate trajectories: Visual inspection of preliminary data suggested an exponential increase in metabolic rate during the diapause termination phase. We constructed a three-parameter function describing this trajectory:

$$R = e^{(t-a)^b} + c,$$

where *a* corresponds to when the persistent metabolic increase that defines the start of the termination phase begins, *b* relates to the rate of metabolic increase during diapause termination, and *c* corresponds to the initial metabolic rate during the diapause maintenance phase. A simplified model in which parameters were fixed could have been chosen, but the three-parameter exponential provided needed flexibility to test our a priori hypotheses about which phase or phases of diapause development have diverged between the two strains and the model performed well when fit to the data. Each individual's respirometric trajectory was fit separately using a least-squares estimation procedure in PRISM 5.0 (GraphPad Software, La Jolla, CA, USA).

RESULTS

To confirm the efficacy of our photoperiod treatments to induce diapause, we compared the metabolic rates of putatively diapausing 5th instar caterpillars exposed with short-day conditions with non-diapausing caterpillars exposed to

long-day conditions. After exposure to 35 days of short-day photoperiod, diapausing larval CO_2 production levels (n = 19) were approximately 45% of non-diapause larvae (n = 14) ($t_{19.12}$ = -7.03, P = 1.05 x 10^{-6}, Fig. 2.3). This result confirms prior studies in which metabolic rate (CO_2 consumption) of diapausing ECB was suppressed by ~50% (Beck & Hanec, 1960; McLeod & Beck, 1963).

To characterize metabolic rate trajectories from diapause to pupation for Z and E-strains of ECB, resting metabolism was measured every other day for 67 days from a total of 17 later-emerging Z-strain/Pdd^L and 18 earlier-emerging E-strain/Pdd^S caterpillars. CO_2 production was similar for diapausing insects under short-day conditions in both strains and for the following 2 days after moving individuals into long-day conditions to end diapause ($P > 0.05$, Fig. 2.3). Over this interval, metabolic rates of individuals in both strains were relatively constant and low, suggesting that larvae do not initiate diapause termination immediately when placed in conditions permissive for ending diapause.

Although initially very similar, rates of CO_2 production began to diverge between the two strains by day 5 after transfer into long-day conditions. On day 5, respiratory metabolism for several earlier-emerging E-strain/Pdd^S insects began to rapidly increase (e.g. Fig. 2.4a), and as a group, levels of CO_2 were significantly elevated compared with the later-emerging Z-strain/Pdd^L insects ($t_{29.73}$ = 2.51, P = 0.012, Fig. 2.3). A larger proportion of earlier-emerging E-strain/Pdd^S genotypes initiated the transition into a metabolically active state indicating the transition from diapause maintenance into the diapause termination phase, leading to more pronounced differences between strains on later days (e.g. day 9 and 11, Fig. 2.3).

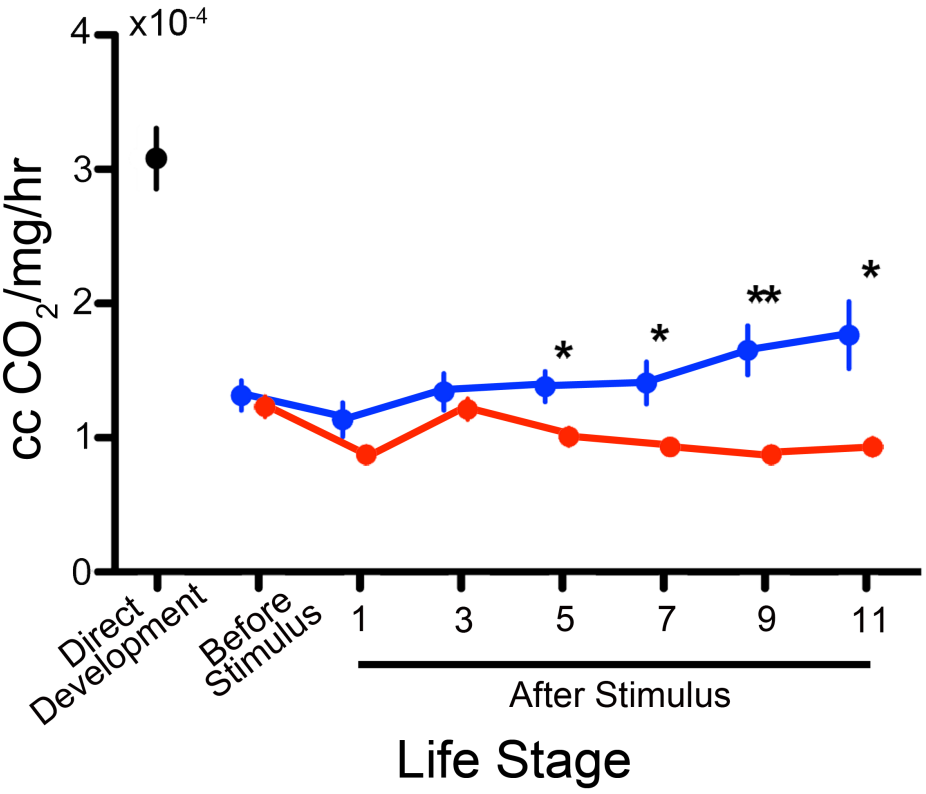

Figure 2.3 Metabolic rate for Z-strain/Pdd^{L} and E-strain/Pdd^{S} insects. Mean metabolic rate is reported for E-strain/Pdd^{S} (n = 18, blue) and Z-strain/Pdd^{L} (n = 17, red) through a developmental time course before and after long-day photoperiod to stimulate diapause termination. Nondiapausing larvae are shown for comparison. Error bars reflect standard errors. *P < 0.05. **P < 0.01.

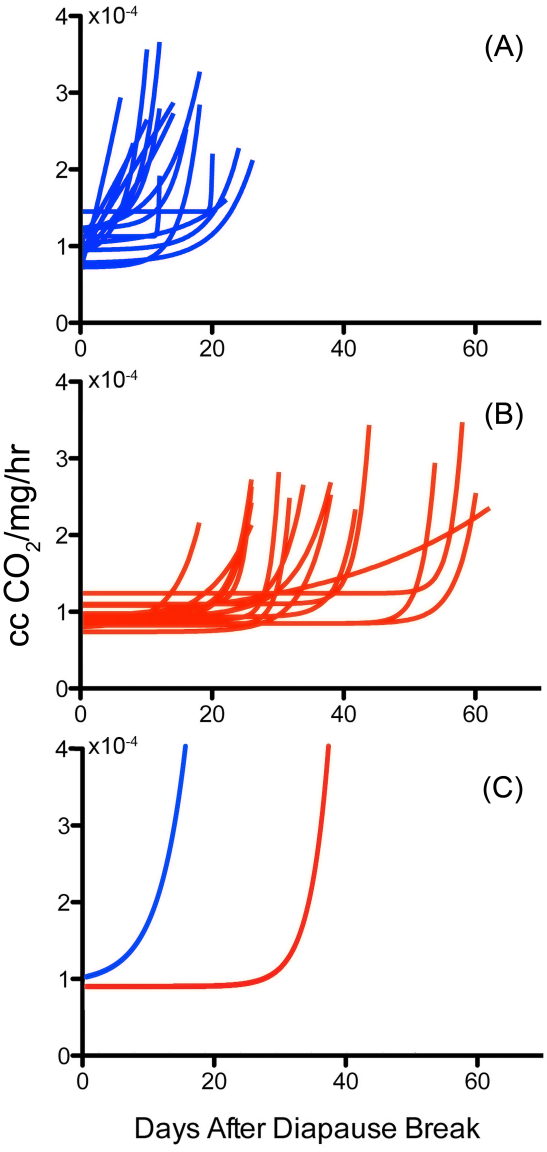

Figure 2.4 Metabolic rate trajectories after diapause break, (a) E-strain/Pdd^{S} larvae (n = 18), (b) Z-strain/Pdd^{L} larvae (n = 17), and (c) plot of median regression parameter values for E-strain/ Pdd^{S} (blue) and Z-strain/Pdd^{L} (red) moths. Average r^2 values for fitted curves were 0.8 and 0.76 for Z-strain/Pdd^{L} and E-strain/Pdd^{S} moths, respectively.

We tested which phase or phases of the diapause developmental programme have diverged between the genetically distinct bivoltine and univoltine ECB strains using least-squares regression with a 3-parameter exponential model. As anticipated based on preliminary results, the identified nonlinear model provided a good fit to individual daily respiration rates. Average r^2 values were 0.76 and 0.8 for earlier-emerging E-strain/Pdd^S and later-emerging Z-strain/Pdd^L, respectively. By day 20 after the shift to long-day photoperiods, all larvae having short development time alleles entered an exponential phase of respiration that ended at pupation several days later (Fig. 2.4a). Long diapause insects showed a similar exponential increase in metabolic rate leading to pupation soon after, but most of these long-diapausing larvae did not enter the exponential phase of metabolic rate increase that indicates the diapause termination phase until day 30 or later (Fig. 2.4b).

There was some variation in the shape of respirometric trajectories among individual moths within each strain during diapause termination and initiation of pupal morphogenesis (Fig. 2.4a,b). However, only minor differences were observed within and between ECB strains in initial baseline metabolism during the diapause maintenance phase and in the rate of release from metabolic depression during the termination phase. Indeed, later-emerging Z-strain/Pdd^L and earlier-emerging E-strain/Pdd^S genotypes did not significantly differ in estimated parameter values corresponding to the intercept (c) or rate of exponential increase (b) in the nonlinear model (Table 2.1). Thus, neither the initial degree of metabolic suppression during the diapause maintenance phase nor the rate of

Table 2.1: Estimated parameters from nonlinear regression of daily metabolic rate for E-strain/*Pdd*^{S} and Z-strain/*Pdd*^{L} moths

Parameter	E-strain/*Pdd*^{S} (n=18)	Z-strain/*Pdd*^{L} (n=17)	*P*-value
a	12.90 (19.71)	34.47 (16.39)	2.99×10^{-7}
b	0.26 (0.27)	0.38 (0.27)	0.15
c	0.95 (1.17)	0.90 (0.13)	0.92

E-strain/*Pdd*^{S} and Z-strain/*Pdd*^{L} show median (interquartile range) parameters values from a 3-parameter exponential model (see Materials and Methods), where *a* corresponds to when the persistent metabolic increase that defines the start of the termination phase begins, *b* relates to the rate of metabolic increase during diapause termination, and *c* corresponds to the initial metabolic rate during the diapause maintenance phase. *P*-value reports results from Wilcoxon rank-sum tests of Z-strain/*Pdd*^{L} and E-strain/*Pdd*^{S} parameters. Average r^2 values for fitted curves were 0.8 and 0.76 for Z-strain/*Pdd*^{L} and E-strain/*Pdd*^{S} moths, respectively.

completion of the metabolically elevated diapause termination phase contribute significantly to adaptive divergence in the timing of the end of larval diapause and the beginning of pupal development between the two ECB strains. In contrast, substantial variation within and between strains was observed in the timing of the exponential increase in metabolic rate (parameter *a*) that indicates the initiation of the diapause termination phase. The median value for parameter *a* in the model was ~2.5-fold larger in the long diapause time group ($W = 233$, $P = 2.99 \times 10^{-7}$, Table 2.1). Plotting representative metabolic trajectories for each strain using median parameter values clearly illustrated strain differences in the timing of the persistent increase in metabolic rates that signals the initiation of the diapause termination phase, and a lack of difference in either the initial metabolic rates during the diapause maintenance phase or in the rate of release from metabolic suppression once the diapause termination phase had begun (Fig. 2.4c).

DISSCUSSION

The ability to synchronize dormant life stages with adverse climatic conditions and active stages with favourable ones is a key adaptive strategy for animals and plants living in seasonal environments. Changes in the timing of dormant and active life stages have been implicated as playing a major role in allochronic isolation and species diversification (Alexander, 1968; Tauber et al., 1977; Filchak et al., 2000; Ording et al., 2010; Dopman et al., 2010) as well as local adaptation along environmental gradients, like latitude and altitude, and adaptation to contemporary climate change (Tauber et al., 1986; Bradshaw & Holzapfel, 2001; Bradshaw et al., 2004; Schmidt et al., 2005; Bradshaw &

Holzapfel, 2006; Gomi et al., 2007). However, the developmental, genetic and physiological mechanisms underlying adaptive divergence in the timing of transitions between dormant and active life stages are poorly understood in animals. Using metabolism as an indicator of the previously covert phases of diapause development, we were able to clearly identify three important parameters of the diapause developmental programme and test for divergence in them between the genetically distinct earlier-emerging E and later-emerging Z-strains of the European corn borer. We clearly show that a change in the timing of the initiation of the diapause termination phase is responsible for the nearly month-long shift in diapause timing that produces asynchronous breeding cycles and temporal reproductive isolation, whereas there appears to be no difference between the earlier-emerging E and later-emerging Z-strains in either the initial degree of metabolic suppression during the diapause maintenance phase or in the duration or rate of progression of the diapause termination phase. To our knowledge, this is the first study to clearly identify which of the multiple phases of the dormancy developmental programme differ between genetically distinct lineages and contribute to the evolution of insect seasonality, allochronic isolation, and seasonal adaptation.

From an adaptive standpoint, altering only the timing of diapause termination to change adult emergence timing and not the level of metabolic suppression during the diapause maintenance phase would seem to make good functional sense. In spite of the clear advantages of diapause related to avoidance of stressful periods and life-cycle synchronization, dormancy is quite costly.

Reductions in both fecundity and survival often occur in diapausing generations (Denlinger, 1981; Bradshaw et al., 1998; Munyiri et al., 2004; Matsuo, 2006), likely as a result of physiological stress (e.g. desiccation or cold shock) and/or depletion of metabolic reserves (review in Hahn & Denlinger, 2007). For example, flesh flies experience a ~75% decrease in both their metabolic reserves and in fertility when exposed to natural winter conditions in diapause compared to those that do not undergo diapause (Denlinger, 1981; Adedokun & Denlinger, 1985). In the case of the ECB, larvae do not feed during or after diapause and the univoltine Z-strain both enters diapause earlier and ends diapause later than the bivoltine E-strain. Because late-emerging Z-strain individuals must experience greater overwintering energy demands of diapause, an obvious physiological strategy would be to remain in a deep state of metabolic and respiratory arrest to save energy until the time of diapause termination and then to complete the energy-intensive diapause termination phase as quickly as possible (Fig. 2.2b).

Although we did not observe any differences in the degree of metabolic suppression between the Z and E-strains of ECB during the diapause maintenance phase (Fig. 2.2a), these values vary widely among taxa based on their diapause life-history strategy and even between populations within a species. For example, diapausing pupae of the flesh fly spend the winter inactive underground and suppress their metabolism up to 90% compared with non-diapausing pupae (Denlinger et al., 1972). In contrast, monarch butterflies overwinter in a state of reproductive diapause wherein they retain the ability to fly and suppress their metabolism by only 10% compared with non-diapausing, reproductively active

individuals (Chaplin & Wells, 1982). Within species, diapausing larvae of the dusky-wing butterfly from populations that experience warmer winters, and thus greater metabolic demand during diapause, have lower metabolic rates during the diapause maintenance phase (Williams et al., 2012). More comparative work across additional species pairs and populations is needed to test the extent to which differences in the degree of metabolic suppression during diapause may contribute to differences in diapause life-history timing and to seasonal adaptation in response to global climate change.

The duration and rate of completion of the diapause termination phase could also feasibly contribute to shifts in dormancy timing as a result of temporal adaptation. Specifically, populations active early in the season may experience a more rapid release from metabolic and developmental arrest, shortening the diapause termination phase, relative to populations that do not become active until later in the year (Fig. 2.2c). Testing this model requires an ability to detect subtle differences in daily metabolism with individual resolution. Although many studies have now quantified patterns of increased metabolism during diapause termination and post-diapause development, estimates have often been made for groups of individuals (Koštál et al., 1998; Singtripop et al., 2007; but see Ragland et al., 2009). One exception was a recent study on *Rhagoletis pomonella* fruit flies (Ragland et al., 2009), in which respiratory patterns of single pupae were tracked from diapause maintenance through the termination phase and into active post-diapause adult morphogenesis. Although substantial variation in the shape of metabolic rate trajectories was uncovered, it remains to be tested whether

differences in the rate of pupal emergence from diapause explains a ~3 week shift in adult emergence time between the apple and hawthorn races in nature (Feder & Filchak, 1999). Like *R. pomonella*, metabolic trajectories among *O. nubilalis* larvae are variable within strain (Fig. 2.4a,b), but differences in the rate of emergence from metabolic suppression or the length the diapause termination phase between strains were not detected. Although adaptive changes in the rate of release from developmental arrest would seem to be relatively unimportant in the ECB, developmental rates can differ among species of insects, between populations within species, between sexes within populations and even plastically in response to environmental cues (Nylin & Gotthard, 1998). Thus, critical tests of the extent to which differences in the rates of diapause termination might contribute to life-cycle evolution and species diversification are warranted.

An interesting parallel to the ECB system occurs in North American *Papilio* swallowtail butterflies (Scriber & Ording, 2005; Ording et al., 2010; Kunte et al., 2011; Scriber, 2011). In this system, the northern-distributed species *P. canadensis* is univoltine with an obligate pupal diapause that produces a single early summer flight, whereas the southern-distributed species *P. glaucus* is bivoltine with a facultative pupal diapause that produces both early (diapausing) and late (non-diapausing) summer flights. Hybridization between these two species has lead to the recent formation of a third species, *P. appalachiensis*, which occurs in cooler habitats along the Appalachian Mountains in Virginia and West Virginia in the United States. *P. appalachensis* is univoltine with an obligate pupal diapause and adults fly later in the summer than either the univoltine *P.*

canadensis or the first generation of the bivoltine *P. glaucus*, but before the second generation of *P. glaucus*, effectively isolating the hybrid species from both parents. Several studies have shown that pupal diapause and flight timing variation across these *Papillio* species is Z-linked, like in ECB (Scriber & Ording, 2005; Ording et al., 2010; Kunte et al., 2011; Scriber, 2011). As the extent of similarity of the Z-liked loci associated with seasonality in the highly divergent ECB and swallowtail butterfly systems is still unclear, comparative study of the genetic and physiological mechanisms of diapause and life-cycle timing should be particularly fruitful.

Although characterizing the distinct phases of the dormancy developmental programme across diverse organisms will generate new hypotheses about the nature of dormancy, results presented here and in the literature on the ECB moth supports a model for the role of diapause in insect seasonality in which major life-cycle shifts stem from simple genetic and developmental changes. Our results suggest that progress in understanding two of the most compelling issues in biology – the genesis of biodiversity and response to climate change – will require a focus on the evolution of the developmental transition to dormancy termination. Now that we have clearly identified this stage as the critical developmental event underlying asynchrony in adult emergence between bivoltine E and univoltine Z-strains of the ECB moth, our next goal becomes dissecting the causal genetic and physiological mechanisms. How does allelic variation at the previously identified sex-linked locus *Pdd* interact with the metabolic and endocrine machinery that regulates transitions among the phases of

diapause? Determining how naturally occurring polymorphism regulates diapause timing and seasonality represents an important step towards an understanding of how organisms can persist and even prosper in the face of rapid global climate change.

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CHAPTER 3

TRANSCRIPTOME PROFILING REVEALS MECHANISMS FOR THE EVOLUTION OF INSECT SEASONALITY*

ABSTRACT

Rapid evolutionary change in seasonal timing can facilitate ecological speciation and resilience to climate warming. However, the molecular mechanisms behind shifts in animal seasonality are still unclear. Evolved differences in seasonality occur in the European corn borer moth (*Ostrinia nubilalis*), in which early summer emergence in E-strain adults and later summer emergence in Z-strain adults is explained by a shift in the length of the termination phase of larval diapause. Here, we sample from the developmental time course of diapause in both strains and use transcriptome sequencing to profile regulatory and amino acid changes associated with timing divergence. Within a previously defined QTL, we nominate 48 candidate genes including several in the insulin signaling and circadian rhythm pathways. Genome-wide transcriptional activity is negligible during the extended Z-strain termination, whereas shorter E-strain termination is characterized by a rapid burst of regulatory changes involved in resumption of the cell cycle, hormone production, and stress response. Although gene expression during diapause termination in *Ostrinia* is similar to that found previously in flies, nominated genes for shifts in timing are species-specific. Hence, across distant relatives the evolution of insect seasonality appears to involve unique genetic switches that direct organisms into

* *Wadsworth, CB, & Dopman, EB (2015). Transcriptome profiling reveals mechanisms for the evolution of insect seasonality. Journal of Experimental Biology* ***218(22)****: 3611-3622.*

distinct phases of the diapause pathway through wholesale restructuring of conserved gene regulatory networks.

INTRODUCTION

Defined as a state of environmentally induced developmental arrest, dormancy is a physiologically dynamic developmental trajectory and is a nearly ubiquitous life-history strategy for animals and plants to survive inhospitable climatic conditions in temperate and polar environments. Diapause, a widespread form of insect dormancy, is also viewed as the primary mechanism through which the annual rhythm of major life history phases such as reproduction, growth, development, and migration are synchronized with seasonally varying biotic and abiotic requirements (Danilevskii, 1965; Tauber and Tauber, 1981; Danks, 1987; Leopold and Lang, 1996). Diapause occurs at various life-stages in insects (egg, larval, pupal, adult), and proceeds through pre-diapause, diapause, and post-diapause developmental phases (Koštál, 2006). Diapause itself is further decomposed into an initiation phase in which direct development ceases, a maintenance phase in which developmental arrest continues, and a termination phase in which a stimulus triggers an active potential for development although development may not be overtly visible (Tauber et al., 1986; Koštál, 2006).

Species and populations can reveal subtle shifts in the timing of the unique stages of diapause that can substantially alter annual life-history patterns. Whereas the onset of diapause commonly initiates seasonal migration or polyphenism at appropriate times, the termination of diapause synchronizes active growth and reproduction with favorable conditions (Tauber et al., 1986).

Examples of rapid shifts in the timing of the onset or termination of diapause are common, with many believed to be adaptive responses to a range of contemporary environmental perturbations including changing climates, anthropogenic impacts, and species introductions (Bradshaw and Holzapfel, 2001, 2006, 2008; Bradshaw et al., 2004; Filchak et al., 2000; Mathias et al., 2005; Schmidt et al., 2005; Gomi et al., 2007).

To understand how organisms adapt and persist in novel seasonal environments like those experienced during global warming, we must understand the molecular bases for shifts in diapause timing. In several study systems, different populations or species show shifts to their seasonal dormancy timing (Picket and Neary, 1940; Tauber and Tauber, 1976; Bradshaw and Lounibos, 1977; Glover et al., 1992). However, complications in these systems arising from genetic architecture (e.g., polygenic, epistasis) or genomic architecture (e.g., chromosomal rearrangements), have confounded the identification of genes underlying variation in diapause timing (Tauber et al., 1977; Feder et al., 2002; Bradshaw, 2005; Mathias et al., 2007; Emerson et al., 2010; Wadsworth et al., 2015). Despite these difficulties, genes involved in the transitions of diapause phases have been identified. Cell cycling, DNA replication/transcription, and stress response genes have been nominated as important for diapause initiation in moths and mosquitos (Bin Bao and Xu, 2011; Poelchau et al., 2011). Additionally, genes in the Wnt and TOR signaling pathways have been nominated as strong regulatory candidates during diapause termination in *Rhagoletis* flies (Ragland et al., 2011). Perhaps the best single candidate thus far was identified for

the egg diapause of the silkmoth (*Bombyx mori*), in which transgenerational diapause induction involved the temperature-sensitive *transient receptor potential A1* (*Trpa1*) gene (Sato et al., 2014). Nevertheless, in many of these systems it is still unclear how the regulation of diapause genes and pathways are altered to produce ecologically relevant variation in diapause timing.

A useful model for studying the molecular basis of shifts in seasonality is the European corn borer moth (*Ostrinia nubilalis*). Introduced to North American from Europe in the early 20th century, multiple corn borer ecotypes exist that differ by 20-30 days in the time needed for overwintering larvae in diapause to reinitiate development in spring (Glover et al., 1992; Dopman et al., 2005). Over a half century of research has connected variation in diapause timing to latitudinal differences in generation number (Beck and Apple, 1961; Palmer et al., 1985; Levy et al., 2015). More recently, differences in diapause timing have been emphasized because they contribute to asynchronous adult mating flights of two-generation E-strain borers and one-generation Z-strain borers in New York (Dopman et al., 2010). Thus, divergence in the timing of diapause appears relevant for both successful colonization and ecological speciation.

Two observations provide important insight about possible loci responsible for diapause timing change in European corn borers. First, the a major genetic factor (*Pdd*) has been shown to map to a region of the Z (sex) chromosome that may be rearranged between corn borer strains (Glover et al., 1992; Dopman et al., 2004, 2005; Wadsworth et al., 2015). Second, high temperatures and/or long-day photoperiod cues are known to stimulate diapause

termination in corn borers (McLeod and Beck, 1963). Thus, pathways and genes on the sex chromosome that are sensitive to light and temperature represent strong candidate genes for *Pdd*.

In a prior study, metabolic rate trajectories were used to identify a shift in the timing of the end of the diapause termination phase as critical for explaining earlier emergence in the E-strain and later emergence in the Z-strain of the European corn borer moth (Fig. 3.1a) (Wadsworth et al., 2013). Within a week of exposure to diapause-breaking (long-day) conditions, E-strain moths rapidly progress through the diapause termination phase and enter post-diapause development, as indicated by increased respiratory metabolism. In contrast, Z-strain borers exhibit continued suppression of metabolic rate for nearly a month longer, suggesting that timing divergence stems from an extended diapause termination phase. Here, we sample from the developmental time course of diapause in both strains and we profile regulatory and amino acid changes coincident with changes in timing. We then address two main questions. First, what candidate genes might underlie *Pdd* and therefore divergence in diapause timing? Second, what downstream pathways might direct insects to an earlier or later end of diapause?

RESULTS

Transcriptome. For Z and E-strain corn borers, we sampled three time points along the photoperiodically-initiated and terminated diapause developmental pathway (Fig. 3.1b). Larval diapause in corn borers is initiated under short-day photoperiod, even under temperatures that are permissive for

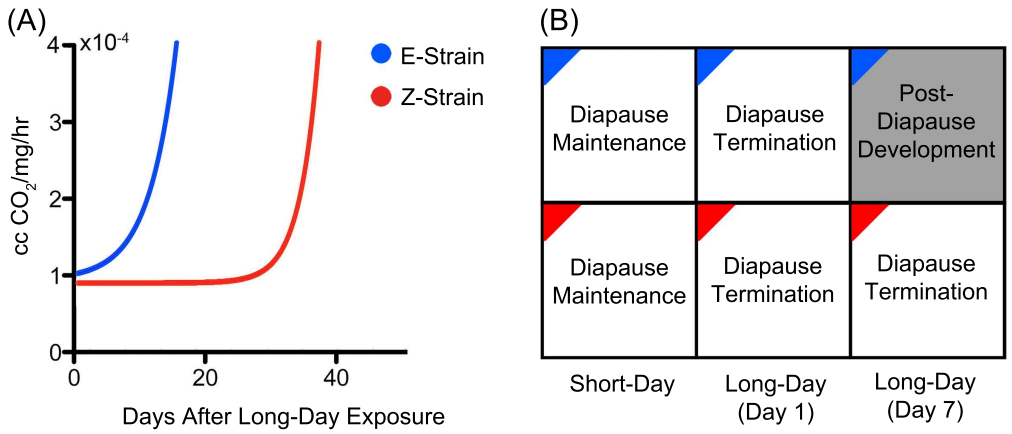

Figure 3.1. Divergence in the timing of photoperiodically-terminated diapause in the European corn borer moth. (A) Non-linear regression of median metabolic trajectories for earlier-emerging E-strain (blue) and later-emerging Z-strain (red) borers after transfer from short-day (diapause inducing) to long-day (diapause breaking) conditions (Wadsworth et al., 2013). Physiological models suggest that the end of diapause, which coincides with metabolic uptick, is shifted by ~30 days in Z-strain borers. (B) Experimental design for transcriptome profiling in which borers were sampled before and after experiencing long-day cues.

development (MeLeod and Beck, 1963; Glover et al., 1992). Therefore, the first sampled time point occurred when both strains were in the diapause maintenance phase under short-day diapause inducing conditions (12:12 L/D). Exposure to long-day conditions stimulates corn borers to enter diapause termination and downstream pupal development (MeLeod and Beck, 1963; Glover et al., 1992). Therefore, the second sampled time point occurred on day 1 after long-day exposure (16:8 L/D), but before a physiological change in respiratory metabolism in either strain (hereafter, "day 1"). The final time point was on day 7 after long-day exposure and after physiological divergence between strains (hereafter, "day 7"). Within this sampling scheme, we expected changes on day 1 to be more reflective of strain specific differences in upstream causal factors. Day 7 in contrast might be more informative of downstream developmental consequences of *Pdd* such as the transition from diapause termination to post-diapause development (as indicated by a metabolic uptick, Fig. 3.1a). Nevertheless, within-strain variation in the length of the diapause termination phase exists (Wadsworth et al., 2013), and therefore patterns of gene expression on day 1 and day 7 likely represent a mixture of cause and effect.

As the most likely tissues involved in photoperiodic responses have been hypothesized to be located in the brain, eyes, or major neuroendocrine glands (Koštál, 2011), at each of the three time points we sampled larval head tissues (n=5 individuals for n=5 replicates per strain). A total of ~562 million 50-bp single-end reads were generated across 30 libraries. Each library had on average 19.46 ± 8.99 million reads. After removal of adapters, mtDNA, and rRNA

contaminants, average library size decreased to 18.38 ± 8.24 million reads. Our *de novo* assembly consisted of 47,565 transcripts (mean length=686, N50=1,025). Of these, 14,177 were annotated via TBLASTX to FlyBase and 23,077 were annotated via TBLASTX to the NCBI non-redundant (nr) database.

There were a total of 15,208 transcripts that were significantly differentially expressed in at least one comparison involving strains or time-points (Table 3.1). We removed some transcripts from our analysis to minimize the number of genes that were differentially expressed between strains because of population divergence (strains diverged approximately 100k years ago, Malausa et al., 2007) and not because of associations with differences in the timing of diapause termination. With E and Z-strain corn borers being physiologically indistinguishable under short-day diapause inducing conditions (Wadsworth et al., 2013), we considered differentially expressed transcripts between strains at this time-point as a control for regulatory divergence unrelated to photoperiodic response or divergence in length of the diapause termination phase. We therefore removed these transcripts from differential expression comparisons between strains at other time points (day 1 and day 7) to help control for false positives.

Gene Candidates. To nominate transcripts as candidates for the 30-day shift in the end of diapause, we initially considered genomic position. *Pdd* maps to a region of the European corn borer Z chromosome between *Ket* and *Ldh* (Dopman et al., 2005). Therefore, an initial candidate list was nominated by TBLASTX hits of corn borer transcripts to the macrosyntenic interval of the *Bombyx mori* Z chromosome containing these genes (6.5 Mb-17.4 Mb) (Kroemer

Table 3.1. Number of significantly differentially expressed (DE) transcripts between and within strain (FDR < 0.01)

Comparison	Total DE	DE Corrected for Population Divergence
Diapause E vs. Z	3077 (71)	-
Day 1 E vs. Z	6615 (135)	4160 (77)
Day 7 E vs. Z	4633 (114)	2337 (60)
Within Z Diapause vs Day 1	23 (0)	20 (0)
Within Z Diapause vs Day 7	1 (0)	1 (0)
Within E Diapause vs Day 1	764 (9)	625 (6)
Within E Diapause vs Day 7	95 (1)	77 (1)

Values in parentheses show the subset of DE genes within each comparison that are putatively Z-linked

et al., 2011). Of the corn borer transcripts that mapped to this interval, two forms of evidence were evaluated. First, the causal factor(s) might be regulatory. Therefore, patterns of differential expression either within strain from diapause maintenance to a later developmental time-point, or between strain on day 1 or day 7, were considered. Second, as differences in diapause timing could be due to structural mutation(s), we considered the presence of fixed amino-acid changing substitutions between strains.

Of the 563 (~1%) transcripts with putative locations between *Ket* and *Ldh*, a total of 48 were nominated as candidates for *Pdd*. 41 were nominated based on patterns of significant differential expression between strains, or significant differential expression within strains across the developmental time course (Fig. 3.2; Table 3.2). Six broad patterns of expression differences were observed between strains: (A) 5 genes were upregulated in the Z-strain on day 1 relative to the E-strain, (B) 3 genes were upregulated in the Z-strain on day 7 relative to the E-strain, (C) 3 genes were upregulated in the Z-strain on day 1 and day 7 relative to the E-strain, (D) 10 genes were upregulated in the E-strain on day 1 relative to the Z-strain, (E) 8 genes were upregulated in the E-strain on day 7 relative to the Z-strain, and (F) 4 genes were upregulated in the E-strain on days 1 and 7 relative to the Z-strain. Within the E-strain there were two observed expression patterns across the time course: (G) 4 downregulated genes from diapause maintenance to day 1, and (H) 1 upregulated gene from diapause maintenance to day 7. Of the transcripts nominated by regulatory patterns, all had some associated annotation information (Table 3.2).

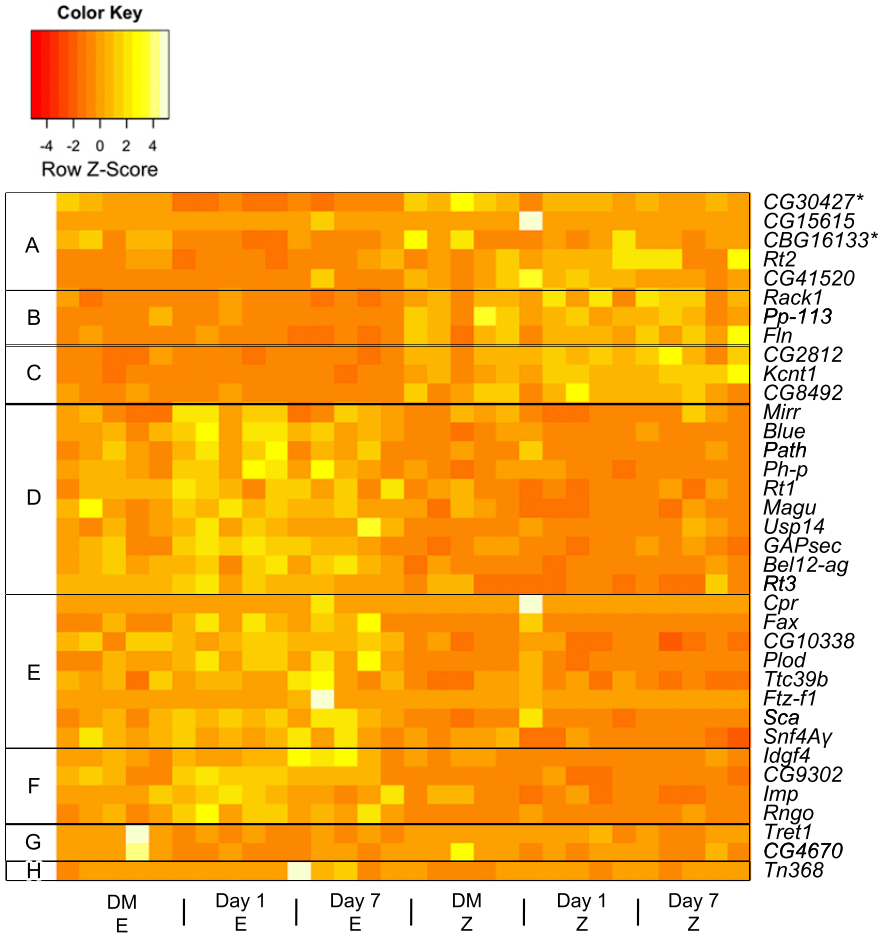

Figure 3.2. Heatmap of differentially expressed genes within the *Ket-Ldh* genomic interval. (A) 5 genes upregulated in the Z-strain on day 1 relative to the E-strain; (B) 3 genes upregulated in the Z-strain on day 7 relative to the E-strain; (C) 3 genes upregulated in the Z-strain on days 1 and 7 relative to the E-strain; (D) 10 genes upregulated in the E-strain on day 1 relative to the Z-strain; (E) 8 genes upregulated in the E-strain on day 7 relative to the Z-strain; and (F) 4 genes upregulated in the E-strain on days 1 and 7 relative to the Z-strain. Within the E-strain there were two observed expression patterns: (G) 4 downregulated genes from diapause maintenance to day 1 (*including two genes in A) and (H) 1 upregulated gene from diapause maintenance to day 7. Duplicate annotations were averaged for expression. All were FDR < 0.01.

Table 3.2. Significantly differentially expressed transcripts (FDR < 0.01) located in the *Ket-Ldh* interval

Description	Gene Symbol	FlyBase ID	NCBI ID	Location B.mori (Mb)	FDR	Expression Pattern*
Between Strains Day 1						
Mirror	*Mirr*	FBgn0014343	XP_004929953	7.8	2.30E-03	D
Fatty acyl-CoA reductase CG30427	*CG30427*	FBgn0043792	EHJ78784	9.0	1.66E-05	A
Ferritin-like CG15615	*CG15615*	FBgn0034159	XP_004929844	9.2	1.83E-04	A
Bluestreak	*Blue*	FBgn0041161	XP_004929978	10.6	2.52E-04	D
Pathetic	*Path*	FBgn0036007	XP_004929913	11.6	7.78E-03	D
Polyhomeotic proximal	*Ph-p*	FBgn0004861	NP_001188364	11.8	7.61E-03	D
Reverse transcriptase	*Rt1*	-	AAQ09229	11.9	3.16E-03	D
CBG16133	*CBG16133*	-	XP_004929918	12.0	3.76E-03	A
Magu	*Magu*	FBgn0262169	XP_004929925	12.4	8.66E-04	D
Ubiquitin carboxyl-terminal hydrolase 14	*Usp14*	FBgn0032216	XP_004927226	12.4	7.85E-03	D
GTPase activating protein, SECIS-dependent read-through	*GAPsec*	FBgn0035916	XP_004927187	14.2	4.56E-03	D
Reverse transcriptase	*Rt2*	-	AAQ09229	14.4	3.23E-05	A
BEL12-AG transposon	*Bel12-ag*	-	XP_004927201	14.5	1.26E-04	D
CG41520	*CG41520*	FBgn0087011	XP_004933130	15.4	2.85E-04	A
Endonuclease and reverse transcriptase	*Rt3*	-	CAX36787	16.4	4.12E-06	D
Between Strains Day 7						
Cuticular protein	*Cpr*	-	AK401102.1	9.1	4.36E-03	E
Failed axon connections	*Fax*	FBgn0014163	XP_004929846	9.3	9.64E-04	E
CG10338	*CG10338*	FBgn0032700	XM_004929795.1	9.4	1.22E-03	E
Receptor of activated protein kinase C 1	*Rack1*	-	EHJ71933	10.9	4.27E-03	B
Procollagen lysyl hydroxylase	*Plod*	FBgn0036147	XP_004929891	10.9	4.79E-03	E
Tetratricopeptide repeat protein 39B	*Ttc39b*	-	XP_004929983	11.1	2.86E-03	E
Unknown secreted protein	*Pp-113*	-	AK405478.1	11.5	4.38E-03	B
Nuclear hormone receptor betaFTZ-F1	*Ftz-f1*	-	AAL50351	11.7	1.32E-03	E
Flightin	*Fln*	FBgn0005633	NP_001130045	12.9	3.67E-05	B
Scabrous	*Sca*	FBgn0003326	XP_004930012	13.5	4.79E-03	E
SNF4/AMP-activated protein kinase γ subunit	*Snf4Aγ*	-	NM_001126248.1	15.6	3.11E-03	E

Between Strains Day 1 and Day 7

WD repeat-containing protein	*CG2812*	FBgn0034931	EHJ79015	7.1	4.66E-03	C
Imaginal disc growth factor 4	*Idgf4*	FBgn0013763	ACW82749	8.5	6.44E-04	F
Protein disulfide-isomerase	*CG9302*	FBgn0032514	XP_004929835	9.0	4.21E-03	F
Potassium channel subfamily T member 1	*Kcnt1*	-	XP_004929982	11.0	8.29E-05	C
IGF-II mRNA-binding protein	*Imp*	FBgn0262735	EHJ69667	11.4	5.42E-03	F
Rings lost	*Rngo*	FBgn0030753	NM_001046849.1	16.6	2.28E-03	F
Lysozyme	*CG8492*	FBgn0035813	ADA67927	16.9	1.80E-03	C

Diapause Maintenance to Later Time-point Within E Strain

Fatty acyl-CoA reductase CG30427	*CG30427*	FBgn0043792	EHJ78784	9.0	2.75E-04	G
Facilitated trehalose transporter 1	*Tret1*	FBgn0051100	XP_004929980	10.8	5.35E-03	G
CBG16133	*CBG16133*	-	XP_004929918	12.0	3.22E-03	G
Sulfhydryl oxidase	*CG4670*	FBgn0033814	XP_004929937	13.4	2.94E-03	G
Retrotransposon TED	*Tn368*	-	M32662.1	14.5	5.81E-03	H

- Expression pattern refers to Figure 2.
- Modified table, see *Journal of Experimental Biology for full table*.

We next considered candidates in the *Ket—Ldh* interval that harbored fixed amino-acid changes between Z and E-strains. Of the total number of variable SNPs between strains (n=1,347,090), 250 were located in European corn borer transcripts with significant TBLASTX hits to the *Ket—Ldh* interval in *B. mori*. These were distributed among 114 unique transcripts, 11 of which contained at least one predicted amino acid change that was fixed between strains. Eight of the 11 transcripts had annotation information (Table 3.3). Four amino acid changes were switches in polarity, two were switches in polarity and charge, and one was a switch in charge.

Global Transcriptional Patterns. To characterize regulatory mechanisms that might represent consequences of differences at *Pdd*, we focused on differential expression of transcripts between strains after exposure to diapause-breaking cues (Table 3.1). For Gene Ontology (GO) and KEGG enrichment (*P*-value cutoff ≤ 0.001) of differentially expressed gene lists, on day 1 there were 14 enriched "biological process" GO terms, 4 "molecular function" terms, and 5 KEGG pathways (Fig. 3.3; supplementary material Table 3.S1). Two terms associated with Wnt signaling were also enriched ($P \leq 0.05$). Between strains on day 7 there were 24 enriched "biological process" terms, 9 "molecular function" terms, and 1 KEGG pathway (Fig. 3.3).

To identify pathways under the control of similar regulatory mechanisms, for each strain we evaluated correlated changes in gene expression across the three time points. Normalized expression data from the diapause maintenance

Table 3.3. All fixed SNPs resulting in predicted amino acid changes between E and Z-strain borers within the *Ket-Ldh* interval

Transcirpt ID	Description	Gene Symbol	FlyBase ID	Location B.mori (Mb)	SNP Z/E	Position in Transcript	Predicted AA Change Z/E
comp34828_c0_seq1	*Fatty acyl-CoA reductase CG30427*	*CG30427*	FBgn0043792	9.0	C/T	1378	Ala/Thr
comp25898_c0_seq1	*Belphegor*	*Bor*	FBgn0040237	9.9	T/G	1004	Ser/Ala
comp117726_c0_seq1	-	-	-	10.2	A/G	280	Cys/Arg
comp67276_c0_seq1	-	-	-	10.6	A/C	486	Ile/Cys
comp67276_c0_seq1	-	-	-	10.6	T/A	487	Ile/Cys
comp18031_c0_seq1	*Neprilysin 4*	*Nep4*	FBgn0038818	10.6	T/A	210	Phe/Leu
comp26697_c0_seq1	*Pico*	*Pico*	FBgn0261811	10.7	A/G	1072	Ile/Val
comp122129_c0_seq1	*Acetyl Coenzyme A synthase*	*AcCoAS*	FBgn0012034	11.9	G/T	258	Ala/Glu
comp29232_c0_seq2	*CG10226*	*CG10226*	FBgn0035695	12.2	C/T	1014	Ser/Asn
comp18621_c0_seq1	*CG10226*	*CG10226*	FBgn0035695	12.2	G/A	233	Ser/Asn
comp97089_c0_seq1	*Period*	*Per*	FBgn0003068	13.0	C/G	232	Gly/Arg
comp81055_c0_seq1	*Activated Cdc42 kinase-like*	*Ack-like*	FBgn0263998	16.2	T/G	384	Ser/Ala

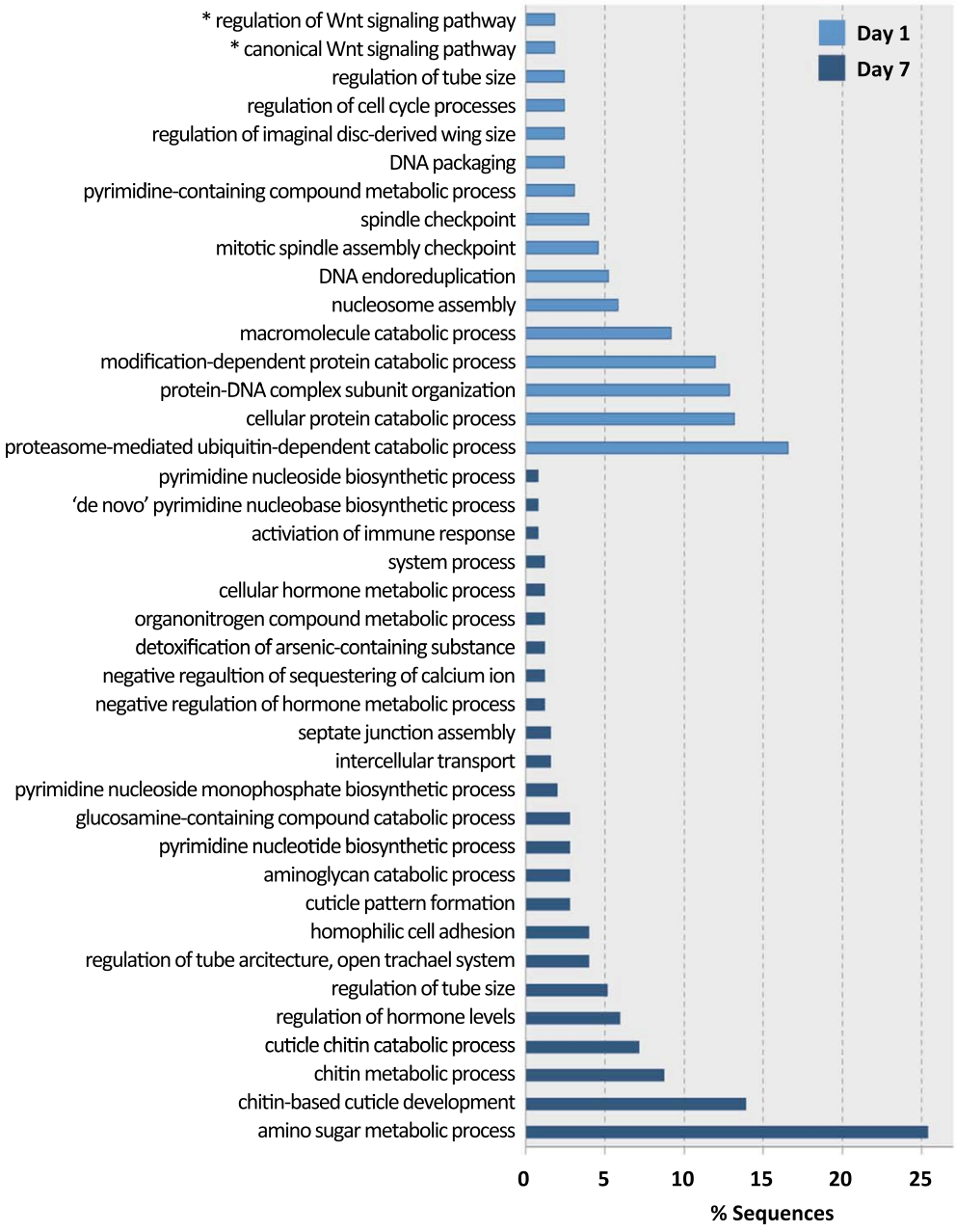

Figure 3.3. Enriched Biological Process GO terms for differentially expressed genes on day 1 (light blue) and day 7 (dark blue) after exposure to long-day conditions. *P*-value cutoff < 0.001 or < 0.05 (*).

time point were randomly coupled to later developmental time points and then $\log_2$ normalized to simulate microarray M values. Short Time-Series Expression Miner (STEM) (Ernst et al., 2005) was then used to discover 5 significant gene expression profiles in the E-strain and 2 in the Z-strain ($P \leq 0.01$) (Fig. 3.4). Pattern A had 105 transcripts assigned to it, pattern B had 109, pattern C had 108, pattern D had 67, pattern E had 64, pattern F had 38, and patterns G had 32. The most extreme expression changes were seen from diapause maintenance to day 1. Groups of genes with similar expression patterns across the time course in the E-strain were enriched for GO categories including development, metabolism, protein catabolism, and oxidoreductase activity (supplementary material Table 3.S2). In contrast, there were no significantly enriched GO terms for the two temporal expression profiles in the Z-strain. Time series expression profiles were also plotted for individual transcripts in pathways of interest. Pathways included cell cycling, ecdysone, circadian rhythms, heat shock, Wnt signaling, and insulin signaling (Fig. 3.5; supplementary material Fig. 3.S1). At least one transcript within each of these pathways showed significant differential expression between the strains (supplementary material Table 3.S3).

DISCUSSION

In insects, divergence in seasonal life-cycle timing may commonly involve changes in the duration of the diapause termination phase in spring. Z and E-strain European corn borers differ by ~30 days in the length of the diapause termination phase as indicated by a shift in the timing of metabolic release coincident with the end of diapause (Wadsworth et al., 2013) (Fig. 3.1a). Although strains are

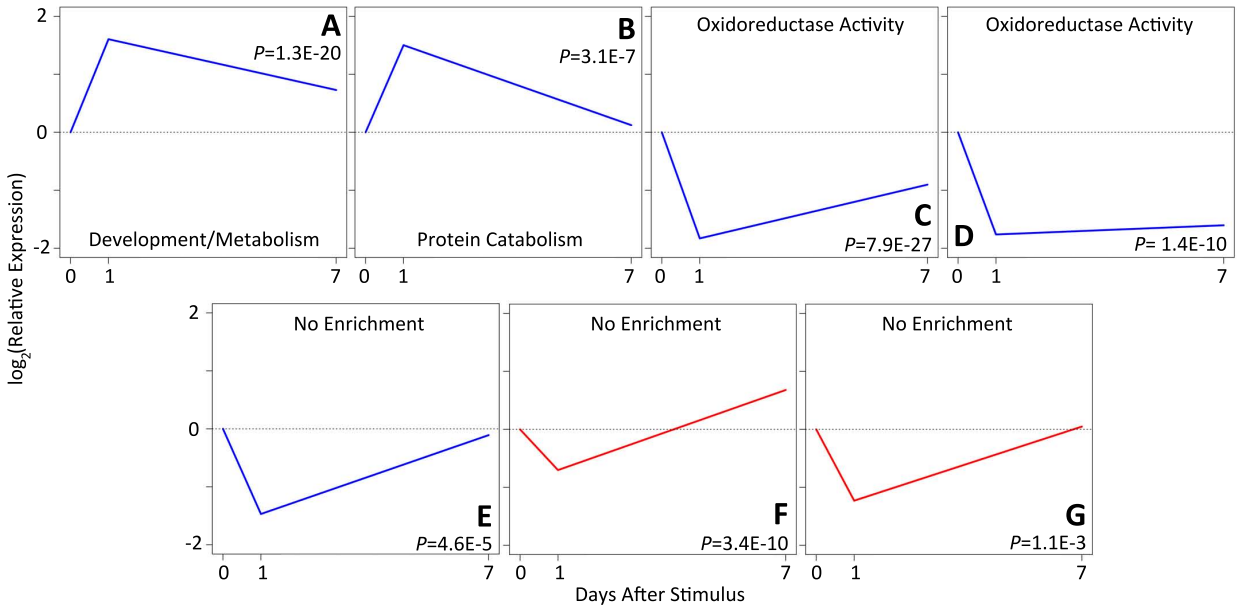

Figure 3.4. Pathways with correlated changes in gene expression during the diapause developmental pathway. Gene expression was log2 normalized to the diapause maintenance time-point for each strain. (A-E) are significant (*P* 0.01) trajectories identified in the E-strain (blue) and (F-G) are significant (*P* 0.01) in the Z-strain (red). Labels represent enriched GO terms for each profile (supplementary material Table 3.S2).

physiologically indistinguishable in early stages of diapause termination, RNA sequencing shows this to be a period of intense transcriptional activity in the E-strain, as indicated by a large number of differentially expressed transcripts from diapause maintenance to day 1 (Table 3.1). Conversely, the Z-strain shows negligible change from diapause maintenance to day 1 or 7 (Table 3.1). Thus, the major genetic cause of altered seasonal diapause timing, *Pdd,* appears to act as a genetic switch operating shortly after the beginning of the growing season in the E-strain, allowing for rapid development, while being delayed in the Z-strain.

Gene Candidates. Specific gene candidates for shifts in insect seasonal timing have been difficult to accumulate via QTL mapping due to complex genetic bases or genomic architectures between populations (Tauber and Tauber, 1977; Feder et al., 2002; Bradshaw et al., 2005; Mathias et al., 2007; Wadsworth et al., 2015). In such systems, transcriptome profiling can be a useful alternative to QTL mapping to nominate candidate genes. For example, in the pitcher-plant mosquito (*Wyeomyia smithii*), Northern and Southern populations differ in the critical day length required for diapause termination, but the genetic basis is complex involving many loci and epistasis (Bradshaw et al., 2005; Mathias et al., 2007). Using microarrays, a total of 29 genes were nominated as candidates with one, a cuticular protein with cornea-specific expression, falling within a previously defined QTL for altered termination response (Emerson et al., 2010).

In European corn borers, early results implicated a major Mendelian factor underlying differences in diapause termination timing (Glover et al., 1992; Dopman et al., 2004, 2005). However, recent findings show that *Pdd* and roughly

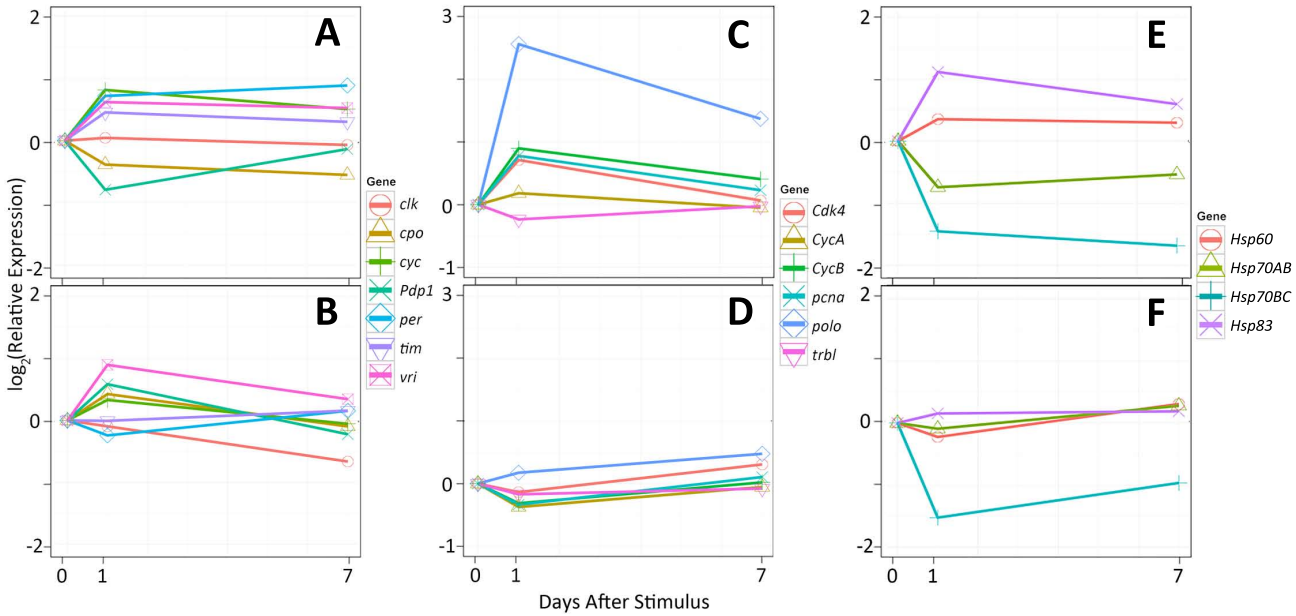

Figure 3.5. Trajectories of gene expression during the diapause developmental pathway. Shown are circadian genes in (A) the E-strain and (B) the Z-strain, cell cycle genes in (C) the E- strain and (D) the Z-strain, and heat shock genes in (E) the E-strain and (F) the Z-strain. Gene expression data were normalized to begin at zero during diapause maintenance where day 1 and day 7 time-points show simulated M values representing the log2 (diapause promoting/diapause breaking conditions).

20% of the sex chromosome may be caught up in a large inversion (Wadsworth et al., 2015). As inversions can cause large swaths of genes to be inherited together and thus act as "supergenes", there is potential for *Pdd* to be composed of multiple linked loci. Of the genes that are predicted to be within the chromosomal interval containing *Pdd*, 48 have fixed amino acid changes between strains or experience a change in expression through diapause termination (Table 3.2, 3.3). Candidate gene lists did not overlap between *W. smithii* and European corn borers (Emerson et al., 2010). Within our nominated gene lists, transcripts involved in insulin signaling and circadian rhythms are some of the most promising candidates for *Pdd* because both pathways are sensitive to light and temperature (Suttie et al., 1991; Chan et al., 1999; Shingleton et al., 2005; Taylor et al., 2005; Luckenbach et al., 2007; Sim and Denlinger, 2009), environmental factors that are known to stimulate diapause termination in corn borers (McLeod and Beck, 1963; Glover et al., 1992). These two pathways are also proposed regulators of dormancy or diapause in other species (Ikeno et al., 2010; Hahn and Denlinger, 2011). Of the 48 candidates, we focus on four with direct involvement in these pathways.

The γ subunit (*Snf4A*γ, 15.6 Mb in *B. mori*) of AMP-activated protein kinase (AMPK) is a highly conserved gene that makes up the energy-sensing component of the AMPK protein (Kahn et al., 2005). AMPK is a member of the insulin, target of rapamycin (TOR), and cell death pathways (Kahn et al., 2005; Lippai et al., 2008), all of which are known to be important in the progression of development and molting in Lepidoptera (Lippai et al., 2008; Gu et al., 2009;

Smith et al., 2014). In our transcriptome, *Snf4Aγ* was significantly downregulated in the later-emerging Z-strain compared to the earlier-emerging E-strain (FDR < 0.001 on day 7) (Table 3.2; Fig. 3.2). In *Drosophila*, loss of *Snf4Aγ* expression has been shown to inhibit the ability of 20-hydroxyecdysone (20HE) to promote cell death, a major component in metamorphosis in insects (Lippai et al., 2008). Thus, perhaps suppression of *Snf4Aγ* in Z-strain moths promotes a state of delayed development through inhibition of cell death and turnover, processes that occur ~20-30 days earlier in the E-strain.

Two members of the insulin growth factor (IGF) pathway were identified as candidate genes. The first, *IGF-II mRNA binding protein* (*Imp*, 11.4 Mb in *B. mori*), is associated with growth and development, and like the insulin signaling pathway, the IGF pathway commonly shows sensitivity to both temperature and photoperiod (Suttie et al., 1991; Dahl et al., 1997; Luckenbach et al., 2007). *Imp* was found to be downregulated (day 1 and 7) in the later-emerging Z-strain larvae (FDR < 0.001) (Table 3.2; Fig. 3.2). This result is notable because suppression of the IMP homolog in mice prevents IMP from binding to insulin growth factors, resulting in a reduction in growth (Hansen et al., 2004). In both mice and nematodes, disruption of other genes within the IGF pathway result in phenotypes reminiscent of longer dormancy such as growth reduction and extend lifespan (Liu et al., 1993; Kenyon, 2010). Hence, downregulation of *Imp* in corn borers may promote later emergence from diapause in the Z-strain through decreased IGF signaling.

The second IGF candidate, *Receptor for activated C kinase 1* (*Rack1*, 10.9 Mb in *B. mori*), interacts with the IGF pathway through the insulin-like growth factor I receptor (IGF-IR). In corn borers, *Rack1* was upregulated in the later-emerging Z-strain on day 7 (FDR < 0.001) (Table 3.2; Fig. 3.2). *Rack1* shows a similar association with diapause maintenance in the Asian tiger mosquito (*Aedes albopictus*), where it was one of 314 transcripts to be upregulated upon exposure to short-day conditions and entrance into diapause (Poelchau et al., 2011). Overexpression of RACK1 protein has also been shown to lead to reduced cell growth in mammals (Hermanto et al., 2002). With the upregulation of *Rack1* in the Z-strain, and known upregulation in other species delaying growth or promoting diapause, this gene is an interesting candidate for *Pdd*.

A final gene candidate for *Pdd* is the clock gene *Period* (*Per*, 13.0 Mb in *B. mori*). *Per* shows a predicted glycine to arginine amino acid change between Z and E strain insects (Table 3.3) and is modestly upregulated in the E-strain on both day 1 and day 7 (FDR = 0.58 and 0.89 respectively) (Figs 3.5a,b). Evidence is accumulating that clock genes like *Per* are important for photoperiodic responses and diapause transitions. Within the Z-strain of corn borers, a different amino-acid substitution in *Per* varies with diapause emergence timing and latitudinal changes in generation number (Levy et al., 2015). In *D. melanogaster*, geographic variation in a threonine-glycine repeat within *Per* associates with diapause incidence (Kyriacou et al., 2008). Laboratory studies also support a role for *Per* in diapause. RNA interference of *Per* in the bean bug (*Riptortus pedestris*) disrupts both circadian rhythms and photoperiodic response (Ikeno et al., 2010),

and artificial selection for enhanced diapause in grey flesh fly (*Sarcophaga bullata*) is associated with a deletion at the *Per* locus that is 33 amino acids long (Han and Denlinger, 2009).

Global Transcriptional Patterns. Our results show that the diapause termination phase and the transition into post-diapause development involves stress response, hormone processing, cell cycling, developmental patterning, and metabolic pathways (Figs 3.3, 3.4). Similar results were found in flies (Emerson et al., 2010; Poelchau et al., 2011; Ragland et al., 2010, 2011). This speaks to a conserved transcriptional basis for the end of the diapause developmental pathway, despite ~200 million years of divergence (Pringle et al., 2007), and distinct diapausing life-stages (larval *vs*. pupal) in different environments (host plant *vs*. soil).

Release From Developmental Arrest. Previous results in flies suggest that a common characteristic of diapause is cell-cycle arrest in the G0/G1 or G2 phases, with transition into cell cycle progression being indicative of the end of diapause (Koštál et al., 2009; Ragland et al., 2010, 2011). Gene expression profiles in corn borers support a transition into active cell cycling upon exposure to long-day cues in the E-strain but not the Z-strain (Figs 3.5c,d), suggesting E-strain borers are rapidly exiting diapause. For example, *Proliferating cell nuclear antigen* (*Pcna*), a DNA replication related protein that is active in the S phase of the cell cycle, is upregulated during the rapid diapause termination phase in E-strain European corn borers and also diapause termination of *Rhagoletis* flies (Ragland et al., 2011), but it is downregulated in Z-strain borers (FDR < 0.00001

on day 1). Differentially expressed genes between Z and E-strains were highly enriched for cell cycling GO terms (Fig. 3.3; supplementary material Table 3.S1). Additionally, genes involved in the progression of the cell cycle were upregulated in the E-strain, whereas they were downregulated in Z-strain (e.g., *CycA*, *CycB* [FDR < 0.001 days 1 and 7]; *Cdk4*, *Polo* [FDR < 0.01 on day 1]). Overall, patterns suggest that active cell cycling occurs within days of exposure to long-day conditions in the E-strain allowing for release from diapause-induced metabolic depression and progression into post-diapause development, whereas cellular development remains suppressed for ~30 additional days in the Z-strain.

Transcripts involved in development showed congruent patterns to those involved in mitotic progression. Wnt signaling pathways promote proper developmental patterning in insects (e.g., metamorphosis) through cellular communication, resumption of the cell cycle, and also may regulate growth through the canonical growth-regulatory pathways (Logan and Nusse, 2004; Gokhale and Shingleton, 2015). The Wnt signaling and canonical Wnt signaling pathways showed enrichment of GO terms on day 1 between strains (Fig. 3.3; supplementary material Table 3.S1). Many transcripts involved in these pathways were downregulated in the Z-strain and upregulated in the E-strain (e.g., *Fz3*, *Arm*, *Smo*, *Wg*, and *Pan* [FDR < 0.01 on day 1]) (supplementary material Figs 3.S1a,b; Table 3.S3). Coupled with the regulation of members of the cell cycling pathway, upregulation of members of Wnt signaling pathways in the E-strain indicate a reversal of arrest on both the cellular and developmental levels.

Stress Response. Studies in flies have documented shifts from anaerobic to aerobic metabolism during the diapause termination phase as the hypoxic stress of winter is lifted (Emerson et al., 2010; Ragland et al., 2010, 2011). In moths, we find evidence for an equivalent shift in the E-strain but not in the Z-strain. Members of the anaerobic pyruvate metabolic pathway experienced a ~2-fold decrease in expression within the E-strain from diapause maintenance to day 1 or day 7 (e.g., *Aldh, CG6084*, *CG9629*, *Men, CG31674*, *Hex-t2* [FDR < 0.001]), and the pathway was upregulated in the Z-strain compared to the E-strain during diapause termination (e.g., *Aldh, CG9629, CG31075* [FDR < 0.01 on day 1]). These results suggest that a switch to aerobic metabolism is not associated with the diapause termination phase in moths *per se*, but is primarily associated with the metabolic uptick that marks the end of diapause termination phase.

Diapause is commonly associated with cold-hardiness across many insect species, yet it is unclear if the cold-hardy state persists through all of the phases of diapause. Our results suggest that in moths the reduction in cold-hardiness out of winter is primarily a function of a change in photoperiod (short to long-days) and entrance into diapause termination phase rather than the end of diapause. One class of proteins associated with cold-hardiness and diapause are heat shock proteins (HSPs) that act as molecular chaperones (Rinehart et al., 2007; Koštál and Tollarová-Borovanská, 2009; Ragland et al., 2010, 2011). Proteins within the HSP70 family are essential to recovery after chill shock treatments with the silencing of these proteins increasing mortality in flesh flies and linden bugs (Rinehart et al., 2007; Koštál and Tollarová-Borovanská, 2009). Our results show

significant downregulation of genes in this family within the E-strain, both in the diapause termination phase and at the end of diapause (e.g., *Hsp70AB*, *Hsp70BC* [FDR < 0.01 on day 1 and 7]). Although not significant, a similar pattern is seen for *Hsp70BC* within the Z-strain, even though they remain in the diapause termination phase for much longer (Figs 3.5e,f). Consistent downregulation of HSPs in both strains prior to the end of diapause implies that cold-hardiness is a property of diapause maintenance in moths, rather than the entire diapause developmental pathway.

Encocrine. Once hormones are released from neurosecretory cells in the brain, downstream development and metamorphosis become inevitable. One important hormone, 20-hydroxyecdysone (20HE), plays a role in inducing insect molting and metamorphosis at appropriate developmental stages (Denlinger, 2002). 20HE commonly breaks diapause in many insect species when injected, fed, or topically applied (Žďárek and Denlinger, 1975; Gadenne et al., 1990; Kidokoro et al., 2006; Yamamoto et al., 2008). Therefore, extension of the diapause termination phase should be correlated with suppressed transcription of ecdysone-related genes through decreased availability of 20HE or the inability to bind it. Indeed, *Neverland* (*Nvd*), *Spook* (*Spo*), and *Phantom* (*Phm*) are involved in 20HE production and were upregulated in the E-strain compared to the Z-strain (e.g., *Nvd* [FDR < 0.01 day 1 and 7]; *Spo*, *Phm* [FDR < 0.0001 on day 1 and 7]) (supplementary material Figs 3.S1c,d; Table 3.S3). Loss of function of *Nvd* is known to cause developmental arrest and reduced growth in *D. melanogaster* through reduced ecdysteroid production (Yoshiyama et al., 2006). 20HE receptors

were also upregulated in the E-strain (e.g., *EcR* [FDR < 0.01 on day 1]; *Ftz-f1* [FDR < 0.01 on day 7]). Surprisingly, one upregulated receptor, *Ftz transcription factor 1* (*Ftz-f1*), controls stage-specific responses to 20HE (Broadus et al., 1999) and was located within the QTL for *Pdd* (Table 3.2).

The Circadian Clock. It has long been hypothesized that the circadian clock is intimately connected to photoperiodic responses like diapause (Bünning, 1936). Supporting this idea, many latitudinal clines show co-variation between clock genes and diapause response (Mathias et al., 2005; Schmidt et al., 2008; Tauber et al., 2007; Cogni et al., 2013; Levy et al., 2015). In addition to the possible involvement of *Per*, we found the circadian gene *PAR-domain protein 1* (*Pdp1*) to be significantly differently expressed between the strains (e.g., FDR < 0.01 on day 1) (Figs 3.5a,b; supplementary material Table 3.S3). However, although predicted to be Z-linked, *Pdp1* is not located in the QTL (21.9 Mb in *B. mori*). In *Drosophila*, *Pdp1* encodes a transcription factor that promotes the transcription of the gene *Clock* (*Clk*) (Cyran et al., 2003). In the apple maggot fly, *Pdp1* is significantly downregulated from diapause maintenance to the diapause termination phase (Ragland et al., 2011). Similarly, in the E-strain *Pdp1* is downregulated from diapause maintenance to day 1 (Fig. 3.5a). Although the functional significance of this expression change is unclear, conserved expression of this gene between species may be indicative of an important role for *Pdp1* in the transition to the end of diapause.

Conclusions. Although diapause is deployed at various life stages in insect taxa, studies in flies and moths now point to deep conservation of

transcriptional pathways during the phases of diapause (Fig. 3.3; supplementary material Table 3.S3) (Emerson et al., 2010; Ragland et al., 2010, 2011). However, there appears to be little evidence in these organisms for shared genetic control of natural variation in diapause timing (Table 3.2, 3.3) (Emerson et al., 2010). The situation might be different within the Lepidoptera, such as *Ostrinia* moths and swallowtail butterflies, in which variation in diapause termination timing is associated with a common region of the sex chromosome (Hagen and Scriber, 1989; Ording et al., 2010; Dopman et al., 2005; Wadsworth et al., 2015). Hence, independent physiological and genetic mechanisms appear to govern the evolution of diapause timing across distantly related taxa, whereas a common origin may characterize more closely related lineages.

Elucidating the regulatory mechanisms of diapause evolution has long been challenging because of a lack of anatomical landmarks and a complex genetic basis. Our results demonstrate the combined power of high-throughput transcriptome and physiological screens for understanding the evolution of diapause. We anticipate that expanding this framework to other diapause phases (i.e., diapause induction and maintenance), taxonomic groups, and at various stages of evolutionary divergence will provide a foundation to understand how animals adapt to climate change and synchronize major life-history events with seasonally varying environments.

MATERIALS AND METHODS

Sample preparation. Diapause was induced in larvae using short-day conditions (12:12 LD at 23°C). After 35 days of exposure to these conditions,

diapausing borers remain at the 5th instar whereas direct developing insects will have already pupated or enclosed as adults (Glover et al., 1991; Dopman et al., 2005; Wadsworth et al., 2013). Diapause was terminated in 36 day old larvae using long-day conditions (16:8 LD at 26°C) (Glover et al., 1991; Dopman et al., 2005; Wadsworth et al., 2013). Alternative developmental genetic pathways underlying differences in diapause termination timing seem to be triggered by *Pdd* within the first 7 days after insects experience diapause-breaking cues, as indicated by elevated respiratory metabolism in the E-strain but not the Z-strain (Wadsworth et al., 2013). Therefore, we collected tissues at three time points from both strains within this interval (Fig. 3.1b): in diapause maintenance prior to a long-day (diapause breaking) cue, 1 day after transfer to long-day conditions when strains are physiologically indistinguishable but have entered the diapause termination phase, and on day 7 after transfer when the Z-strain remains in diapause termination (previously referred to as "diapause maintenance" in Wadsworth et al., 2013) and most E-strain moths have entered post-diapause development. Head tissues were sampled to capture changes in major neuroendocrine glands and the primary endogenous clock (Tauber et al., 1986; Koštál, 2011). Five individuals were pooled for each of 5 replicates per strain per time point. All samples were collected at the same time of day (14:00-16:00 hrs.) to avoid spurious effects of circadian rhythmicity. Tissues were ground in liquid nitrogen and total RNA was prepared for multiplexed Illumina sequencing using the Qiagen RNeasy kit (Qiagen Inc., Germantown, MD) followed by the TruSeq RNA Prep kit (Illumina Inc., San Diego, CA). Six libraries were multiplexed per

lane and subjected to single-end 50 bp sequencing on an Illumina HiSeq 2000 (Illumina Inc., San Diego, CA) at Tufts University Core Facility.

Data processing. Low quality reads and adapter sequences were removed using Trimmomatic v.0.27 (Lohse et al., 2012). Retained reads were ≥ 36bp after eliminating reads with an average quality of < 15, and trimming off ends with a quality of < 5 in leading and trailing bases. Mitochondrial (NC_003367.1) and ribosomal contaminants (all *Ostrinia* ribosomal sequences in NCBI) were removed using Bowtie2 v.2.1.0 (Langmead and Salzberg, 2012). Library quality was subsequently assessed using FastQC v.0.10.1 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Prior to *de novo* assembly, duplicate reads were collapsed to reduce library complexity using FASTX-Toolkit v.0.0.13 (Gordon and Hannon, 2010). Trinity was chosen for *de novo* assembly because it has been shown to recover more full length genes than other single-kmer *de novo* assembly programs (Grabherr et al., 2011; Zhao et al., 2011). It was run at default parameters (kmer=25, minimum contig length=48) on all libraries and the longest transcript per transcript cluster was used for subsequent analyses. Annotation of genes was by TBLASTX search to FlyBase and the NCBI nr database and retention of transcripts with e-values ≤ 10^{-5}. Putative locations for corn borer transcripts was determined using TBLASTX (E-value ≤ 10^{-5}) and the genome of *B. mori* (KAIKObase v.3.2.2) (Shimomura et al., 2009), which shows conserved macrosynteny with *O. nubilalis* (Kroemer et al., 2011). The *Pdd* QTL is known to be between *Ket* and *Ldh* (Dopman et al., 2005) or between 6.5 and 17.3 Mb on the *B. mori* Z chromosome. We used Bowtie 2

v.2.1.0 with *--end-to-end* and *--very-sensitive* options, which resulted in a higher percentage of uniquely mapped reads than any other combination of preset options. Count data were generated using the single best alignment for each read. To help mitigate G-C bias during PCR amplification, within and between sample data were corrected for G-C content using EDAseq (supplementary material Fig. 3.S2) (Risso et al., 2011).

Gene Ontology Analysis. Annotation of genes was conducted by TBLASTX search to both the FlyBase and NCBI nr databases, retaining annotations with e-values ≤ 10^{-5} as putative orthologs. GOstats v.2.12 conditional hypergeometric test (Falcon and Gentleman, 2007) was used to test for "Biological Process", "Molecular Function", and "KEGG pathway" enrichment using the ontology terms from the Bioconductor package org.Dm.eg.db v.2.10.1 with the *P*-value cutoff of ≤ 0.001. The Short Time-series Expression Miner v.1.3.8 (Ernst et al., 2005) was used to look for groups of genes that showed coordinated expression change from diapause maintaining to diapause breaking conditions. Normalized expression data were randomly coupled from diapause maintenance to later developmental time-points and then $\log_2$ normalized to simulate microarray M-values. Multiple transcripts with duplicate annotations were averaged. STEM Miner determines all possible iterations gene expression change through time, with the diapause maintenance time-point normalized to 0, increasing or decreasing up to a user defined step size (=2). Each gene in the dataset is then assigned to a given model based on its correlation coefficient, which was defined as 1. *P*-values were determined by calculating the observed

number of genes assigned to each profile compared to the expected number, which is calculated by permuting the fit of each gene to the model 1000 times ($P < 0.01$). Significantly enriched Gene Ontology terms for each time-series was determined using Bonferroni corrected P-values (≤ 0.0001).

Differential expression. Differential expression analyses used edgeR (Robinson and Oshlack, 2010), which employs an empirical Bayes method to estimate gene-specific biological variation. A multi-factor analysis was used to account for both day and strain factors. Per-gene dispersions were estimated using the Cox-Reid profile-adjusted method and a negative binomial generalized log-linear model was fit to identify significantly differentially expressed transcripts (supplementary material Figs. 3.S3, 3.S4). Subsequently, gene lists were corrected for multiple testing using a false discovery rate (FDR) (cutoff of ≤ 0.01). Candidates within the predicted QTL interval were selected if they showed no differential expression between strains during diapause maintenance, were differentially expressed between strains on day 1 or 7, or were differentially expressed within strain through time.

SNP Analysis. We identified fixed SNPs between strains by pooling all libraries by strain, mapping reads, and estimating SNP frequencies using Popoolation2 v1.201 (Kofler et al., 2011). This method has been shown to be effective at recovering the majority of true allele frequencies in populations (Konczal et al., 2014). A minimum coverage of 8 reads per population and a pool size of 75 was used to calculate F_{ST} for every SNP. To determine if fixed SNPs coded for an amino acid change in the subset of transcripts whose putative

locations were within the QTL, ESTScan was used to predict the reading frame (Iseli et al., 1999).

DATA ARCHIVING

Libraries are archived in Genbank (Bioproject ID: PRJNA294976; for accession numbers see supplementary material Table 3.S4).

SUPPLMENTAL MATERIAL

Table 3.S1. Enriched GO terms and KEGG pathways for differentially expressed genes between E and Z strains (*P*-value < 0.001)

Category	Term	Count	Size	*P*-value
Day1				
GO: biological process	proteasome-mediated ubiquitin-dependent protein catabolic process	30	83	8.39E-06
GO: biological process	cellular protein catabolic process	43	146	4.24E-05
GO: biological process	protein-DNA complex subunit organization	17	39	5.12E-05
GO: biological process	modification-dependent protein catabolic process	42	145	7.95E-05
GO: biological process	macromolecule catabolic process	54	210	2.44E-04
GO: biological process	nucleosome assembly	8	13	2.92E-04
GO: biological process	DNA endoreduplication	8	13	2.92E-04
GO: biological process	mitotic spindle assembly checkpoint	6	8	3.88E-04
GO: biological process	spindle checkpoint	6	8	3.88E-04
GO: biological process	pyrimidine-containing compound metabolic process	10	20	4.92E-04
GO: biological process	DNA packaging	19	54	5.57E-04
GO: biological process	regulation of imaginal disc-derived wing size	8	14	5.85E-04
GO: biological process	regulation of cell cycle process	39	146	8.57E-04
GO: biological process	regulation of tube size	13	32	8.64E-04
GO: biological process	canonical Wnt signaling pathway	8	21	*1.35E-02**
GO: biological process	regulation of Wnt signaling pathway	15	54	*2.29E-02**
GO: molecular function	glucose transmembrane transporter activity	8	11	5.78E-05
GO: molecular function	copper ion binding	10	17	9.61E-05
GO: molecular function	monosaccharide transmembrane transporter activity	9	17	6.31E-04
GO: molecular function	calcium ion binding	37	135	9.43E-04
KEGG Pathway	Proteasome	23	33	6.69E-12
KEGG Pathway	Ascorbate and aldarate metabolism	9	13	2.82E-05
KEGG Pathway	Retinol metabolism	8	13	2.76E-04
KEGG Pathway	DNA replication	12	26	3.13E-04
KEGG Pathway	Non-homologous end-joining	4	4	6.94E-04
Day7				
GO: biological process	amino sugar metabolic process	18	55	2.27E-07
GO: biological process	chitin-based cuticle development	22	79	2.62E-07

GO: biological process	chitin metabolic process	15	46	2.48E-06
GO: biological process	cuticle chitin catabolic process	3	3	5.82E-04
GO: biological process	regulation of hormone levels	10	43	2.34E-03
GO: biological process	regulation of tube size	10	44	2.81E-03
GO: biological process	regulation of tube architecture, open tracheal system	13	67	3.17E-03
GO: biological process	homophilic cell adhesion	7	25	3.48E-03
GO: biological process	cuticle pattern formation	7	25	3.48E-03
GO: biological process	aminoglycan catabolic process	5	14	4.25E-03
GO: biological process	pyrimidine nucleotide biosynthetic process	4	9	4.33E-03
GO: biological process	glucosamine-containing compound catabolic process	4	9	4.33E-03
GO: biological process	pyrimidine nucleoside monophosphate biosynthetic process	3	5	5.12E-03
GO: biological process	intercellular transport	3	5	5.12E-03
GO: biological process	septate junction assembly	7	28	6.88E-03
GO: biological process	negative regulation of hormone metabolic process	2	2	6.98E-03
GO: biological process	negative regulation of sequestering of calcium ion	2	2	6.98E-03
GO: biological process	detoxification of arsenic-containing substance	2	2	6.98E-03
GO: biological process	organonitrogen compound metabolic process	64	572	7.93E-03
GO: biological process	cellular hormone metabolic process	7	29	8.43E-03
GO: biological process	system process	35	279	9.17E-03
GO: biological process	activation of immune response	3	6	9.60E-03
GO: biological process	'de novo' pyrimidine nucleobase biosynthetic process	3	6	9.60E-03
GO: biological process	pyrimidine nucleoside biosynthetic process	3	6	9.60E-03
GO: molecular function	structural constituent of chitin-based cuticle	6	7	2.32E-06
GO: molecular function	chitin binding	13	39	1.22E-05
GO: molecular function	extracellular matrix structural constituent	7	13	3.64E-05
GO: molecular function	structural constituent of chitin-based larval cuticle	11	32	4.16E-05
GO: molecular function	serine-type endopeptidase activity	17	69	5.16E-05
GO: molecular function	serine hydrolase activity	20	91	6.74E-05
GO: molecular function	iron ion binding	16	67	1.28E-04
GO: molecular function	monosaccharide transmembrane transporter activity	7	17	3.04E-04
GO: molecular function	molybdenum ion binding	3	3	6.34E-04
KEGG Pathway	Drug metabolism - other enzymes	8	29	4.35E-04

* Asterix indicates *P*-value <0.05

Table 3.S2. Gene enrichment analysis table for the seven significant STEM profiles (Bonferroni corrected *P*-value cutoff ≤ 0.0001)

Strain	Profile	GO Category Name	#Genes Enriched	Corrected p-value	Fold
E	1	single-organism developmental process	52.2	1.10E-11	2.7
E	1	macromolecule metabolic process	51.4	2.70E-11	2.7
E	1	regulation of biological process	49.3	2.60E-09	2.4
E	1	regulation of cellular process	46.7	7.30E-09	2.4
E	1	organic substance metabolic process	50.3	2.30E-08	2.2
E	1	endopeptidase activity	16.4	1.20E-07	7.2
E	1	proteolysis	20.8	1.30E-07	5
E	1	anatomical structure development	32.4	2.50E-07	2.9
E	1	homophilic cell adhesion via plasma membrane adhesion molecules	8.5	1.80E-06	19.8
E	1	developmental process involved in reproduction	22.9	2.00E-06	3.8
E	1	anatomical structure morphogenesis	27.9	3.80E-06	3
E	1	proteasome regulatory particle	7.7	4.00E-06	23.3
E	1	plasma membrane	20.5	4.30E-06	4.1
E	1	cellular macromolecule metabolic process	36	6.90E-06	2.3
E	1	single organism reproductive process	22.4	7.00E-06	3.6
E	1	macromolecule catabolic process	15.7	7.70E-06	5.7
E	1	negative regulation of cellular process	25.2	1.00E-05	3.1
E	1	peptidase activity, acting on L-amino acid peptides	16.8	1.10E-05	5
E	1	primary metabolic process	41.4	1.10E-05	2.1
E	1	protein binding	31.1	1.20E-05	2.6
E	1	ubiquitin-dependent protein catabolic process	13	1.20E-05	7.3
E	1	peptidase activity	16.7	1.60E-05	4.9
E	1	modification-dependent macromolecule catabolic process	12.9	2.00E-05	7.1
E	1	modification-dependent protein catabolic process	12.9	2.00E-05	7.1
E	1	proteolysis involved in cellular protein catabolic process	12.9	2.00E-05	7.1
E	1	cellular component organization	33	2.10E-05	2.4
E	1	protein catabolic process	11.5	2.50E-05	8.4
E	1	proteasome-mediated ubiquitin-dependent protein catabolic process	10.7	3.10E-05	9.2
E	1	proteasomal protein catabolic process	10.7	3.60E-05	9.1
E	1	cellular developmental process	29.5	4.60E-05	2.5
E	1	intracellular part	45.3	5.00E-05	1.9
E	1	negative regulation of biological process	24.8	5.90E-05	2.9
E	1	calcium-dependent cell-cell adhesion via plasma membrane cell adhesion molecules	6.7	9.30E-05	21.3
E	2	nucleus	30.9	2.00E-06	2.8
E	2	protein catabolic process	12.4	3.90E-06	8.7
E	2	single-organism developmental process	39.1	5.10E-06	2.2
E	2	modification-dependent macromolecule catabolic process	12.8	3.30E-05	6.8
E	2	modification-dependent protein catabolic process	12.8	3.30E-05	6.8
E	2	proteolysis involved in cellular protein	12.8	3.30E-05	6.8

		catabolic process			
E	2	proteasome-mediated ubiquitin-dependent protein catabolic process	10.7	4.70E-05	8.9
E	2	proteasomal protein catabolic process	10.6	5.40E-05	8.8
E	2	anatomical structure development	27.7	5.90E-05	2.6
E	2	cellular response to DNA damage stimulus	15.2	6.10E-05	5
E	2	macromolecule catabolic process	14.5	8.40E-05	5.2
E	3	oxidoreductase activity	35.9	1.30E-18	6.9
E	3	oxidation-reduction process	27.8	4.30E-13	6.3
E	3	single-organism metabolic process	40.1	1.90E-09	2.9
E	3	integral component of membrane	26.2	2.50E-07	3.7
E	3	organic acid metabolic process	18.8	3.80E-07	5.4
E	3	oxoacid metabolic process	18.8	3.80E-07	5.4
E	3	carboxylic acid metabolic process	18	7.40E-07	5.5
E	3	small molecule metabolic process	22	5.20E-06	3.8
E	3	carbohydrate metabolic process	14.7	4.10E-05	5.4
E	3	organonitrogen compound metabolic process	19.1	6.20E-05	3.8
E	4	oxidoreductase activity	18.2	3.20E-07	5.8
E	4	oxidation-reduction process	16.8	7.80E-07	6.2
E	4	single-organism catabolic process	12.1	4.10E-05	7.4

Table 3.S3. Significance (FDR) values for differentially expressed genes between E and Z strain moths in long-day conditions

Gene Symbol	Transcripts	FDR Day 1	FDR Day 7
Ecdysone			
Usp	comp37290_c0_seq1*; comp10851_c0_seq1; comp108932_c0_seq1	8.26E-03	5.41E-03
Spo	comp30567_c0_seq1	4.63E-07	9.21E-10
Phm	comp24584_c0_seq1	3.76E-06	2.69E-05
Eip75B	comp18210_c0_seq1; comp11010_c0_seq2*	6.48E-03	-
Eip78C	comp122877_c0_seq1; comp151979_c0_seq1	-	-
EcR	comp34369_c0_seq1	1.01E-03	-
Nvd	comp8057_c0_seq2	8.88E-03	2.62E-03
Circadian			
Clk	comp120765_c0_seq1	-	-
Cpo	comp89983_c0_seq1	-	-
Cyc	comp120765_c0_seq1	-	-
Pdp1	comp36140_c0_seq1*; comp15799_c0_seq1*	9.33E-03; 8.35E-04	-
Tim	comp27153_c0_seq1	-	-
Vri	comp23405_c0_seq1	-	-
Per	comp7426_c0_seq1; comp8184_c0_seq1; comp97089_c0_seq1	-	-
Cell Cycle			
Cdk4	comp127995_c0_seq1*; comp233925_c0_seq1*	2.61E-03; 1.22E-04	-
CycB	comp21605_c0_seq1	1.32E-07	5.73E-04
Pcna	comp9566_c0_seq1	2.38E-06	-
Polo	comp21903_c0_seq1*; comp10877_c0_seq1*	2.22E-03; 1.56E-03	-
CycA	comp29198_c0_seq1	-	-
Trbl	comp20015_c0_seq1; comp25406_c0_seq1	-	-
Heat Shock			
Hsp83	comp22537_c0_seq1	5.03E-04	-
Hsp60	comp19138_c0_seq1	8.82E-03	-
Hsp70Bc	comp418731_c0_seq1; comp427912_c0_seq1	-	-
Hsp70Ab	comp9675_c0_seq1	-	-
Canonical Wnt Signaling			
Fz3	comp15297_c0_seq1	3.93E-03	-
Arm	comp17057_c0_seq1	9.38E-03	-
Smo	comp14234_c0_seq1	7.71E-03	-
Wg	comp13981_c0_seq1	1.91E-03	-
Wnt6	comp25466_c0_seq1	-	-
Hs6st	comp78453_c0_seq1	-	-

Gro	comp8218_c0_seq1		-	-
Pan	-		7.04E-03	-
Insulin				
Akt1	comp18543_c0_seq1; comp53008_c0_seq1; comp10465_c0_seq1	comp38567_c0_seq1;	-	-
Foxo	comp19046_c0_seq1		-	-
InR	comp25612_c0_seq1; comp16806_c0_seq1; comp223642_c0_seq1	comp14287_c0_seq1; comp22132_c0_seq1;	-	
Pi3k	comp190582_c0_seq1; comp31462_c0_seq1	comp28365_c0_seq1;	-	-
Pten	comp6369_c0_seq1; comp7648_c0_seq1		-	-
SNF4y	comp26437_c0_seq1		-	3.11E-03
Tor	comp16536_c0_seq1; comp19143_c0_seq1; comp26489_c0_seq1	comp17899_c0_seq1;	-	-

**Some genes had multiple transcripts, asterix denotes which transcript shows differential expression*

Table 3.S4. Short read archive accession numbers

Strain	Days After Long-day Exposure	Sample Name	SRA Accession
E	0	Shortday_E_biologicalrep1	SRX1206058
E	0	Shortday_E_biologicalrep2	SRX1206872
E	0	Shortday_E_biologicalrep3	SRX1211504
E	0	Shortday_E_biologicalrep4	SRX1211546
E	0	Shortday_E_biologicalrep5	SRX1211572
Z	0	Shortday_Z_biologicalrep1	SRX1211612
Z	0	Shortday_Z_biologicalrep2	SRX1211648
Z	0	Shortday_Z_biologicalrep3	SRX1211686
Z	0	Shortday_Z_biologicalrep4	SRX1211701
Z	0	Shortday_Z_biologicalrep5	SRX1211703
E	1	Longday1_E_biologicalrep1	SRX1211704
E	1	Longday1_E_biologicalrep2	SRX1211706
E	1	Longday1_E_biologicalrep3	SRX1211707
E	1	Longday1_E_biologicalrep4	SRX1211711
E	1	Longday1_E_biologicalrep5	SRX1211709
Z	1	Longday1_Z_biologicalrep1	SRX1211714
Z	1	Longday1_Z_biologicalrep2	SRX1211718
Z	1	Longday1_Z_biologicalrep3	SRX1211716
Z	1	Longday1_Z_biologicalrep4	SRX1211720
Z	1	Longday1_Z_biologicalrep5	SRX1211723
E	7	Longday7_E_biologicalrep1	SRX1211725
E	7	Longday7_E_biologicalrep2	SRX1211741
E	7	Longday7_E_biologicalrep3	SRX1211744
E	7	Longday7_E_biologicalrep4	SRX1211746
E	7	Longday7_E_biologicalrep5	SRX1211748
Z	7	Longday7_Z_biologicalrep1	SRX1211750
Z	7	Longday7_Z_biologicalrep2	SRX1211752
Z	7	Longday7_Z_biologicalrep3	SRX1211782
Z	7	Longday7_Z_biologicalrep4	SRX1211784
Z	7	Longday7_Z_biologicalrep5	SRX1211786

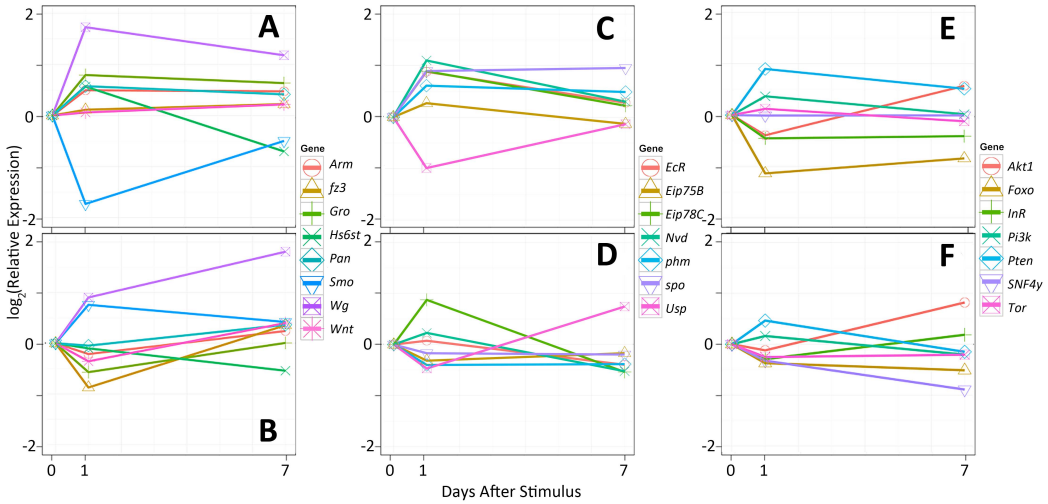

Figure 3.S1. Gene expression trajectories from short-day to long-day conditions for (A) E-strain Wnt signaling genes, (B) Z-strain Wnt signaling genes, (C) E-strain ecdysone-related genes, (D) Z-strain ecdysone-related genes, (E) E-strain insulin signaling genes, and (F) Z-strain insulin signaling genes.

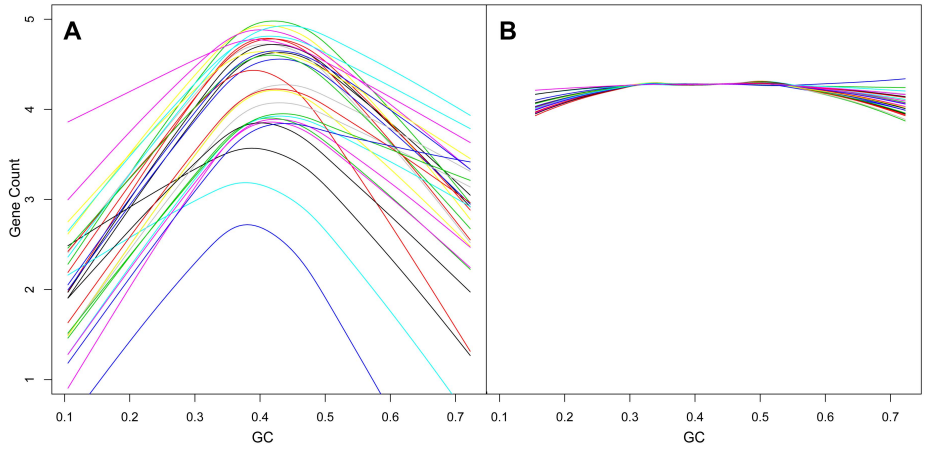

Figure 3.S2. GC normalization in EDAseq. (A) Lowess regression of log gene-level counts on GC content for each library. (B) Full quantile within and between lane normalization for each library.

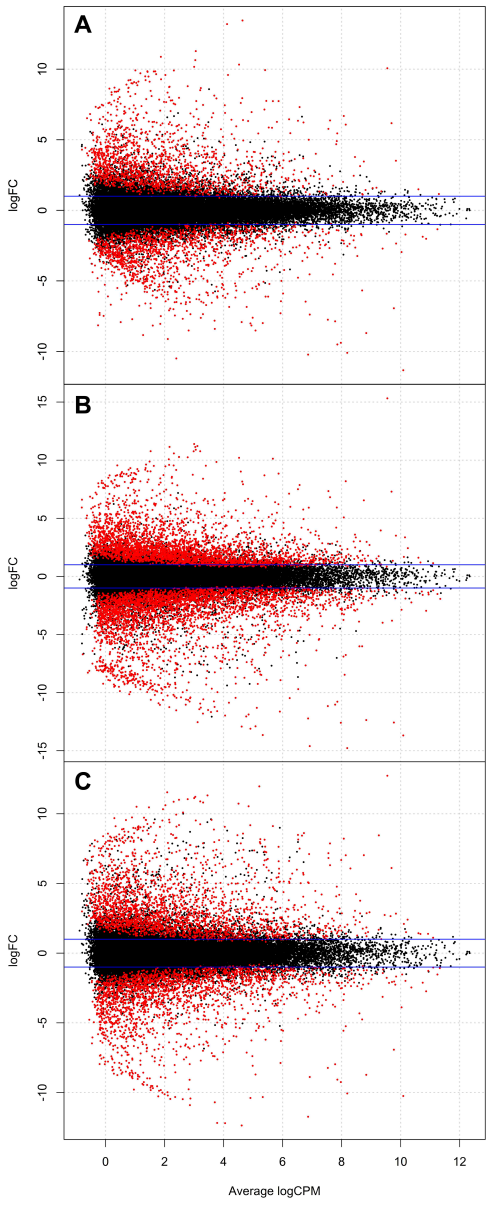

Figure 3.S3. MA plot of differential expression within strain shown as log-fold change of expression versus average log counts per million. (A) E-strain diapause maintenance to day 1, (B) E-strain diapause maintenance to day 7, (C) Z-strain diapause maintenance to day 1, (D) Z-strain diapause maintenance to day 7. Red dots are significantly differentially expressed at FDR < 0.01.

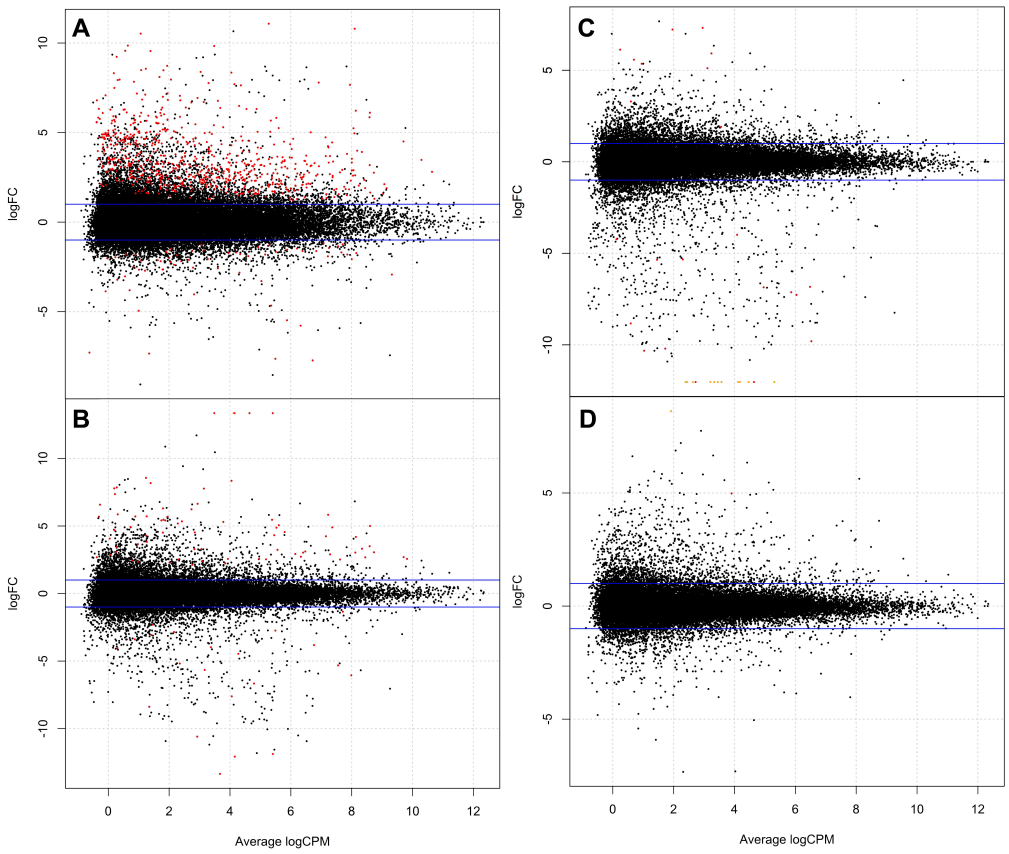

Figure 3.S4. MA plot of differential expression between strains (A) in diapause maintenance, (B) on day 1, and (C) on day 7, shown as log-fold change of expression versus average log counts per million. Red dots are significantly differentially expressed at FDR < 0.01.

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CHAPTER 4

A RECOMBINATION SUPPRESSOR CONTRIBUTES TO ECOLOGICAL SPECIATION IN *OSTRINA* MOTHS*

ABSTRACT

Despite unparalleled access to species' genomes in our post-genomic age, we often lack adequate biological explanations for a major hallmark of the speciation process—genetic divergence. In the presence of gene flow, chromosomal rearrangements such as inversions are thought to promote divergence and facilitate speciation by suppressing recombination. Using a combination of genetic crosses, phenotyping of a trait underlying ecological isolation, and population genetic analysis of wild populations, we set out to determine whether evidence supports a role for recombination suppressors during speciation between the Z and E strains of European corn borer moth (*Ostrinia nubilalis*). Our results are consistent with the presence of an inversion that has contributed to accumulation of ecologically adaptive alleles and genetic differentiation across roughly 20% of the *Ostrinia* sex chromosome (~4 Mb). Patterns in *Ostrinia* suggest that chromosomal divergence may involve two separate phases—one driving its transient origin through local adaptation and one determining its stable persistence through differential introgression. As the evolutionary rate of rearrangements in lepidopteran genomes appears to be one of the fastest among eukaryotes, structural mutations may have had a

* Wadsworth, CB, Li, X, & Dopman, EB (2015). A recombination suppressor contributes to ecological speciation in *Ostrinia* moths. *Heredity*, **114(6)**: 593-600.

disproportionate role during adaptive divergence and speciation in *Ostrinia* and in other moths and butterflies.

INTRODUCTION

A key feature of the speciation process is the evolving structure of genetic differentiation between populations that culminates in genetic divergence across much of the genomes of daughter taxa. Indeed, genomic patterns of evolutionary divergence across the speciation continuum promise to reveal not only the functional nature of adaptive evolution and reproductive isolation (Storz, 2005) but also the processes underlying speciation (Pinho and Hey, 2010). Consequently, a major research theme has emerged that aims to characterize genome-wide patterns of differentiation between diverging populations, races and species. Pockets of divergence or 'genomic islands of speciation,' as well as larger 'continents,' have been uncovered and could reflect the effect of genes contributing to reproductive isolation ('barrier loci') (Dopman et al., 2005; Turner et al., 2005; Feder, Egan et al., 2012). However, connecting these fundamental genetic signatures with their biological cause remains elusive (Noor and Bennett, 2009; Dopman, 2011; Nachman and Payseur, 2012; Cruickshank and Hahn, 2014).

Chromosomal rearrangements such as inversions are thought to facilitate speciation and promote the origin of evolutionary divergence by suppressing recombination. When speciation occurs in the face of gene flow, suppressed recombination in a heterokaryotypic hybrid offspring may cause the entire inverted segment to behave as a single linked unit (Noor et al., 2001; Rieseberg,

2001). Consequently, only a small number of barrier loci might be required to protect a large chromosomal band, possibly several megabases in length, from gene flow. Because of restricted gene flow, barrier and locally adaptive alleles are thought to accumulate within inversions leading to increasing divergence until speciation is complete (Navarro and Barton, 2003). Finally, adaptive alleles that are captured by inversions may rapidly drive their fixation, creating long-range hitch-hiking across the nonrecombining rearranged interval (Kirkpatrick and Barton, 2006; Kirkpatrick, 2010; Feder et al., 2011). In the absence of structural mutations, reduced recombination in collinear regions could also lead to evolutionary divergence; however, the footprint of genetic differentiation and opportunity for the accumulation of barrier loci might be limited (Butlin, 2005; West and Via, 2008; Feder et al., 2012). Thus, changes in recombination rate because of structural mutations might be the key to speciation with gene flow, and they may therefore help to explain many instances of genomic islands of speciation.

In this study, we evaluate the role of suppressed recombination for the evolution of a modestly sized genomic island of speciation between a pair of moth lineages. The 'Z' and 'E' strains of European corn borer (ECB, *Ostrinia nubilalis*) are incipient species that colonized North America ~100 ya without an appreciable bottleneck from allopatric locations in Italy (mixture of E and Z strains) and Hungary (exclusively Z-strain; Smith, 1920; Caffrey, 1927; Klun and Cooperators, 1975; Dopman, 2011). Strains are recently diverged (~100,000 ya; Malausa et al., 2007) and are textbook examples of speciation (Coyne and Orr,

2004), in which one species is splitting into two through the evolution of many forms of reproductive isolation (Dopman et al., 2010). The sex (Z) chromosome harbors several known genetic factors for adaptation and/or barrier traits, including those underlying behavioral isolation (Roelofs et al., 1987; Dopman et al., 2004) and temporal isolation (Glover et al., 1992; Dopman et al., 2005). Current and historical gene exchange between ECB strains is likely, as indicated by hybridization in Europe and North America (Dopman et al., 2010; Coates et al., 2013), geographic variation in strength of reproductive isolation (ranging from 0.91 to 0.99; Dopman et al., 2010) and molecular evidence for an isolation-with-migration model of divergence (2 $Nm \sim 10$; Malausa et al., 2007). Whereas most loci show extensive shared polymorphism (for example, $F_{ST} < 0.05$), a possible island of speciation along an ~ 1 cM swath of the Z chromosome consists of four genes (*Tpi* and three olfactory receptors, *OR*s) and reveals ECB strains as nearly reciprocally monophyletic (for example, $F_{ST} > 0.7$; Dopman et al., 2005; Dopman, 2011; Lassance et al., 2011). Evolutionary divergence can be explained by several tightly linked barrier loci on the sex chromosome or by a regional selective sweep at these or other loci. However, such interpretations must be viewed as incomplete without proper tests of suppressed recombination and its repercussions for the genomic zone of influence for adaptation and/or barrier loci. Indeed, a possible signature of an inversion between Z and E strains was detected on the Z chromosome (Dopman et al., 2004), in which the lowest estimated recombination rate across the entire 1697-cM genetic linkage map was found in the vicinity of the *Tpi*/*OR*s loci (Fig. 4.1).

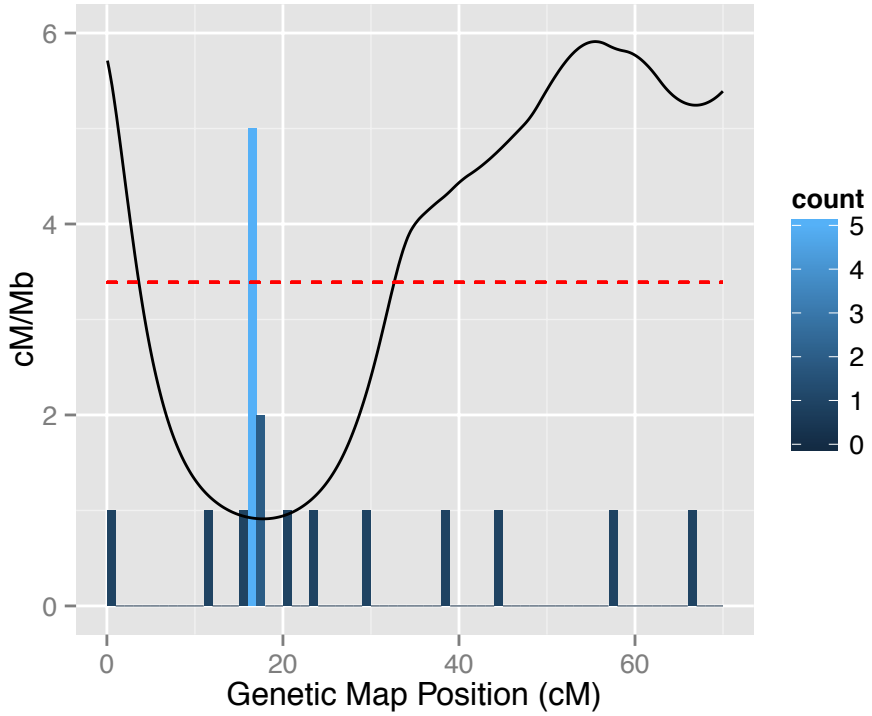

Figure 4.1. Local recombination rates (cM/Mb, black line) and marker densities (count) on the ECB sex (Z) chromosome. The dashed red line indicates the genome-wide average. Genetically differentiated loci *Tpi* & *Ors* ($F_{ST} > 0.7$) are located in the low-recombination region (~17 cM). Adapted from Dopman et al. 2004.

If inversions are important during speciation and the evolution of genetic differentiation, at least three testable predictions have been made (Faria and Navarro, 2010). First, patterns of gene flow should be higher within collinear versus rearranged regions (Rieseberg, 2001; Feder et al., 2005; Kulathinal et al., 2009; McGaugh and Noor, 2012). Second, traits involved in reproductive isolation should map to regions located within chromosomal rearrangements (Noor et al., 2001; Feder et al., 2005; Lowry and Willis, 2010). Finally, genotyping hybrid offspring in the laboratory ought to reveal evidence for suppressed recombination (Feder et al., 2003; Kulathinal et al., 2009). By using a combination of genetic crosses, phenotyping of a trait contributing to reproductive isolation, and population genetic analysis, we set out to test these predictions for the ECB sex chromosome.

MATERIALS AND METHODS

Recombination suppression: To estimate variation in recombination frequencies surrounding the genetically differentiated region of the ECB sex chromosome, we generated three-point test crosses within and between Z and E strains. Crosses used insects collected as caterpillars, pupae and adults from the New York State, USA (*n* ~500 males and *n* ~500 females each from East Aurora, Geneva and Bouckville). Laboratory populations were maintained by mass rearing ~100 males and ~100 females each generation. The female sex-pheromone blend is strain-specific. Z-strain females produce a 3:97 ratio of E/Z11-14:OAc, whereas E-strain females use a 99:1 ratio. Strain identities of all parental stocks were confirmed by genotyping *pgFAR*, the autosomal locus that

determines the female sex-pheromone blend (Lassance et al., 2010).

Recombination is restricted to males in many Lepidoptera (Dopman et al., 2004). Therefore, in backcross pedigrees only F1 males are informative. Here, between-strain backcrosses (referred to as 'Z × E') involved a Z grandmother, an E grandfather, an F1 male and a recurrent Z-strain mother. Within-strain crosses (referred to as 'Z × Z') had a similar design but were exclusively from the Z-strain.

The ECB diverged from *Bombyx mori* (the silk moth) ~100 Mya (Pringle et al., 2007); however, the species pair shows a high degree of conservation in gene order like many other Lepidoptera (Pringle et al., 2007; d'Alencon et al., 2010; Kroemer et al., 2011). Therefore, we relied on the *B. mori* genome to identify evenly spaced sex-linked mapping loci for the ECB, whose genome is incomplete. Markers consisted of *Tpi* at 9 Mb, *Per* at 13 Mb and *Ldh* at 17.4 Mb (International Silkworm Genome Consortium, 2008). We adopted methods developed elsewhere for *Tpi* and *Ldh* (Dopman et al., 2005), whereas initial Per sequence for the ECB was obtained using degenerate PCR.

DNA from mapping family individuals was extracted from adult legs following using a DNeasy Blood and Tissue kit (QIAGEN, Valencia, CA, USA). Genotyping assays for offspring were developed from segregating polymorphisms and consisted of diagnostic PCR products that amplified polymorphic indels and/or restriction enzyme cut sites (*EcoR1*, *Alu1* and *Bsr1* for *Tpi*, *Per* and *Ldh*, respectively). Only female offspring were genotyped and, because female Lepidoptera are the heterogametic sex and possess only one Z chromosome, one

allele per locus was detected. Genotyping results were confirmed by Sanger sequencing (*n* ~100 sequenced alleles per marker). One Z × E cross had already been genotyped for *Tpi* and *Ldh* in a prior study (Dopman et al., 2005).

Colocalization of reproductive isolation: As prior results (Fig. 4.1) and preliminary data suggested evidence for reduced recombination near *Tpi* in F1 hybrids, we tested for co-localization of traits involved in reproductive isolation. A subset of Z × E backcross offspring was mapped for a major sex-linked factor (named *Pdd*) controlling diapause emergence time (Glover et al., 1992; Dopman et al., 2005), defined as the time to pupation for over-wintering caterpillars under environmental conditions conducive to breaking diapause. Differences at *Pdd* contribute to temporal isolation between Z and E strains in North America by conferring an ~30-day shift in emergence and thus adult-mating flights (Wadsworth et al., 2013). Shifts in adult flights between univoltine Z and bivoltine E populations eliminate as much as 85% gene flow in nature (Dopman et al., 2010).

We measured diapause timing variation by inducing and then breaking diapause in recombinant ECB caterpillars. Following earlier studies (Glover et al., 1992; Dopman et al., 2005), diapause was induced by a 12:12 light:dark photoperiod and then broken 35 days later with a 16:8 light:dark cycle. Diapause emergence time was noted every 2 days. As females are hemizygous and *Pdd* is inherited as a Mendelian locus, backcross females show patterns of emergence consistent with either their grandfather (here, E-strain) or grandmother (here, Z-strain; Glover et al., 1992; Dopman et al., 2005). Hence, recombinant backcross

females show E-like earlier emergence times and pupate in less than 22 days (range = 8–22 days, mean = 15.5 days, s.e. ± 0.55), or they show Z-like later emergence times and pupate in more than 18 days (range = 18–80, mean = 43.94 days, s.e. ± 1.64; Glover et al., 1992).

Higher introgression in collinear regions: We evaluated patterns of genetic variation among field-caught female ECB: 18 insects were E-strain, 18 were Z-strain and at least one insect was from an outgroup species, the Asian corn borer (ACB, *O. furnacalis*). ECB caterpillars, pupae or adults were collected over a large geographic range, and thus genetic similarities should be viewed as indicating a combination of ongoing or recent gene exchange. Samples included insects from USA (n = 7 E-strain and n = 5 Z-strain from New York; n = 7 E-strain from North Carolina and n = 4 Z-strain from North Carolina; n = 4 Z-strain from Iowa), Italy (n = 4 E-strain and n = 3 Z-strain) and Hungary (n = 2 Z-strain). Moths were classified into strain by diagnostic gas chromatographic profiles of female sex-pheromone blend (that is, 99:1 versus 3:97 ratio of E/Z11-14:OAc; for example, Dopman et al., 2004) and/or by genotyping the *pgFAR* locus.

Molecular markers were developed from genes that were evenly distributed across the *B. mori* sex chromosome. Using ECB transcripts that were developed from a separate study, reciprocal BLASTs were performed to obtain ECB–*B. mori* gene pairs with predicted locations on the ECB sex chromosome (*B. mori* nucleotide CDS, BLASTn, *e*-value 1e – 40). ECB transcripts were then searched with tBLASTx against the matching *B. mori* genomic sequence to identify predicted introns. Primer pairs for 23 loci that had inter-locus intervals

ranging from 0.1 to 2.5Mb (mean = 0.96Mb, s.e. ± 0.16) were designed to amplify ECB introns using PrimerBlast (NCBI). Sequences from field-caught insects were aligned and edited by eye using Genious Pro 5.5 (Biomatters Inc., San Francisco, CA, USA). Markers were named based on *Bombyx* gene identifiers.

Measures of genetic variation (π, θ_W), genetic differentiation (d_a, d_{xy}, F_{ST} (Hudson et al., 1992), S_{nn} (Hudson, 2000)) and the allele-frequency spectrum (Tajima's *D* (Tajima, 1989b), Fay and Wu's *H* (Fay and Wu, 2000)) were calculated using DnaSP (Rozas et al., 2003). The genealogical sorting index (*gsi*; Cummings et al., 2008) was calculated to quantify the degree of exclusive ancestry of Z- and E-strain moths. *gsi* ranges from 0 (no exclusivity) to 1 (monophyletic), and was calculated in R (Team RDC, 2013) on the Tufts high-performance computing research cluster using the genealogicalSorting library (www.genealogicalsorting.org). Genealogies were constructed using neighbor-joining with a Tamura-Nei 93 model of evolution to calculate distances. Statistical significance of *gsi* was assessed through permutation tests (n = 10,000) on each of 1,000 bootstrap replicates for a total of 10,000,000 replicated genealogies. Indel polymorphisms were considered a fifth base when calculating genetic differentiation and genetic exclusivity. All figures were constructed using the ggplot2 library (Wickham, 2009) in R.

RESULTS

Recombination suppression: We used three-point crosses within- and between-strain to test for evidence of suppressed recombination associated with a high-frequency chromosomal rearrangement between Z and E strains of ECB. No

significant differences in recombination frequency were observed among different within-strain backcross families (Z × Z, *n* = 2 families) or among between-strain backcross families (Z × E, *n* = 7 families) and therefore families were combined for each backcross type. Within strain, we observed six recombinants between the *Tpi* and *Per* pair (7%, *n* = 86 offsprings), six between the *Per* and *Ldh* pair (7%, *n* = 86 offsprings) and twelve between *Tpi* and *Ldh* (14%, *n* = 86 offsprings; Fig. 4.2a). Hence, in agreement with that found in *B. mori*, the predicted gene order within the Z-strain of ECB is *Tpi— Per—Ldh* (Fig. 4.2b).

Between-strain crosses revealed heterogeneity in recombination frequency compared with that seen within-strain (Fig. 4.2a). Z x E crosses yielded slightly elevated recombination frequencies for the *Per– Ldh* gene pair (seven recombinants, 9.1%, *n* = 77 offsprings; Fisher's exact test, *P*-value = 0.7739) and slightly reduced recombination between *Tpi* and *Ldh* (seven recombinants, 9.1%, *n* = 77 offsprings; Fisher's exact test, *P*-value = 0.4643). However, a statistically significant suppression in recombination was observed between *Tpi* and *Per*. Zero recombinants were found in over 600 offsprings (*n* = 666 offsprings; Fisher's exact test, *P*-value = 1.91e – 06).

Colocalization of reproductive isolation: We phenotyped a subset of backcross offspring to determine the linkage relationship between the nonrecombining *Tpi–Per* gene pair and *Pdd*. Diapause emergence timing of 333 hemizygous females from backcross families ranged from 2 to 100 days; yet, a clear bimodal distribution emerged (Fig. 4.3). Eight offspring (2.4%) had emergence times that fell within the period of overlap of parental Z and E strains

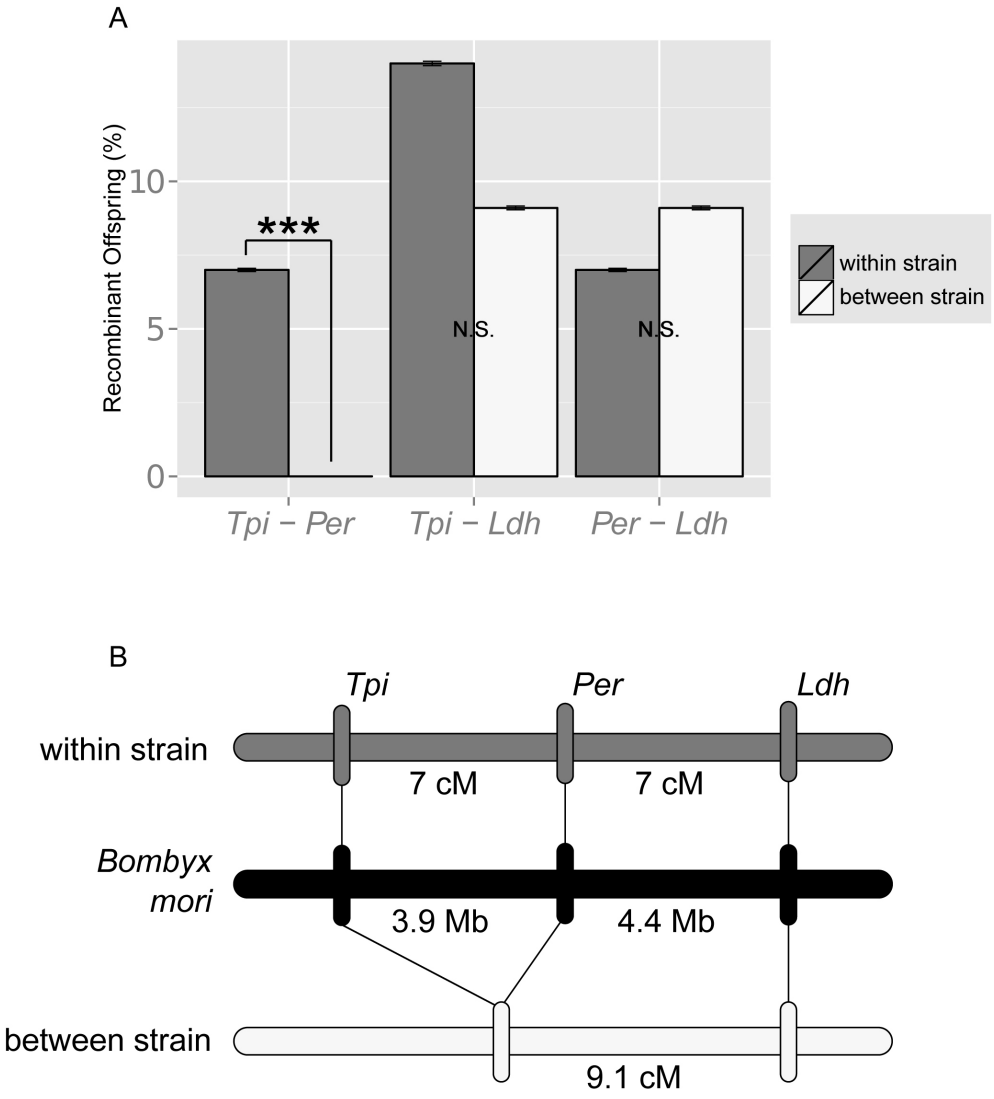

Figure 4.2. Suppressed recombination on the sex (Z) chromosome in ECB hybrids. A. Percent recombinant offspring between *Tpi*, *Per*, and *Ldh* in within-strain (Z x Z) and between-strain (Z x E) crosses. B. Inferred linear order of markers in Bombyx mori and the two ECB crosses. *** *P* value < 0.001.

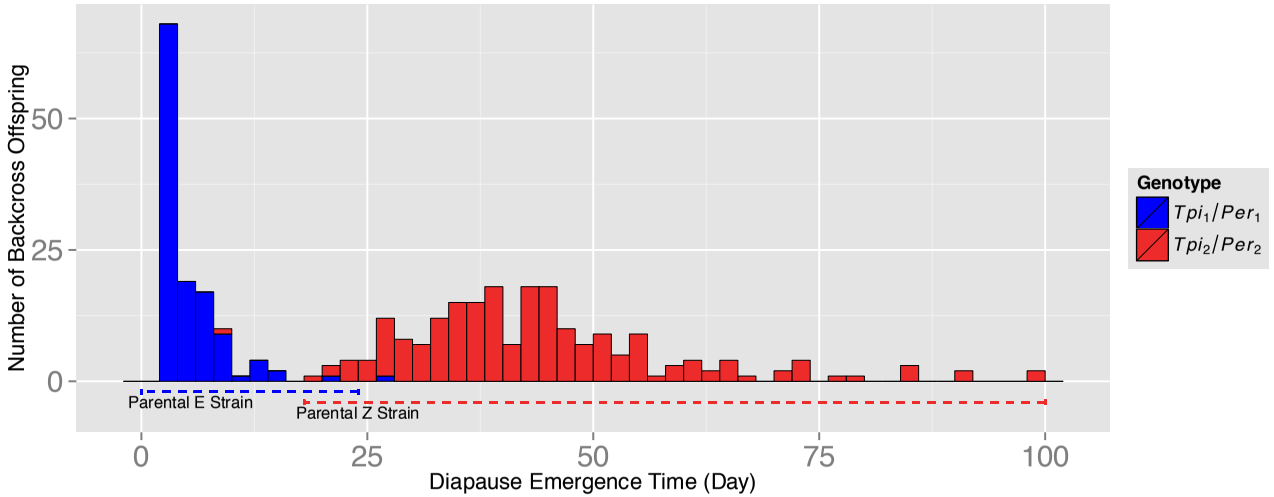

Figure 4.3. Diapause emergence time maps to sex (Z) linked chromosomal rearrangement. Bimodal distribution of diapause emergence time for female backcross offspring genotyped for *Tpi* and *Per*. Dashed lines indicate parental distribution values.

(day 18–22) and their *Pdd* genotype was considered indeterminate. The remaining 325 offspring had emergence times consistent with pure-strain insects. Of these, one female with E-strain markers at the *Tpi–Per* gene pair displayed Z-strain like diapause and emerged later, and one female with Z-strain markers displayed E-strain like diapause and emerged earlier. Thus, two females (0.62%) were apparent recombinants between the *Tpi–Per* gene pair and the *Pdd* locus. In the other 323 offspring (99.38%), *Pdd* was perfectly associated with their genotype at the *Tpi–Per* gene pair.

Higher introgression in collinear regions: We characterized genetic variation for 23 loci to test for differences in patterns of genetic variation between loci with predicted locations inside (referred to as 'rearranged') and outside (referred to as 'collinear') of the nonrecombining *Tpi–Per* chromosomal region. A total of 779 sequences were obtained, resulting in ~9 kb of aligned data and 224 segregating sites. Seven loci fell within the *Tpi–Per* interval and 16 were outside (8 upstream and 8 downstream).

We tested for variation in patterns of genetic differentiation using four different indices. Rearranged loci exhibited statistically significantly elevated evolutionary divergence for both F_{ST} (median rearranged = 0.14990, median collinear = 0.06418, W = 88, P-value = 0.01764) and S_{nn} (median rearranged = 0.6569, median collinear = 0.5742, W = 90, P-value = 0.01258; Fig. 4.4). Similarly, the number of net substitutions per site between strains (d_a, median rearranged= 0.09%, median collinear = 0.029%, W = 74, P-value = 0.121) and the average number of substitutions per site between strains (d_{xy}, median rearranged =

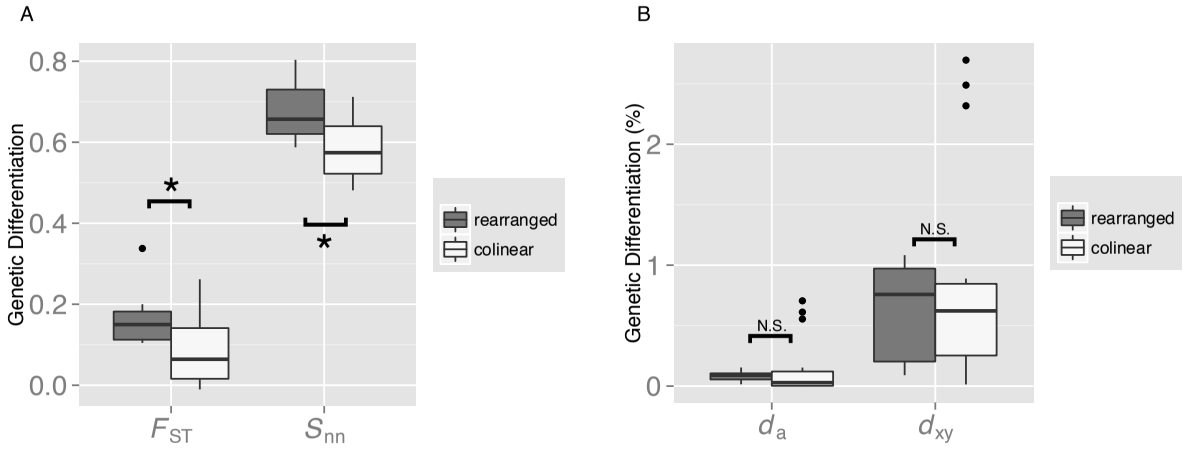

Figure 4.4. Genetic differentiation between ECB strains for collinear and rearranged loci. (A) F_{ST} and S_{nn}. (B) d_a and d_{xy}. * P value < 0.05.

0.76%, median collinear = 0.66%, W = 54, P-value = 0.5647) were both higher for rearranged loci (Fig. 4.4), although these differences were not statistically significant. We also used *gsi* to explore gene tree topology measures of genetic differentiation (Table 4.1). Loci with significant values of *gsi* ranged from 0.01 to 0.68 with an average of 0.22. E-strain ECB had seven loci with significant genealogical exclusivity, whereas the Z-strain had 15. There were no significant differences in the magnitude of *gsi* for rearranged and collinear loci (t = – 1.3504, degrees of freedom = 19.019, P-value = 0.1927); however, all rearranged loci possessed significant genealogical exclusivity in either one or both strains, in contrast to ~50% of collinear loci (Fisher's exact test, P-value = 0.0574).

To evaluate patterns of nucleotide polymorphism, we compared π and θ_W between groups. Average genetic variation for rearranged loci was approximately one-half of that for collinear loci (Table 4.2), although only statistically significant (or trending) for E-strain moths (mean rearranged π = 0.386%, mean collinear π = 0.812%, t = – 1.7158, degrees of freedom = 16.839, P-value = 0.05227; mean θ_W = 0.347%, mean collinear θ_W = 0.916%, t = – 2.3966, degrees of freedom = 15.098, P-value = 0.01496). For both strains Tajima's D showed a slight skew toward an excess of intermediate-frequency alleles compared with low-frequency alleles in rearranged loci (that is, more positive values; Table 4.2), whereas Fay and Wu's H revealed a relative excess of high-frequency derived alleles compared with intermediate-frequency alleles (that is, more negative values; Table 4.2). However, neither estimate was statistically significant.

Table 4.1. Genetic exclusivity between ECB strains for collinear and rearranged loci

Location	Locus	gsi_E	P value[a]	gsi_Z	P value[a]
rearranged	*Tpi*	0.14	N.S.	0.26	***
rearranged	*Per*	0.11	N.S.	0.21	*
rearranged	680	0.28	**	0.35	***
rearranged	524	0.18	N.S.	0.35	***
rearranged	710	0.17	N.S.	0.26	**
rearranged	1368	0.19	N.S.	0.29	**
rearranged	683	0.18	*	0.12	N.S.
colinear	2071	0.23	N.S.	0.23	N.S.
colinear	2076	0.13	N.S.	0.17	N.S.
colinear	2104	0.10	N.S.	0.12	N.S.
colinear	2005	0.17	N.S.	0.28	*
colinear	622	0.14	N.S.	0.20	N.S.
colinear	643	0.26	***	0.23	***
colinear	647	0.33	**	0.32	**
colinear	563	0.02	N.S.	0.50	***
colinear	9691	0.68	***	0.52	***
colinear	13318	0.01	N.S.	0.23	**
colinear	13333	0.20	N.S.	0.22	N.S.
colinear	8010	0.28	***	0.33	***
colinear	12292	0.08	N.S.	0.04	N.S.
colinear	13832	0.19	**	0.28	***
colinear	587	0.12	N.S.	0.20	**
colinear	2781	0.07	N.S.	0.12	N.S.

[a] Significance determined by permutation (n=10,000) of bootstrap replicates (n=1,000). *** P value < 0.001. ** P value < 0.01. * P value < 0.05. $^{N.S.}$ Not significant.

Table 4.2. Patterns of genetic variation among rearranged and collinear loci

		Loci	Length[a]	S[b]	$\pi^c(\%)$	$\theta^d(\%)$	D^e	H^f
E Strain	rearranged	7	3541	47	0.386	0.347	0.214	-0.229
	colinear	16	5172	107	0.812	0.916	-0.492	-0.178
	P-value				0.052	0.015	0.888	0.473
Z Strain	rearranged	7	3549	60	0.486	0.514	-0.165	-3.266
	colinear	16	5172	106	0.812	0.849	-0.379	-0.883
	P-value				0.127	0.102	0.646	0.131

[a] Base pairs.
[b] Segregating sites.
[c] Heterozygosity from average number of differences per site.
[d] Heterozygosity from number of polymorphic sites.
[e] Tajima (1989).
[f] Fay and Wu (2000).

DISCUSSION

Although our post-genomic age has produced billions of base pairs of DNA sequence data for many organisms across the speciation continuum, we often lack adequate biological explanations for the evolution of fundamental genomic features such as genetic divergence. Inversions are thought to have far-reaching impacts on evolutionary divergence during speciation, especially when divergence occurs in the face of gene flow. However, demonstrating the importance of inversions to the speciation process requires evidence for (i) recombination suppression in hybrid offspring, (ii) localization of barrier or adaptation loci in rearranged regions and (iii) enhanced sequence divergence (Faria and Navarro, 2010). In this study, we combined genetic crosses (Fig. 4.2), phenotyping of traits underlying reproductive isolation (Fig. 4.3) and observational studies of DNA polymorphism in wild populations (Fig. 4.4, Table 4.1) to show that Z and E strains of ECB meet all three criteria. Although we cannot physically confirm the presence of an inversion yet through cytology, these results are consistent with the presence of an inversion that has contributed to accumulation of ecologically adaptive alleles and genetic differentiation across roughly 20% of the ECB sex chromosome (~4 Mb).

Inversions and reproductive isolation: A major appeal of chromosomal rearrangement models of speciation is their ability to help explain apparent examples of speciation with gene flow (Pinho and Hey, 2010). Indeed, a serious theoretical weakness of non-allopatric speciation is that recombination randomizes associations between genes conferring local adaptation (hybrid

unfitness) and assortative mating (Felsenstein, 1981; Gavrilets, 2003; Butlin, 2005). Although the specifics of chromosomal rearrangement models of speciation differ (Noor et al., 2001; Rieseberg, 2001; Navarro and Barton, 2003; Kirkpatrick and Barton, 2006; Faria and Navarro, 2010), a common feature is the removal of this antagonism and a buildup of adaptation and barrier loci within rearrangements during a non-allopatric phase(s) that then allows for reinforcement and the completion of speciation. Thus, a critical prediction for speciation with gene flow is that rearrangements such as inversions should be present and contain multiple prezygotic and/or postzygotic barrier loci with major effects on reproductive isolation. Determining the degree to which these expectations are borne out in nature is challenging. First, we have limited data on quantitative measures of reproductive isolation, defined as $RI = 1 -$ (between species fitness) ÷ (within species fitness) and ranging from 0 (no effect) to 1 (complete *RI*; Ramsey et al., 2003; Coyne and Orr, 2004). Second, the genomic positions of the underlying barrier loci is often unknown. Monkeyflowers are a rare exception, in which loci of large reproductive isolation effect that contribute to floral traits, flowering time and hybrid male sterility map to chromosomal rearrangements in the *Mimulus* genome (Ramsey et al., 2003; Lowry and Willis, 2010; Fishman et al., 2013).

The putative sex-linked inversion we document in *Ostrinia* appears consistent with the large-effect, multicomponent prediction of chromosomal rearrangement models of speciation. First, multiple 'speciation phenotypes' (Shaw and Mullen, 2011) in ECB have known sex-linked factors, including male

pheromone response and diapause timing (McLeod, 1978; Dopman et al., 2004; 2005; Wanner et al., 2010; Ikten et al., 2011). Second, a recent study indicated that large isolation effect sizes are common between ECB strains ($RI \geq 0.5$ for 4 of 7 forms of isolation; Dopman et al., 2010). Given this prior work and the large size of the putative inversion (7 cM or ~4 Mb; Fig. 4.2b), there would appear to be good reason to expect diverse and potent barrier loci in an inversion on the ECB sex chromosome. Indeed, we found that only two of 325 offspring were recombinants (0.62%) between the nonrecombining region (*Tpi–Per* loci, Fig. 4.2b) and the factor(s) determining diapause emergence time (*Pdd*; Fig. 4.3). Thus, a major cause of temporal isolation (mean $RI = 0.65$) is likely inside or near the chromosomal rearrangement. Similarly, the sex-linked *ORs* are known to underlie strain-specific male antennal response to Z versus E-strain female pheromones (that is, 3:97 versus 99:1 ratio of E/Z11-14:OAc) and they are tightly linked to *Tpi* and thus the sex-linked rearrangement (Wanner et al., 2010; Lassance et al., 2011). Hence, a rearrangement may have promoted coupling between multiple genes conferring ecological (*Pdd*) and behavioral (*OR*s) divergence, thereby facilitating speciation. Clearly, there is a need for formal experimentation to determine whether chromosomal rearrangements in *Ostrinia* and in other systems harbor multiple loci of large isolation effect that contribute to varied components of reproductive isolation.

Evolution of genetic divergence: Results from several systems including *Ostrinia* and *Drosophila* (McGaugh and Noor, 2012) imply high genetic divergence within inversions and minimal divergence outside of them, and a

reciprocal relationship for polymorphism. Such patterns may be common in the early stages of speciation because of the coupling of selection and recombination. Globally beneficial mutations and adaptive barrier alleles that arise within fixed inversions are expected to get trapped and cannot easily migrate between lineages (Navarro and Barton, 2003). Further, according to the Kirkpatrick–Barton model (Kirkpatrick and Barton, 2006), selective establishment and fixation of inversions occurs because locally adaptive alleles at loci within inversions (for example, underlying ecological and behavioral divergence) cannot recombine with chromosomes from other populations and thus they are favored because they do not suffer the 'Achilles' heel' of being located on the same chromosome as immigrant disadvantageous allele(s). Hence, genetic hitch-hiking associated with either selective fixation of inversions or their internal mutations might lead to inversion-specific reductions in polymorphism within one or both lineages, while also creating enhanced differentiation for relative divergence measures that are sensitive to within lineage variation (for example, F_{ST}; Charlesworth, 1998).

An additional role of suppressed recombination for evolutionary divergence involves repeated bouts of gene exchange and disruptive selection. Specifically, barrier loci within inversions under the Navarro–Barton model (Navarro and Barton, 2003) might produce a molecular signal of differentiation that is spread across the inversion or at least near its breakpoints (Rieseberg, 2001; Noor et al., 2001; Strasburg et al., 2009; McGaugh and Noor, 2012) because of selective purging of maladapted (and nonrecombining) regions encompassed by inversions upon their introduction into sister taxa. Such a process

might be analogous to 'differential introgression,' in which gene flow is restricted at individual barrier loci but is high elsewhere (Barton and Bengtsson, 1986; Wu, 2001; West and Via, 2008; Harrison, 2012). Over the long term, differential introgression is expected to produce specific signatures. For example, increases in absolute genetic divergence (for example, d_{xy}) as new mutations arise and fix independently in daughter taxa (Charlesworth et al., 1997; Charlesworth, 1998; Nachman and Payseur, 2012; Cruickshank and Hahn, 2014).

Despite limitations of our current sampling scheme (18 samples per strain from both allopatric and sympatric localities), results from *Ostrinia* suggest that selection and suppressed recombination were important for evolutionary divergence. Compared with 'collinear' loci, patterns of genetic variation at 'rearranged' loci are consistent with a selective sweep(s), including 50% lower genetic variation (primarily in E-strain moths; Table 4.2) and 2–3 × greater differentiation for measures that are sensitive to selection at linked sites (F_{ST} and d_a; Fig. 4.4). Support for differential introgression is less obvious. The putative inversion shows modest increases in absolute divergence (1.15 × greater values of d_{xy}) and also genealogical exclusivity (1.14 × greater values of S_{nn}). Moreover, although patterns in the allele frequency spectrum (*D* and *H*) are consistent with rare migration within the inversion and higher migration elsewhere (Table 4.2; Tajima, 1989a; Simonsen et al., 1995; Przeworski, 2002; Fay and Wu, 2005), these changes are relatively minor. Lack of genetic evidence for differential introgression runs counter to observations in nature. An appreciable number of hybrid offsprings occur at sampled sympatric localities in New York (5–15%;

Dopman et al., 2010; Coates et al., 2013) and yet differentiation between Z and E strains at *Tpi* has been maintained at these sites for more than 40 generations (Glover et al., 1991; Dopman et al., 2005; Dopman, 2011). Disruptive selection following introgression of the nonrecombining region that includes *Tpi* might be important in maintaining divergence at New York localities, where sweeps and incomplete lineage sorting alone cannot easily account for patterns of nucleotide variation across loci. Equivocal genetic support for differential introgression should not be surprising because statistical power is limited during early stages of speciation (for example, for d_{xy}; Cruickshank and Hahn, 2014). Hence, lineages that are in the early stages of divergence like ECB strains may be too closely related to show strong statistical signs of differential gene flow even at genome regions where it occurs.

More extensive investigation of *Ostrinia* moths and other systems might validate the scenario we propose in which evolutionary divergence during speciation occurs by two complementary but distinct mechanisms—one driving the transient origin of differentiation through local adaptation and selection on linked sites, and one determining its stable persistence through reproductive isolation and/ or differential introgression (see also Dopman, 2011; Nachman and Payseur, 2012; Cruickshank and Hahn, 2014). If an inversion is present as a low-frequency polymorphism in allopatry and greatly differentiates lineages only in geographic regions where they co-occur (for example, as in the 'mixed-geographic model' (Feder et al., 2011)), this might suggest a direct role of recombination suppression in promoting evolutionary divergence by local

adaptation and selective sweeps (for example, to eliminate the creation of less fit hybrids or to keep high fitness loci together). In contrast, the presence of an older, high-frequency inversion that differentiates lineages regardless of geography could indicate an indirect role of recombination suppression in facilitating accumulation of additional genetic changes once inversions establish or fix. That is, the rearrangement could protect chromosomal regions from gene flow and allow for additional genetic changes to accrue (for example, through local sweeps of internal beneficial mutations or differential introgression of the region encompassed by the rearrangement). Broader comparisons between recently sympatric Z and E strains of *Ostrinia* living in North America and their allopatric, European ancestors may help distinguish between direct and indirect roles of recombination suppression during divergence.

CONCLUSIONS

Our studies of recombination, reproductive isolation and genetic variation in the wild imply that structural mutations have facilitated speciation with gene flow between Z and E strains of ECB. Our results add to the limited number of studies that provide empirical support for this notion, including apple maggot flies (Feder et al., 2003; 2005; Michel et al., 2010; Powell et al., 2013), fruit flies (Noor et al., 2001; Kulathinal et al., 2009), stickleback fishes (Kitano et al., 2009), sunflowers (Rieseberg, 2001; Strasburg et al., 2009) and monkeyflowers (Lowry and Willis, 2010; Fishman et al., 2013). Our finding that structural mutations contribute to speciation may be the first of many for Lepidoptera. The rate of rearrangement in lepidopteran genomes appears to be one of the fastest

among eukaryotes at ~2 breakages/Mb/My, or ~3 × faster than nematodes and more than an order of magnitude faster than flies, mammals and plants (Ranz et al., 2001; Coghlan and Wolfe, 2002; d'Alencon et al., 2010). As lepidopterans (and nematodes) have diffusely organized centromeres (holocentric), these high evolutionary rates may stem from an increased likelihood of reintegration of double-strand break fragments (d'Alencon et al., 2010). Hence, tens or even hundreds of fixed rearrangements could characterize moth or butterfly species pairs that diverged just several hundred thousand years ago, implying that structural mutations may have had a disproportionate role over the evolutionary history of the second largest order of insects, and may commonly promote adaptive divergence and speciation in moths and butterflies.

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CHAPTER 5

GENETIC DIFFERENCES IN BASAL COLD-TOLERANCE CORRELATED WITH PHENOLOGICAL SHIFTS IN *OSTRINIA* MOTHS

ABSTRACT

Phenological shifts contribute to ecological speciation and persistence during global warming. However, shifts in seasonal timing may also result in populations being exposed to higher or lower temperatures than those experienced within the ancestral environment due to thermal variation across a season. Therefore, we would expect that persistence in novel temporal habitats may be associated with adaptive divergence in thermal tolerance. The European corn borer moth (*Ostrinia nubilalis*) has two distinct temporal ecotypes that occur in sympatry in upstate NY. The E-strain exits larval diapause and pupates in the spring, while the Z-strain exits larval diapause and pupates in the summer. Here, we determine that this difference in seasonal timing results in a ~6°C colder environment experienced by earlier-emerging E-strain pupae compared to later-emerging Z-strain pupae. We find that in parallel with this temperature difference, the E-strain has greater capacity to survive cold-shock treatments during this life-stage. Our results suggest a genetically based difference in basal cold-tolerance between strains, and that this difference is due to thermal niche diversification due to phenological shifts.

INTRODUCTION

Organisms live in heterogeneous thermal environments that vary spatially, by latitude and altitude, and also temporally throughout the year. This thermal

variation acts as a selective pressure on the physiological limits of heat and cold-tolerance. Temporal thermal variation across a season creates a landscape of high and low temperature habitats, and as the life-stages of organisms often vary in their thermal tolerances (Kingsolver et al., 2011), populations must time the distinct stages of their ontogeny with the appropriate thermal conditions for survival. Thus, we expect that thermal tolerance of each life-stage will be locally adapted to the conditions at the time of the year they are present. However, contemporary perturbations to seasonal habitats due to global climate change challenges the synchrony of thermally vulnerable life-stages to their ancestral thermal environments as phenology shifts earlier and/or later into the growing season (Roy & Sparks, 2000; Bradshaw & Holzapfel, 2001; Gibbs & Breisch, 2001; Walther et al., 2002; Both et al., 2004; Bradshaw et al., 2004; Gordo et al., 2005; Schmidt et al., 2005; Menzel & Dose, 2005; Menzel, 2005; Bradshaw & Holzapfel, 2006; Gomi et al., 2007; Parmesan, 2006; Bradshaw & Holzapfel, 2008). Therefore, in order for vulnerable life-stages to persist in novel thermal conditions due to phenological shifts, we may expect correlated adaptive evolution in thermotolerance in these stages.

Temperature-mediated selection for populations with enhanced tolerance to local thermal conditions has been extensively documented in both natural and experimental populations. In nature, populations living at different latitudes and altitudes commonly show variation in thermotolerance, with the observation that populations inhabiting colder climates are more cold-tolerant, and populations inhabiting hotter climates are more heat-tolerant. This pattern is wide-spread

across taxa and has been reported in insects (Ayres & Scriber, 1994; Krebs & Loeschcke, 1995; Gibert & Huey, 2001; Hallas et al., 2002; Hoffmann et al., 2002; David et al., 2003; Klok & Chown, 2003; Wang & Kang, 2005; Zani et al., 2005; Ragland & Kingsolver, 2008; Sgró et al., 2010; Kleynhans et al., 2014), crustaceans (Castañeda et al., 2004, 2005; Kelley et al., 2011), gastropods (Dong & Somero, 2009), fish (Fangue et al., 2006), and amphibians (Sørensen et al., 2009). This suggests that distinct thermal habitats select for thermal niche diversification.

Experimental studies have discovered that evolution in thermal tolerance can be rapid (less than 30 generations), and also that shifts in tolerance are heritable. Transplants of threespine stickleback fish (*Gasterosteus aculeatus*) to novel thermal environments in the field, have found rapid evolution in the ability of populations to withstand cold stress in just three generations, suggesting a strong effect of temperature in selecting for more tolerant fish (Barrett et al., 2011). In the lab, artificial selection for individuals with rapid chill-coma recovery times results in populations with an enhanced ability to resist cold in *Bicyclus* anynana butterflies (Dierks et al., 2012), and *Drosophila sp*. lines (Anderson et al., 2005; Mori & Kimura, 2008; Bertoli et al., 2010; Udaka et al., 2010). Chill-coma is caused by cold exposure that leads to a loss of neuromuscular function that although reversible, prevents mating, foraging, and predator avoidance, and therefore may have a large impact on fitness in field conditions (Denlinger & Lee, 2010). Lines selected for decreased chill-coma recovery also had increased survival to severe cold-shocks suggesting similar

mechanisms underlie both chill-coma and cold-tolerance phenotypes (Anderson et al., 2005; Udaka et al., 2010).

While there is ample evidence for thermal niche adaptation in both the lab and the field, there are only a handful of studies that have linked diversity in thermotolerance with phenological shifts. We expect that shifts in phenology will result in exposure to novel thermal environments and subsequently thermotolerance adaptation. Perhaps the best example of thermal niche diversification is from the pine processionary moth (*Thaumetopoea pityocampa*). This Mediterranean moth has two distinct temporally isolated populations that exist in sympatry, with an ancestral ecotype in which the larval stages develop in the winter, and a 'mutant' ecotype in which these same stages develop in the summer (Santos et al., 2007, 2011). This temporal displacement into different thermal habitats has led to adaptive evolution in heat-tolerance in the summer ecotype, where at temperatures greater than 35 °C summer ecotypes had better survival than winter ecotypes (Santos et al., 2011). A second example of thermal niche diversification comes from the apple-maggot fly (*Rhagoletis pomonella*). In this species a host-plant shift has resulted in two temporally isolated populations. Ancestral hawthorn-race larvae occur in the fall, and derived apple-race larvae occur 3–4 weeks earlier in the summer (Filchak et al., 2000). This results in a 4 °C shift in mean temperatures experienced by larval stages between races. Rearing hawthorne-race in common-garden conditions, higher rearing temperatures selected for alleles that were common to the apple-race and lower temperatures selected for hawthorne-race alleles (Filchak et al., 2000). This

suggests thermal niche diversification due to adaptation to different temporal environments in this system. While these are two very good examples of correlated evolution in thermotolerance and phenology, it has yet to be determined if lower thermal limits can shift as a consequence of evolution in phenology.

In insects, overwintering diapause is a mechanism for insects to 'escape' extreme cold temperatures through enhancement of cold-hardiness, with direct developing life-stages often being less thermally resistant (Tauber et al., 1986; Ragland & Kingsolver, 2008; Denlinger & Lee, 2010). Diapause is also often viewed as one of the primary mechanisms through which the annual rhythm of major life history phases such as reproduction, growth, development, and migration are synchronized with seasonally varying biotic and abiotic requirements (Danilevskii, 1965; Tauber and Tauber, 1981; Danks, 1987; Leopold & Lang, 1996). Phenological shifts are often associated with shifts in the timing of the diapause developmental pathway (Bradshaw and Holzapfel, 2001, 2006, 2008; Bradshaw et al., 2004; Filchak et al., 2000; Mathias et al., 2005; Schmidt et al., 2005; Gomi et al., 2007). Earlier release from diapause in the spring-time or later entrance into diapause in the fall, have the potential to expose insects to novel colder climatic conditions. Therefore, persistence of new temporal populations due to shifts in diapause timing may be correlated with evolution in thermotolerance of the direct developing life-stages that proceed or immediately follow diapause.

The European corn borer moth is an excellent system to explore the relationship between shifts in spring-time phenology timing and the evolution of thermotolerance. In upstate New York, the 'E' and 'Z' strains of the European corn borer moth (*Ostrinia nubilalis*) define two temporally distinct populations. E-strain corn borers have two generations per season (bivoltine) and Z-strain corn borers have one generation per season (univoltine). Corn borers diapause overwinter as 5th instar larvae. Thus, to fit two generations into a single growing season, the first generation of the E-strain larvae must emerge from diapause earlier than the Z-strain. The second generation of E-strain larvae must also enter diapause later in the fall than Z-strain larvae. Previous work has found that the E-strain and the Z-strain show genetically based variation in diapause termination timing of overwintering larvae. The E-strain takes on average 14 days to terminate diapause and become non-diapausing pupae, and the Z-strain takes on average 44 days (Roelofs et al., 1985; Glover et al., 1992; Dopman et al., 2005; Wadsworth et al., 2013). This ~30-day difference in diapause termination timing correlates with the ~30-day difference seen in adult mating flights in the field (Dopman et al., 2010).

A predicted consequence of this genetically based difference in diapause termination timing is that earlier-emerging E-strain pupae will likely experience a colder thermal environment than later-emerging Z-strain pupae. These colder thermal environments experienced by E-strain pupae may select for enhanced cold-tolerance in this strain and specifically at this life-stage. Here, we quantitatively define the differences in thermal environments experienced by the

pupal life-stages in these temporally distinct populations. We then ask if the E-strain and the Z-strain differ in cold-tolerance and how cold tolerance varies across life-stage in corn borers. If adaptation in cold-tolerance is correlated with phenological shifts, we predict that E-strain borers will have greater cold-tolerance than the Z-strain, and these differences may be greatest in the pupal life stage. Alternatively, we may not see differences in cold-tolerance between strains if corn borers have a large range of thermal tolerance (and this range is not costly to maintain), or if hybridization occurs between the E and Z-strain and gene flow erodes divergence in this trait (i.e., migration is greater than selection). We finally investigate a possible physiological mechanism of enhanced cold-tolerance across strains and life-stages.

METHODS

Seasonal Predictions: To link the discreet life-stages of E and Z-strain corn borers to temporal niches across a season, data on the timing of adult corn borer flights was collected from the Sweet Corn Pheromone Trapping Network's database from the years 1999-2010 at a representative site in Farmington, NY (USA), where E and Z-strains are sympatric (Dopman et al., 2010). The trapping network monitors corn borer flights at sites throughout New York State from May through October. Cooperating farms set Scentry Heliothis nets (Scentry Biologicals, Inc., Billings, MT) at the edges of corn fields. Separate traps were set with E or Z-strain pheromone lures (Trécé, Inc., Adair, OK), which lures males of each strain to traps containing the female pheromone of their own strain. In order to mitigate false-positives, as a small percentage of males will fly to the opposite

strain's pheromone blend (Roelofs et al., 1985), flights were defined by, 1) catch of at least five males per trap within a week, and 2) outlier weeks with no catches in subsequent weeks were not considered. First and second flights for the E-strain were resolved by defining a minimum threshold of two weeks with less than 5 males in a trap.

The resultant adult flight distributions were used to calculate the occurrence of other life-stages across a season. Daily climate data was collected from the *National Oceanic and Atmospheric Administration's* (NOAA) database for Geneva, NY, for high temperatures, low temperatures, and climate normals. Degree-days (DD) were calculated in *Fahrenheit* units using the standard formula, where the minimum base temperature for corn borer development (10°C/50°F) (Matteson & Decker, 1965; Got & Rodolphe, 1989; Mason et al., 1996; Trnka et al., 2007) was subtracted from the mean daily temperature to estimate the heat units for each day. This calculation excludes days in which the mean temperature was less than the minimum temperature required for development (< 10°C). The known temporal distribution of adult flights and resultant heat units were subsequently used to calculate the distribution of other life-stages using the required number of DD for development of each stage: we used 212 DD from first adult occurrence to predict the first occurrence of 1st instar larvae, 580 DD from the first occurrence of 1st instar larvae to the first of 5th instar larvae, 210 DD from the first occurrence of 5th instar larvae to pupae, and 190 DD from pupae to adult (Mason et al., 1996). Previous studies suggest that the required number of DD for development of particular life-stages is similar for

populations of corn borers across the United States (Calvin et al., 1991), therefore standard DDs for development were used for both E and Z-strains.

Corn Borer Rearing: Experimental corn borers were acquired from Bouckville, NY (univoltine Z-strain) and Geneva, NY (bivoltine E-strain), and were generously donated by Charles Linn and colleagues at the New York State Agricultural Experiment Station (NYSAES). Colonies were maintained *en masse* at a common rearing temperature (26°C), with approximately 100 breeding pairs per generation. As stocks were derived in 1994 and 1996 respectively, plastic or maternal effects can be eliminated as casual to the observed phenotypes. The larval stages were fed a standard artificial diet for corn borers (Southland Products, Lake Village, AR, USA) during the active feeding phases.

Under laboratory conditions, direct development or diapause can be induced in corn borers by long-days (16:8 LD) or short-days (12:12 LD) respectively (Takeda & Skopik, 1985; Skopik & Takeda, 1986; Skopik et al., 1986; Glover et al., 1991, 1992; Dopman et al. 2005; Wadsworth et al. 2013, 2015). Therefore, direct developing life-stages (i.e., 5th instar larvae and pupae) were obtained by rearing corn borers from eggs under 16:8 LD photoperiodic conditions at 26°C. For both strains, larvae were collected after 24 days in these conditions, and pupae were collected 3-4 days after pupation. To simulate winter conditions for 5th instar diapause larvae, 1st instar larvae were transferred just after hatching to 12:12 LD conditions at 23°C. After 24 days corn borers were confirmed to be 5th instar and were transferred to 12:12 LD with a cycling thermoperiod of 10°C during photophase and 0°C during scotophase to cold-

acclimatize the larvae for an additional 20 days. Developmental stage for both direct developing and cold-acclimatized diapause larvae was confirmed by measuring head-capsule width (Beck, 1987) across a subset of individuals for each strain (n=32 per group).

For all experimental treatments, larvae were held for at least 24-hours in individual plastic cups with a moist piece of dental wicking but without food in order to ensure the gut had been purged of ice-nucleators, which have known impacts on cold-hardiness measures in corn borers (Hanec and Beck, 1960). Pupae were also held in individual plastic cups with a moist piece of dental wicking for at least 24-hours. After the holding period, residual moisture, which can influence survival at subzero temperatures (Hanec and Beck, 1960), was removed from the surface of each individual by blotting with tissue before the experimental treatments.

Lethal Temperature (LT): Corn borers were placed into individual gelatin capsules (21 mm x 7 mm) and randomly assigned to a treatment group to be held at various sub-zero temperatures for 2 hours. Treatment groups were divided into the following temperature categories: -2°C to -16°C over 2°C intervals for non-diapause larvae treatment groups (n=4 groups of ~10 individuals per condition per strain), -2°C to -16°C over 2°C intervals for pupal treatment groups (n=4 groups of ~10 individuals per condition per strain), and -4°C to -24°C over 4°C intervals for the cold-acclimatized diapause group (n=3 groups of ~10 individuals per condition per strain). A different thermal range was sampled in cold-acclimatized diapause groups due to an expected lower lethal temperature

for diapause larvae based on previous studies (Barnes and Hodson, 1956; Hanec and Beck, 1960; Nordin et al., 1984; Yi et al., 1987; Grubor-Lajsic et al., 1992; Andreadis et al., 2008). Treatment groups were placed into individual 50 mL conical tubes, which were submerged into a Neslab RTE-140M refrigerated bath circulator (Thermo Neslab, Waltham, MA, USA) containing a 1:1 solution of propylene glycol and water (Sigma-Aldrich, St. Louis, MO, USA). Control groups had no treatment and were maintained under the rearing conditions for each life-stage during the treatment period. Total sample sizes are shown in Table 5.1.

After the cold shock treatment, corn borers were transferred to individuals cups with a moist piece of dental wicking and returned to favorable growing conditions at 26°C in 16:8 LD. Control groups were also moved to 26°C in 16:8 LD. After 10 days of their return to favorable growing conditions corn borers were scored for mortality. For larval groups, death was clearly evident with larvae becoming non-mobile, black, and shriveled (Hanec and Beck, 1960). In the pupal groups, death was also clearly evident with pupae becoming black and shriveled, or with adults failing to emerge completely from their puparium and perishing.

Supercooling Point (SCP): Individuals were placed into separate capsules with a type T copper constant thermocouple (sensitivity rating of ±0.2°C) (Thermoworks, Lindon, UT, USA) fed into the interior. Each capsule was then submerged within a 50 mL conical tube in the refrigerated bath circulator. From 10°C, the bath was cooled at a rate of -1°C per minute. Real-time temperature measurements were taken using a Picotech TC-08 datalogger and the

PicoLog software (Pico Technology, St Neots, Cambridgeshire, UK) for eight samples simultaneously. The lowest temperature reached before a spike in temperature, due to the exothermic nature of ice crystallization, was recorded as the SCP following the methods of prior studies (Goto et al., 2001; Andredis et al., 2008).

Statistical Tests: All analyses were conducted in R v.3.1.1 (R Core Team, 2014). For cold-shock experiments, in order to understand the temperature-specific effects on death due to treatments, mortality was normalized to the control groups using the Henderson-Tilton formula (Henderson and Tilton, 1955):

$$\%Mortality = (1 - \frac{Ta * Cb}{Ca * Tb}) * 100$$

Where *Cb* is the number of individuals in the control group before treatment, *Ta* is the number of individuals in the treatment group after treatment, *Ca* is the number of individuals in the control after treatment, and *Tb* is the number of individuals in the treatment group before treatment. After normalization with this formula, we implemented a hard cap at 0% mortality and 100% survival for treatment groups with better survival than the controls. Resultant normalized count data was rounded to the nearest whole number. Control mortality was < 15%.

An analysis was run to determine the temperature at which 50% of the population perished (LT50) for all life-stages and both strains. Temperature doses were transformed to positive values by adding 25 (where the temperature range

from -24 to -2 was transformed to 1 to 23) and a two-parameter log-logistic function was then fit to the data using the drc package v.2.5-12 (Ritz and Streibig, 2013) for each strain by life-stage:

$$f(x) = \frac{1}{1 + \exp(b(\log(x) - \log(e)))}$$

Where b is the slope, e is the inflection point (LT50), and the upper and lower limits are fixed at 1 and 0 respectively. 95% confidence intervals were estimated using the delta method. Finally, significance between LT50s of groups was evaluated by comparing the ratio of LT50s between populations or life-stages (Wheeler et al., 2006), followed by a correction for multiple testing (Benjamini & Hochberg, 1995).

In order to determine the effects of temperature dose, strain, and life-stage on mortality, multiple generalized linear models (GLMs) were fit to the data. The first GLM investigated the non-diapausing pupal and larval life-stages. A model was fit using the stats' package *glm* function v.3.2.2 (R Core Team, 2014) with temperature dose, strain, and life-stage as factors. Temperature dose was considered a discreet factor within these models. Interaction terms with no significant effects were removed to simplify the model. This was followed by Tukey-adjusted post hoc pairwise comparisons of the least-squares mean survival of each strain and life-stage combination, nested within temperature using the *lsmeans* function v.2.20-23 (Lenth, 2015). Due to different temperature sampling design in the cold-acclimatized larval groups, a second GLM was fit for these

groups with temperature dose and strain as factors, and again, we conducted Tukey- adjusted post hoc comparisons of the strains nested within temperature. In order to determine if cold-acclimatized diapause groups were significantly different from non-diapausing larvae at the lowest common temperature condition measured (-16°C), a Fisher's Exact test (Fisher, 1922) was performed on normalized dead and alive counts within each strain.

For SCP experiments, we looked for significant differences in SCP between life-stages and strains. An analysis of covariance (ANCOVA) with mass as a covariate followed post hoc by Tukey's honest significant difference (HSD) test was run using the stats' package *aov* function v.3.2.2 (R Core Team, 2014).

RESULTS

Seasonal Predictions: The average first occurrence of adult males from 1999-2010 occurred on June 3rd for the BE strain and June 26th for the UZ strain. Using standard required degree-days for development of life-stages, and the first appearance of the adults in the field at Farmington, the resultant temporal distribution of corn borer strains revealed that the first pupal life-stage and last larval life-stages in the E-strain are exposed to colder thermal environments across the season in upstate NY compared to the Z-strain (Figure 5.1). To focus on the consequences of spring-time phenology shifts on adaptive evolution in cold-tolerance, we directly compared the thermal environments of Z and E-strain pupae. The average first occurrence of E-strain pupae was estimated to be May 13th ± 4.04 days, and the average last occurrence of pupae was June 19th ± 3.0 days for the years 1999-2010 (Figure 5.2). For the same years, the average first

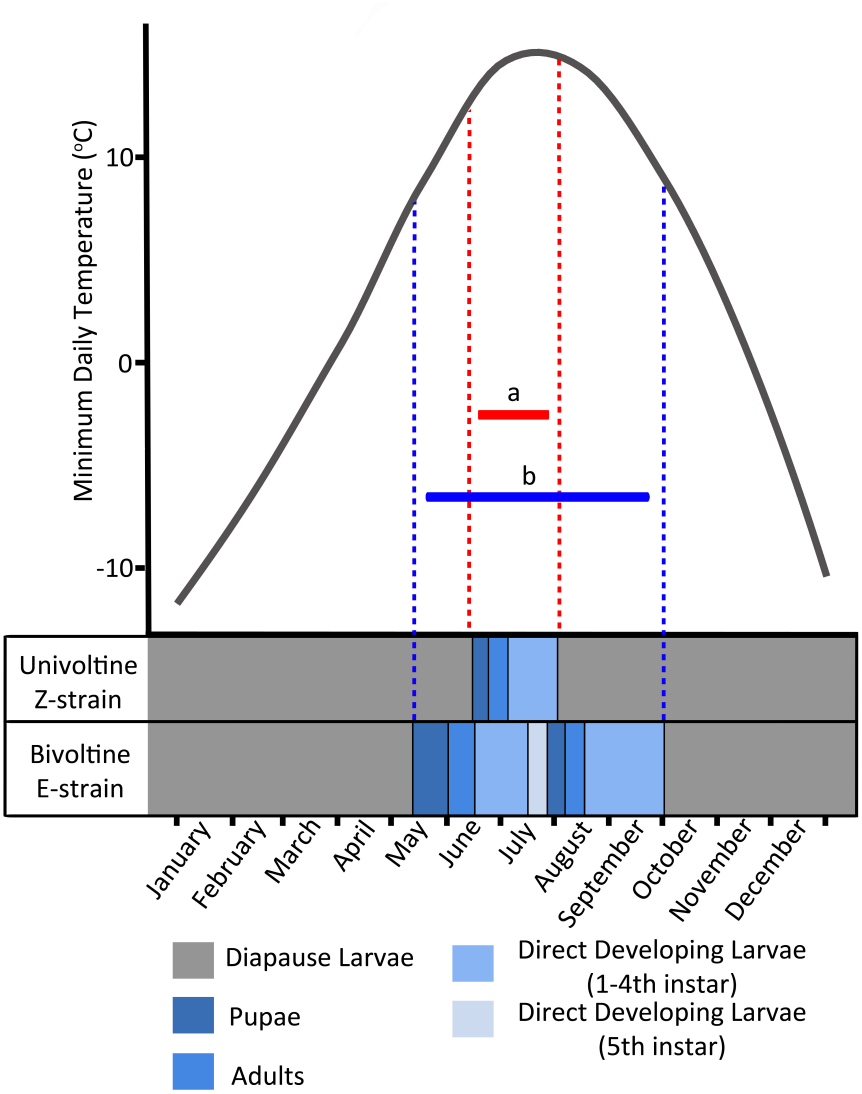

Figure 5.1. Predicted first occurrences of direct developing life-stages for strains of European corn borers in Farmington, NY, where (a) is the average minimum thermal range that direct developing univoltine-Z borers experience (red bar), and (b) is the predicted average minimum thermal range that direct developing bivoltine-E borers experience (blue bar). A smoothing line was fit to minimum daily temperature normals from the 1981-2010 National Oceanic and Atmospheric (NOAA) database for Geneva, NY. The temporal distribution of life-stages was rooted using the average first occurrence of adults from pheromone trapping data collected from 1999-2010, with the first occurrence of other life-stages calculated using degree-days (Mason et al., 1996).

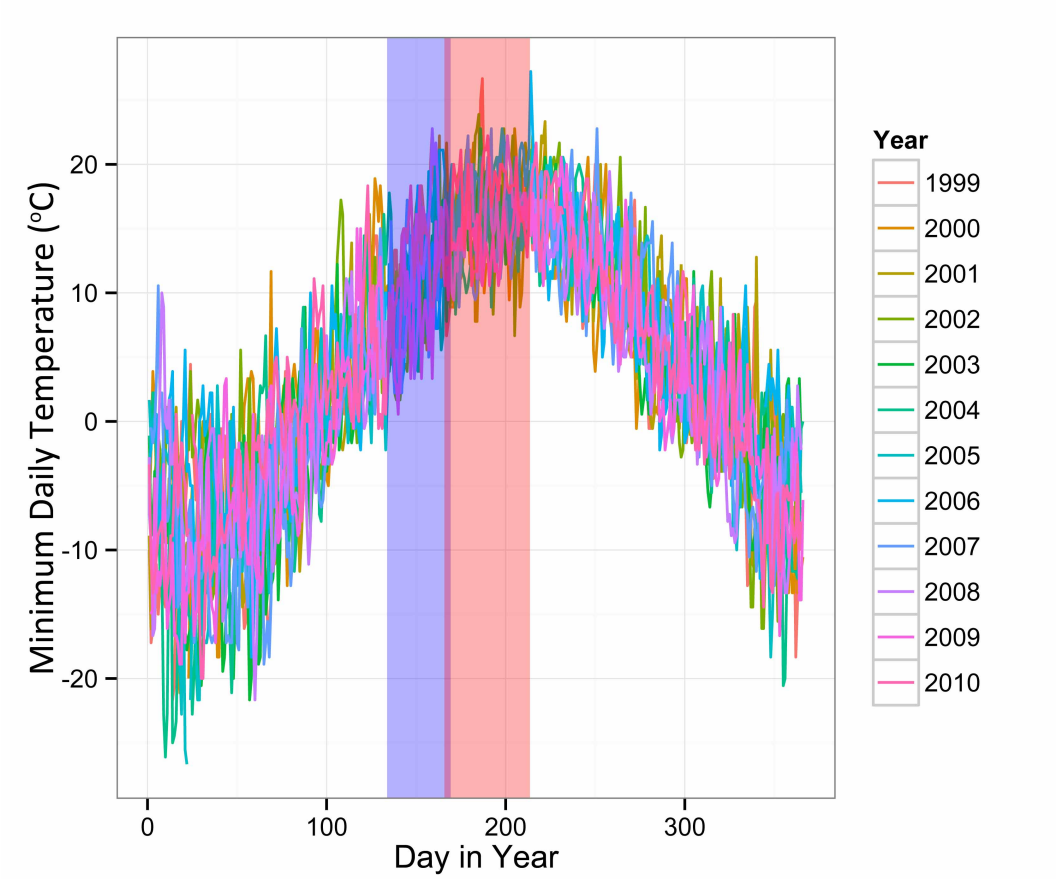

Figure 5.2. Minimum daily temperatures from Geneva Research Farm, NY plotted from 1999-2010. Temperature data was collected from NOAA. The predicted range for the occurrence of direct developing pupae is overlaid for BE (blue) and UZ (red) corn borers averaged over the same years. Adults flight data collected from the Sweet Corn Pheromone Trapping Network, and first and last occurrence of pupae was back-calculated using degree-days needed for pupal development for each year and then averaged (Mason et al., 1996).

occurrence of Z-strain pupae was June 30th ± 5.76 days, and average last occurrence of Z-strain pupae was August 2nd ± 5.79 days (Figure 5.2). Over the 12 years investigated, daily maximum and minimum temperatures for the predicted pupal ranges were collected to define the thermal environments that each strain typically experience (Figure 5.3). The maximum daily temperatures during the pupal stage ranged from 6.67°C to 35.56°C for the E-strain and 9.44°C to 35.0°C for the Z-strain. Over the same period, the minimum daily temperatures during the pupal stage ranged from -2.22°C to 22.78°C for the E-strain and 3.89°C to 27.22°C for the Z-strain. The thermal environments of the first occurring pupal stages between strains were highly significantly different for both the maximum daily temperatures (Wilcox Rank Sum, $W = 80085$, $p < 0.0001$) and minimum daily temperatures (Wilcox Rank Sum, $W = 63516$, $p < 0.0001$).

Lethal Temperature: Ten days after subzero temperature exposure, direct developing individuals had either died, had pupated in larval groups, or eclosed in pupal groups. After the same time-period, cold-acclimated diapause larvae had died, were still alive and had pupated, or remained as robust and clearly viable larvae that would move vigorously upon disturbance. Estimates for the lethal temperature for 50% mortality in each population were lower in E-strain borers across all life-stages (Table 5.1; Figure 5.4a,b). LT50s were significantly different between strains in direct developing larval and pupal stages, but not in the cold-acclimatized diapause stage (Table 5.2). Within the E-strain, LT50 estimates were significantly different between all life-stages (Table 5.2). The Z-strain showed a different pattern where both direct developing life-stages were

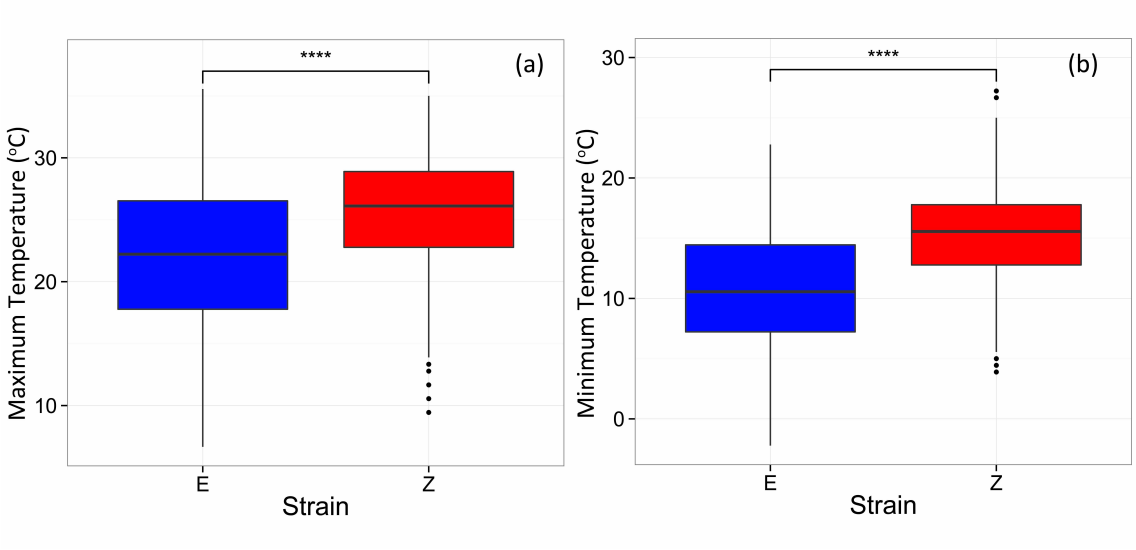

Figure 5.3. For the predicted date range for BE and UZ pupae, the daily maximum and minimum temperatures were concatenated for a 12-year range. The temperature ranges that strains inhabit are highly significantly different for both (a) maximum (Wilcox Rank Sum, W = 80085, p < 0.0001), and (b) minimum temperatures (Wilcox Rank Sum, W = 63516, $p < 0.0001$).

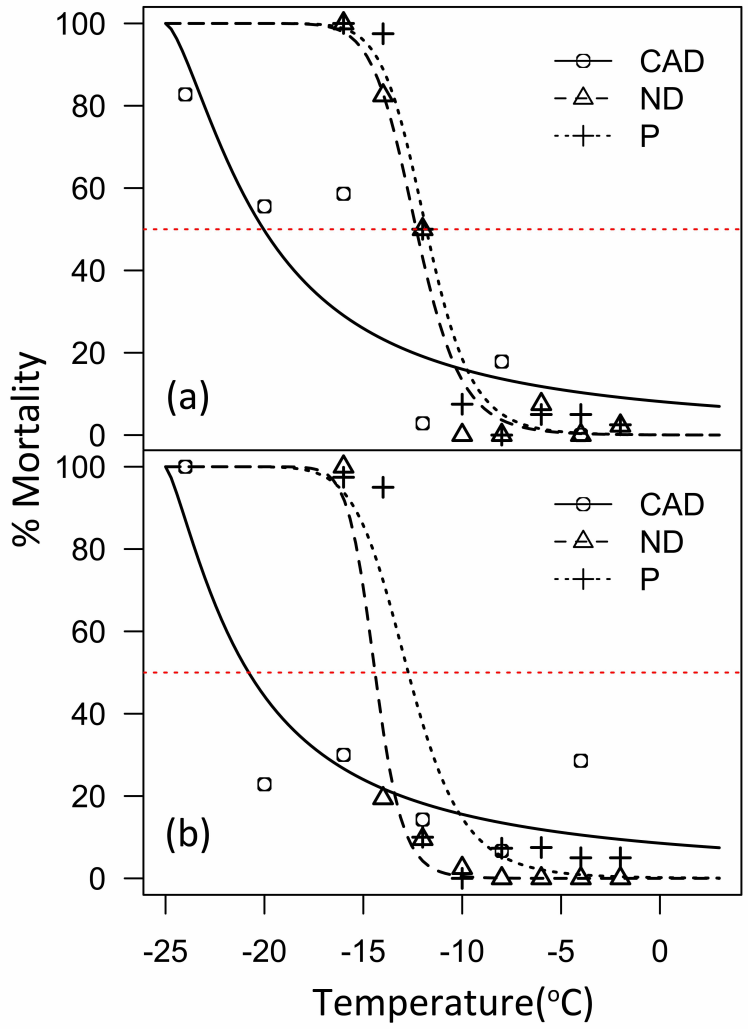

Figure 5.4. Log-logistic function fit to mortality data to predict LT50s. Panel (a) shows fits for the Z-strain, and panel (b) shows fits for the E-strain, for cold-acclimatized diapause larvae (circles, solid line), non-diapausing larvae (triangles, dashed line), and pupae (pluses, dotted line). Red dotted line shows 50% mortality.

Table 5.1. Lethal temperature 50 (LT50) estimates for corn borer strains by life-stage

Strain	Voltinism	Life-stage	Sample Size	LT50	SE	95% CI
Z	Univoltine	Cold-acclimatized diapause larvae	192	-20.04	0.72	-18.63 to -21.46
Z	Univoltine	Non-diapause larvae	327	-12.32	0.24	-11.85 to -12.79
Z	Univoltine	Pupae	320	-11.81	0.24	-11.34 to -12.29
E	Bivoltine	Cold-acclimatized diapause larvae	193	-20.80	0.71	-19.41 to -22.18
E	Bivoltine	Non-diapause larvae	324	-14.41	0.20	-14.02 to -14.79
E	Bivoltine	Pupae	320	-12.73	0.27	-12.20 to -13.26

Table 5.2. Results of lethal temperature 50 (LT50) ratio test

Contrast	Strain	Voltinism	SE	*t*	FDR
Within Strain					
Non-diapause *vs*.cold-acclimatized diapause larvae	Z	Univoltine	0.38	4.15	0.0002
Pupae *vs*. cold-acclimatized diapause larvae	Z	Univoltine	0.39	4.26	0.0002
Pupae *vs*. non-diapause larvae	Z	Univoltine	0.03	1.46	0.17
Non-diapause *vs*.cold-acclimatized diapause larvae	E	Bivoltine	0.43	3.57	0.0006
Pupae *vs*. cold-acclimatized diapause larvae	E	Bivoltine	0.49	3.89	0.0006
Pupae *vs*. non-diapause larvae	E	Bivoltine	0.03	4.74	0.0002
Between Strains					
Cold-acclimatized diapause larvae	Z *vs*. E	-	0.26	0.68	0.50
Non-diapause larvae	Z *vs*. E	-	0.03	6.20	0.0002
Pupae	Z *vs*. E	-	0.03	2.41	0.02

significantly different than the cold-acclimatized diapause group, but were not significantly different from one another (Table 5.2).

For all life-stages investigated, E-strain corn borers had better survival at subzero temperatures than Z-strain corn borers (Figure 5.5a-c). For direct developing life-stages, the main effects of temperature, strain, and life-stage were significant, as were all two-way interactions (Table 5.3). The E-strain had significantly better survival than the Z-strain at -12°C in the direct developing larval (HSD, $z = -4.52$, $p < 0.0001$) and pupal (HSD, $z = -3.58$, $p = 0.0019$) life-stages. E-strain corn borers also had better survival than Z-strain borers at the -14°C treatment in the direct developing larval stage (HSD, $z = -5.03$, $p < 0.0001$). For cold-acclimatized diapause larvae, only temperature and the interaction between strain and temperature were significant (Table 5.3). Between strains, E-strain cold-acclimatized corn borers survived better at the -16°C (HSD, $z = -2.17$, $p = 0.029$) and -20°C (HSD, $z = -2.75$, $p = 0.006$) temperature exposures. At the lowest common temperature measured (-16 °C), cold-acclimatized diapause larvae had significantly better survival than direct developing larvae for both the E (Fisher's Exact test, $p = < 0.0001$) and Z-strains (Fisher's Exact test, $p = < 0.0001$).

Supercooling Point: Life-stage was the only significant explanatory factor for SCP (Table 5.4). Cold-acclimatized diapause larvae had a significantly higher SCP than non-diapause larvae (HSD, $p = 0.04$) and pupae (HSD, $p = 0.00003$). Direct developing life-stages were not significantly different from one another (HSD, $p = 0.10$). The E-strain cold-acclimated diapausing group had a

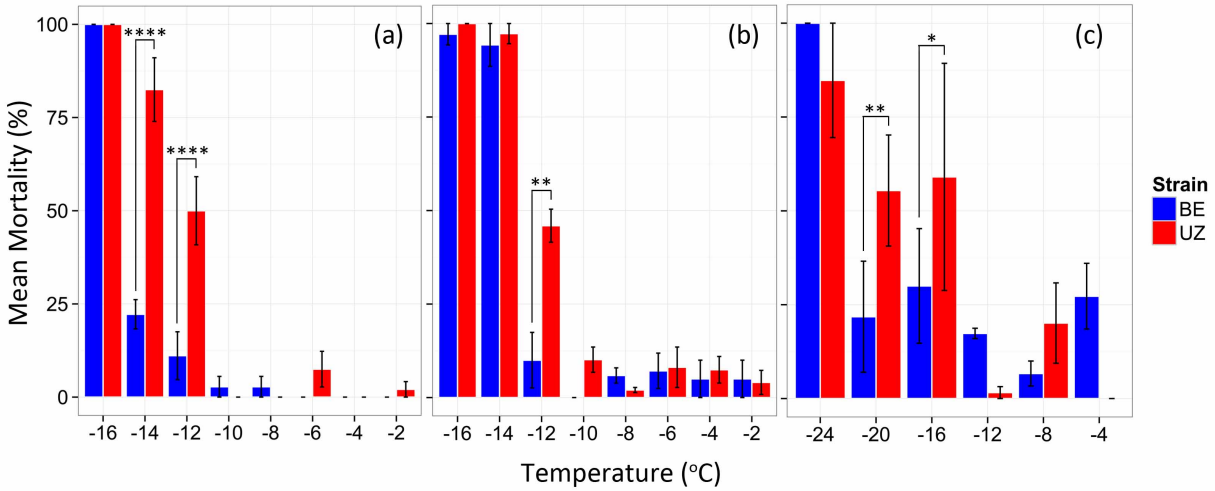

Figure 5.5. Differences in mortality at subzero temperatures between BE and UZ corn borer strains across life-stages, where (a) are the results for direct developing 5th instar larvae, (b) are the results for pupae, and (c) are the results for cold-acclimatized larvae. Bars represent the mean mortality in each group ± SE. Significance between strains at various subzero temperatures was determined using two GLMs followed post-hoc by Tukey's HSD where * = p <0.05, ** = p < 0.01, and **** = p < 0.0001).

Table 5.3. Results of generalized linear models testing the effect of dose, strain, and life-stage on mortality after subzero temperature exposures

	df	Deviance	*P*	Sig. Level
Direct Developing				
strain	1	15.37	8.84E-05	***
life-stage	1	10.89	9.69E-04	***
temperature	7	952.01	< 2.2E-16	***
strain x life-stage	1	16.95	3.84E-05	***
strain x temperature	7	17.62	0.014	*
life-stage x temperature	7	27.32	2.92E-04	***
Cold-acclimatized Diapause				
strain	1	0.332	0.56	
temperature	5	139.877	< 2.2E-16	***
strain x temperature	5	40.446	1.21E-07	***

Significance Level Codes: < 0.001 = ***, < 0.05 = *

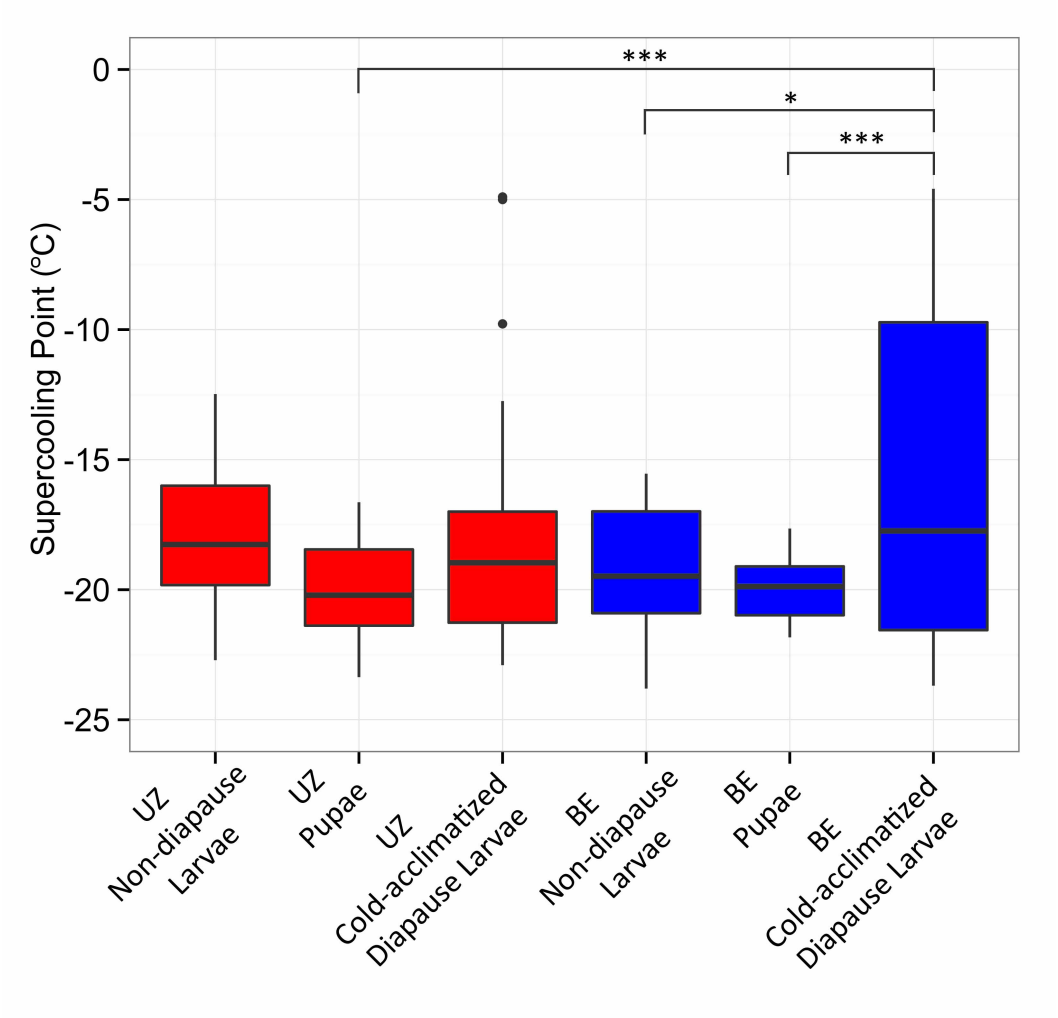

Figure 5.6. Supercooling points for UZ (red) and BE (blue) corn borer strains by life-stage. BE cold-acclimated diapausing had a significantly higher SCP than BE direct developing larvae (Tukey's HSD, $p = 0.01$), BE pupae (Tukey's HSD, $p < 0.001$), and UZ pupae (Tukey's HSD, $p < 0.001$).

Table 5.4. Results of ANCOVA testing the effect of strain, life-stage, and mass on supercooling point (°C)

Factor	**df**	***F*-value**	**P**	**Sig, Level**
strain	1	0.254	0.615	
life-stage	2	10.212	6.21E-05	***
mass	1	0.033	0.855	
strain x life-stage	2	2.713	0.069	.

Significance Level Codes: < 0.001 = ***, <0.1 = .

Table 5.5. Summary of supercooling points (°C) for all life-stages by strain

Strain	Voltinism	Life-stage	Sample Size	Mean SCP (°C ± SD)	Mean Mass (g)
Z	Univoltine	Cold-acclimatized diapause larvae	32	-18.05 ± 4.60 (-4.90 to -22.90)	0.085
Z	Univoltine	Non-diapause larvae	32	-18.17 ± 2.59 (-12.48 to -22.71)	0.116
Z	Univoltine	Pupae	32	-20.02 ± 1.71 (-16.64 to -23.36)	0.099
E	Bivoltine	Cold-acclimatized diapause larvae	32	-16.27 ± 6.12 (-4.59 to -23.69)	0.074
E	Bivoltine	Non-diapause larvae	32	-19.24 ± 2.37 (-15.54 to -23.80)	0.090
E	Bivoltine	Pupae	32	-19.96 ± 1.14 (-17.65 to -21.83)	0.079

Ranges of SCP are given in parentheses.

significantly higher SCP than E-strain direct developing larvae (HSD, p=0.01), E-strain pupae (HSD, p=0.0006), and Z-strain pupae (HSD, p=0.0005). While both strains in the cold-acclimatized diapause groups had higher SCPs than direct developing life-stages, and a similar range from ~-5°C to ~-23°C, E-strain corn borers had more individuals with SCPs greater than -10°C (Table 5.5; supplementary Figure 5.S1), although there was no significant difference between groups (HSD, p=0.33).

DISCUSSION

In seasonal environments, earlier spring-time phenology may expose vulnerable insect life-stages to lower minimum daily temperatures. We show that phenological differences between E and Z-strain corn borers are associated with different thermal environments experienced by the pupal life-stage in the spring and the second generation larvae in the fall. We find that in parallel with these temperature differences, the E-strain has greater cold-tolerance and that these differences occur primarily in the direct developing pupal and larval stages. Differences in cold-tolerance between strains are genetic because experimental corn borers were lab-reared and had been exposed to the same benign temperature environments for more than ten generations, eliminating the possibility of plasticity or maternal effects. Our results suggest that evolutionary changes in cold-tolerance may be associated with persistence of earlier-occurring populations, particularly as organisms shift their phenology in order to take advantage of the longer growing seasons due to global warming.

Phenological shifts can be viewed as natural transplant experiments, where shifts displace life-histories of a population in time, and as a result transplant populations into new thermal environments. Corn borers have a genetically based shift in phenology, where the E-strain has an ~14 day diapause termination phase and the Z-strain has an ~44 day diapause termination phase (Roelofs et al., 1985; Glover et al., 1992; Dopman et al., 2005; Wadsworth et al., 2013). This earlier phenological timing results in the first non-diapausing life-stage (pupae) occurring earlier in the spring in the E-strain than the Z-strain, which first occurs in the summer (Figure 5.1 and 5.2). Comparing our predictions of the thermal environments that the E and Z-strains inhabit in the pupal stage, our results suggest that E-strain pupae experience an environment up to ~6.11°C colder than that of Z-strain pupae. During the 12 years investigated, Z-strain pupae never experienced subzero temperatures whereas E-strain pupae did in both 2006 and 2008.

In order to persist in these significantly different thermal niches, we expected that colder temperatures experienced by E-strain pupae would select for enhanced cold-tolerance. The results of our survival analyses support that in the first direct-developing life-stage, E-strain pupae have better survival at subzero temperatures than Z-strain pupae (Figure 5.4 and 5.5). LT50 results indicate an ~1 °C difference in the ability to withstand cold-temperature shocks between strains (Table 5.1). While the minimum temperature recorded in Geneva, NY over the 12 years investigated did not approach the minimum thermal limit of either strain, other abiotic factors such as precipitation could strengthen the importance of this

slight edge in survival at subzero temperatures in the field. While the survival experiments in this study were conducted under dry conditions, contact moisture is known to have a large impact on the ability of larval corn borers to survive subzero temperatures (Hanec & Beck, 1960). Therefore, in less controlled field settings this 1 °C difference in survival at subzero temperatures could have a large impact on persistence in colder E-strain environments that dip below freezing.

Direct developing 5th instar E-strain larvae are also significantly more cold-tolerant than direct developing 5th instar Z-strain larvae. Our LT50 analysis revealed a ~2°C difference in survival to subzero temperatures between strains at this life-stage. This is somewhat surprising because in upstate New York, the only time 5th instar non-diapausing larvae occur is once in the middle of July for the E-strain, and never in the Z-strain as they go directly into diapause once the 5th instar is reached (Figure 5.1). However, 1st-4th instar larvae in the second generation of the E-strain experience a ~4 °C colder environment than the same life-stages in the only generation of Z-strain borers, perhaps suggesting a generalized mechanism for enhanced cold-tolerance across larval life-stages. While a generalized mechanism for enhanced tolerance across life-stage could contribute to increased survival in populations that occur both earlier and later in the growing season, a generalized mechanism could be costly to maintain in life-stages that will never experience colder temperatures. A costly mechanism would be selected against. Therefore, therefore observing enhanced cold-tolerance in life-stages that do not occur in the cold suggests that there is little to no cost to maintain this particular mechanism in corn borers.

An alternative to consider is that additional generations per year may be selectively favored in order to maximize the intrinsic rate of increase of the populations (*r*) (Fisher, 1958), and therefore some years the E-strain may try to 'fit' in an additional generation per year. This would lead to thermally vulnerable life-stages in the third generation developing through the fall and early winter in the E-strain and exposing non-diapausing 5th instar larvae to colder thermal conditions. Indeed, there is ample evidence for partial generations in many Lepidopterans (Richards, 1940; Geier, 1963; Shapiro, 1975; Blau, 1981; Shreeve, 1986; Bryant et al., 2002; Tobin et al., 2003; Altermatt, 2010), as well as in corn borers in different geographic regions (reviewed by Palmer et al., 1985); lending support to this hypothesis.

Differential thermal adaptation between E and Z-strains may contribute to ecological isolation. In cases with ongoing gene flow, differential thermal adaption has been shown to contribute to the maintenance of stable ecotypes in both plant and animal systems (reviewed by Keller & Seehausen, 2012). One example in sibling species of swallowtail butterflies (*Papilio canadensis* and *P. glaucus*) finds that cold-adapted *P. canadensis* show a significant reduction in survival upon exposure to warmer temperatures in both the spring and autumn as diapausing pupae compared to their warm-adapted *P. glaucus* counterparts (Mercader & Scriber, 2008). Surprisingly, cold-adapted diapausing *P. canadensis* pupae also show reduced survival to subzero cold-shock treatments but this may be due to differences in diapause intensity or differential microhabitats such as the length of snowpack cover which can mitigate extreme temperature fluctuations

(Scriber et al., 2012). Regardless, differential thermal adaptation may contribute to the maintenance of a sharp ecotone between species despite hybridization in this system.

In corn borers, estimated gene flow can reach ~10% between E and Z-strains, and the percentage of hybrids has reported to be greater than 15% at various geographic sites (Roelofs et al., 1985; Klun & Huettel, 1988; Dopman et al., 2010). Given that there is a correlation between enhanced cold-tolerance in the earlier-occurring E-strain, we expect that temperature-mediated selection for tolerant phenotypes is very strong. In this scenario, divergent selection is most likely purging maladaptive intermediate or alternate genotypes from locally adapted populations. For example, in corn borers, an individual with early E-strain phenology but low Z-strain thermotolerance may be unable to survive in the colder E-strain environment. While many studies have focused on the purging of deleterious thermal phenotypes along latitudinal or altitudinal temperature gradients (Graves, 1991; Kozak & Wiens, 2007; McCain, 2009; Cadena et al., 2011), the purging of deleterious thermal phenotypes in the wrong temporal environments has rarely been investigated (e.g., Filchak et al., 2000)

While strong divergent selection could maintain the observed correlation between phenological timing and thermotolerance in corn borers, another possibility to consider is genetic linkage. Corn borers from upstate NY have a large region of suppressed recombination between strains, which is most likely caused by a chromosomal rearrangement (Wadsworth et al., 2015). The rearrangement is sex-linked, encompasses at least 20% of the Z (sex)

chromosome, and harbors the genetic basis for differences in phenological timing between strains (Dopman et al., 2005; Wadsworth et al., 2015). In systems with ongoing gene flow between populations, rearrangements can facilitate speciation by protecting the association of favorable combinations of alleles that enhance fitness due to recombination suppression (reviewed by Hoffman & Rieseberg, 2008). If the gene(s) associated with cold-tolerance are located within this rearrangement between strains, introgression may be prevented despite gene flow. Interestingly in other systems, genes involved in climatic adaptation (e.g., seasonal timing, or thermotolerance) are often associated with chromosomal rearrangements that vary in frequency by latitude (Feder et al. 2003; Rodriguez-Trelles & Rodriguez 1998; Anderson et al. 2005, Santos et al. 2005; Umina et al. 2005; Balanya et al., 2006).

Finally, to test one physiological mechanism that could potentially explain differences in survival between E and Z-strains, we measured SCP which can serve as a proxy to detect any alterations in levels of polyhydric alcohols (polyols) or glycerol, which are common mechanisms in insects to prevent damage to tissues due to freezing (Michaud & Denlinger, 2004). Larval corn borers are freeze-avoidant as indicated by a low SCP (Bale & Hayward, 2010). However, corn borers experience mortality prior to their freezing point, indicating that they experience injury due to chilling (Andreadis et al., 2008). If the mechanism for enhanced survival is due to a shift in the freezing point of corn borers because of up-regulated polyhydric alcohols or glycerol, we would expect to see an alteration in SCP between the strains at any given life-stage. Our results indicted no

difference in supercooling capacity between strains at any life-stage (Figure 5.6), indicating that altered levels of low molecular weight compounds to prevent freezing is not the likely mechanism behind a shift in cold-tolerance between strains. Future studies could focus on other mechanisms such as altered ability to sense cold temperatures, increased ability to refold denatured proteins, or altered ability to change the membrane fluidity of cells, (Michaud & Denlinger, 2004; Bale & Hayward, 2010) to determine the mechanism behind adaptive evolution in cold-tolerance. Currently, we are working on using RNA-seq to identify genes associated with differential cold-tolerance in corn borers.

The ability of organisms to tolerate novel thermal environments will become increasingly important as mean annual temperatures change and phenologies shift in concert, transplanting organisms into new seasonal conditions. To our knowledge, our study contributes to only a handful of studies that have linked shifts in phenology timing to variation in thermotolerance (Filchak et al., 2000; Santos et al., 2007, 2011). We find evidence for correlated evolution between earlier phenology and enhanced thermotolerance in corn borers, most likely due to temperature-mediated selection in distinct thermal habitats across a season. This suggests that despite ongoing gene flow, selection is strong or there is genetic linkage between phenology and thermotolerance variation. Future studies should focus a more in-depth understanding of variation in cold-tolerance in corn borers, for example, do the survival differences to extreme temperature shocks between strains persist under other scenarios known to influence cold-tolerance such as cold-acclimation? Other remaining questions

include, does increasing the duration of cold-exposure change the percentage of surviving individuals, what are the molecular mechanisms responsible for differential survival, and is the genetic basis of cold-tolerance linked to the locus determining phenological timing? Finally, does a particular strain outcompete the other in climate warming scenarios? Answers to all of these questions may provide some additional insight into species persistence during climate change.

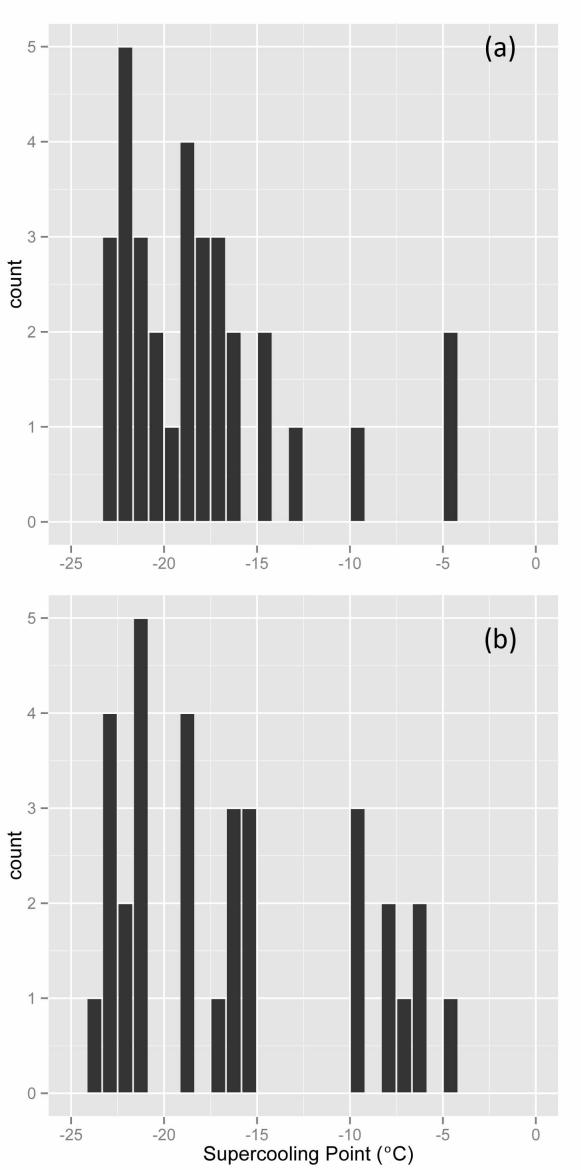

Figure 5.S1. Distribution of SCPs for (a) Z and (b) E cold-acclimatized diapause larvae.

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