One phase of the dormancy developmental pathway is critical for the evolution of insect seasonality

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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. 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 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; Dank, 1987) and is typically regulated by the alteration of a critical developmental hormone, such as juvenile hormone or ecdysteroids (Denlinger, 2002; Denlinger et al., 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 signalling (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 termina-

tion (Denlinger et al., 2005; Kostál, 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; Kostál, 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 developmental 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; Kostál, 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 adaptation 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, 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 popula-
tions 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) (Eckrode et al., 1983; Roelofs et al., 1985; Dopman et al., 2010) (Fig. 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 codominant alleles occur at Pdd. One allele confers earlier emergence from larval diapause and thus earlier adult flight times (PddS), whereas the other confers later emergence and later flight times (PddL). Upon experiencing identical post-winter cues, univoltine Z/PddL moths transition from larval diapause and resume active pupal development 20–30 days later than early emerging bivoltine E/PddS moths under both laboratory and field settings (Glover et al., 1991, 1992; Dopman et al., 2005) (Fig. 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. 1b) has the potential to fully account for genetic differences in adult voltinism among field populations (Fig. 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. 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. 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 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. 2c). We use changes in CO2 production as a metric for metabolic rate to test which of the three components of the diapause programme, or combina-
tions 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 and later-emerging univoltine Z-strain/Pdd 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 nondiapausing 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 (Table S1, S2, in the supporting information). 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 CO2; 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 CO2/H2O 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 CO2 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 CO₂ free air served as a baseline. Empty syringes were used as a control for leakage of CO₂ into the chambers. The manual bolus integration method was used to calculate the rate of CO₂ production per individual per day (Lighton, 2008; Ragland et al., 2009):

\[ R = \int_{t_0}^{t_f} C F dt \]

in which \( R \) is respiration rate (cc CO₂/h), \( C \) is instantaneous CO₂ concentration (cc/L), \( F \) is flow rate (cc/h), \( t \) is elapsed time from purge to injection (h), and \( t_0 \) is the time interval in which CO₂ was integrated (h). Data were corrected for the volume injected vs. the total syringe volume and mass (cc CO₂/mg/h). After correcting for larval mass, there were no significant differences in CO₂ 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:

\[ R = e^{(t - t_0)b} + c, \]

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 nondiapausing caterpillars exposed to long-day conditions. After exposure to 35 days of short-day photoperiod, diapausing larval CO₂ production levels (\( n = 19 \)) were approximately 45% of nondiapausing larvae (\( n = 14 \)) (Fig. 3). This result confirms prior studies in which metabolic rate (O₂ 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\(^*\) and 18 earlier-emerging E-strain/Pdd\(^*\) caterpillars. CO₂ 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. 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 CO₂ 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\(^*\) insects began to rapidly increase (e.g. Fig. 4a), and as a group, levels of CO₂ were significantly elevated compared with the later-emerging Z-strain/Pdd\(^*\) insects (\( F_{29,73} = 2.51, P = 0.012 \), Fig. 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. 3).

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/\(Pdd^S\), 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. 4a). Long diapause insects showed a similar exponential increase in metabolic rate leading to pupation soon after, but most of these long-diapaus ing larvae did not enter the exponential phase of metabolic rate increase that indicates the diapause termination phase until day 30 or later (Fig. 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. 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/\(Pdd^S\) and earlier-emerging E-strain/\(Pdd^S\) genotypes did not significantly differ in estimated parameter values corresponding to the intercept \(a\) or rate of exponential increase \(b\) in the nonlinear model (Table 1). Thus, neither the initial degree of metabolic suppression during the diapause maintenance phase nor the rate of 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 (Figure 4b). Long diapause insects showed a similar exponential increase in metabolic rate \(d\) several days later (Fig. 4a). Long diapause insects showed a similar exponential increase in metabolic rate \(d\) several days later (Fig. 4b).

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### Table 1 Estimated parameters from nonlinear regression of daily metabolic rate for E-strain/\(Pdd^S\) and Z-strain/\(Pdd^S\) moths.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>E-strain/(Pdd^S) ((n = 18))</th>
<th>Z-strain/(Pdd^S) ((n = 17))</th>
<th>(P)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>12.90 (19.71)</td>
<td>34.47 (16.39)</td>
<td>2.99 \times 10^{-7}</td>
</tr>
<tr>
<td>(b)</td>
<td>0.26 (0.27)</td>
<td>0.38 (0.27)</td>
<td>0.15</td>
</tr>
<tr>
<td>(c)</td>
<td>0.95 (1.17)</td>
<td>0.90 (0.13)</td>
<td>0.92</td>
</tr>
</tbody>
</table>

E-strain/\(Pdd^S\) and Z-strain/\(Pdd^S\) 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/\(Pdd^S\) and E-strain/\(Pdd^S\) parameters. Average \(r^2\) values for fitted curves were 0.8 and 0.76 for Z-strain/\(Pdd^S\) and E-strain/\(Pdd^S\) moths, respectively.
abolic rates during the diapause maintenance phase or in the rate of release from metabolic suppression once the diapause termination phase had begun (Fig. 4c).

Discussion

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; Ordning 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. 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. 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 nondiapausing 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 nondiapausing, 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. 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 (Kostal 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 termi-
nation 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. 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 (nondiapausing) summer flights. Hybridization between these two species has lead to the recent formation of a third species, P. appalachensis, 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 Papilio 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-linked 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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References


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

- **Table S1** Respirometry measures for diapause time-point.
- **Table S2** Respirometry measures for diapause termination time-course.

Data deposited at Dryad: doi:10.5061/dryad.1vh94

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