# The diapause response of *Rhagoletis pomonella* to varying environmental conditions and its significance for geographic and host plant-related adaptation

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# Abstract

The recent shift of *Rhagoletis pomonella* Walsh (Diptera: Tephritidae) from its ancestral host hawthorn to apple is a model for incipient sympatric speciation in action. Previous studies have shown that changes in the over-wintering pupal diapause are critical for differentially adapting *R. pomonella* flies to a difference in the fruiting times of apples vs. hawthorns, generating ecologically based reproductive isolation. Here, we exposed pupae of the hawthorn race to various combinations of pre- and over-wintering rearing conditions and analyzed their effects on eclosion time and genetics. We report certain unexpected results in regards to a combination of brief pre-winter and over-wintering periods indicative of gene\*environment interactions requiring a reassessment of our current understanding of *R. pomonella* diapause. We present a hypothesis that involves physiological factors related to stored energy reserves in pupae that influences the depth and duration of *Rhagoletis* diapause. This 'pupal energy reserve' hypothesis can account for our findings and help clarify the role host plant-related life history adaptation plays in phytophage biodiversity.

## Introduction

Phytophagous insects have been argued to be prime candidates for sympatric speciation (divergence in the face of gene flow and in the absence of geographic isolation) via host plant shifting. The sympatric speciation hypothesis stems from reproductive patterns for phytophagous specialists often being intimately tied to their host plants (Bush, 1969a,b, 1975, 1992; Berlocher & Feder, 2002; Drès & Mallet, 2002). As a consequence, insects attacking and specializing on new host plants may become ecologically isolated from populations utilizing ancestral hosts.

When ecological barriers to gene flow arise in sympatry, they often involve shifts in life history timing (Feder et al., 1993, 1998; Abrahamson et al., 1994; Feder & Filchak, 1999; Groman & Pellmyr, 2000; Eubanks et al., 2003; Thomas et al., 2003; Svensson et al., 2005; Antonovics, 2006; Hall & Willis, 2006; Savolainen et al., 2006). These barriers can take the form of allochronic pre-zygotic isolation, when life history adaptations to temporally offset resources or environmental conditions result in populations breeding at different times (Wood & Guttman, 1982; Wood & Keese, 1990; Feder et al., 1993). It is also possible for life history adaptations to generate post-zygotic isolation, when hybrids have developmental profiles that make them ill-suited to survive and reproduce in alternate parental habitats (Dambroski & Feder, 2007).

Differences in life history timing appear to be particularly important ecological barriers separating many host plant-specific taxa of temperate insects (Smith, 1988; Wood & Keese, 1990; Craig et al., 1993, 2001; Feder et al., 1993; Itami et al., 1998; Groman & Pellmyr, 2000). New (derived) host plants often differ in their seasonality from parental (ancestral) hosts. Successful colonization of a new host plant therefore requires that an insect's life cycle be shifted either earlier or later to coincide with the timing of

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a different temporal resource island, which is not trivial, especially for a univoltine insect, considering that favorable conditions for events such as feeding, mating, or oviposition may be of limited duration. Most temperate insects possess a physiological diapause mechanism that allows them to optimize the timing of their life cycles to seasonal events (Tauber et al., 1986). Diapause is a neurohormonally mediated, dynamic state of low metabolic activity associated with reduced morphogenesis, increased resistance to environmental extremes, and altered or reduced behavioral activity. By changing the timing and duration of diapause, it is possible for phytophagous insects to synchronize their life cycles with new plants, with reproductive isolation arising as a by-product of these seasonal shifts.

Here, we examine how changes in the timing of diapause have contributed to sympatric host shifts and ecological reproductive isolation between apple and hawthorn-infesting races of Rhagoletis pomonella Walsh (Diptera: Tephritidae) flies. The recent (<150 years) shift of R. pomonella from its ancestral host hawthorn [Crataegus spp. (Rosaceae)] to introduced, domesticated apple [Malus pumila Mill. (Rosaceae)] in the USA provides an opportunity to study sympatric host race formation in action. An important feature of the shift from apples to hawthorns is that the two trees differ in their fruiting times (Bush, 1966; Feder et al., 1993, 1998; Berlocher & Feder, 2002). At sympatric field sites, domesticated apple varieties favored by the apple host race of R. pomonella generally fruit ca. 3-4 weeks earlier than preferred hawthorn species such as Crataegus mollis (Torr. & A. Gray) Scheele attacked by the hawthorn fly race (Bush, 1966, 1969a,b; Feder et al., 1993, 1998; Berlocher, 2000). Previous studies have shown that shifts in diapause timing are critical for adapting R. pomonella flies to the difference in the fruiting times of apple vs. hawthorn, generating ecological isolation (Feder et al., 1993, 1994, 1997a; Filchak et al., 2000). Two aspects of diapause appear to be particularly relevant for imposing divergent selection pressures. The first is the interaction of pre-winter environmental conditions with the facultative nature of the R. pomonella pupal diapause. The second concerns coordinating the timing of diapause breakage and eclosion to coincide with the end of winter and host fruit availability in the summer.

The facultative nature of *R. pomonella* diapause is important because when pupae are exposed to high temperature for an extended period of time, a significant proportion of flies will forgo a prolonged diapause and immediately develop into adults (Prokopy, 1968; Feder et al., 1997a). Such 'non-diapausing' second generation flies are at a severe fitness disadvantage in nature because they eclose at times in the late fall when little, if any, host fruit are available. Thus, flies attacking earlier fruiting hosts like apple are selected for a deeper initial pupal diapause to withstand the longer periods of higher temperature they experience before winter (Dambroski & Feder, 2007). In contrast, flies infesting later fruiting hosts like hawthorn appear to have faster development (metabolic) rates to quickly attain the proper pupal over-wintering state in the face of lower fall temperatures and, as a result, have been found to be more prone to non-diapause development (Dambroski & Feder, 2007).

Diapause breakage and eclosion represent a second important element of *R. pomonella* life history. Because *R. pomonella* is univoltine and has a limited adult life span of only up to 1 month in the field (Dean & Chapman, 1973; Boller & Prokopy, 1976), flies must terminate diapause and eclose in the summer within a specific time period to coincide with maximal host fruit availability for mating and oviposition. Standardized rearing experiments have implied that in addition to having a deeper initial pupal diapause resulting in their being less prone to direct, non-diapause development, apple-fly pupae also appear to terminate diapause and eclose as adults earlier than hawthorn flies following the end of winter, as a consequence of the earlier fruiting time of apple (Dambroski & Feder, 2007).

Latitudinal variation in host fruiting time introduces another level of complexity to the R. pomonella system, especially with respect to hawthorn flies (the apple race is sympatric with the hawthorn race throughout the northeastern and midwestern regions of the USA and Canada, but does not extend into the deep south or Mexico, as hawthorn flies do; Bush, 1966). Moving south, host plants tend to fruit later in the season. As a result, the period between the end of winter and ripe fruit set is longer for southern than for northern fly populations. In addition, southern flies experience higher temperature conditions for longer periods of time before the onset of milder winters than northern flies. Thus, initial diapause depth appears to be deeper for hawthorn flies in the South, just as it would be locally for hawthorn flies if they were to shift and infest a sympatric apple tree (Dambroski & Feder, 2007). However, southern hawthorn flies are also selected for delayed diapause termination and eclosion time to match the later fruiting phenology of plants in the South (Dambroski & Feder, 2007). Consequently, certain aspects of geographic life history adaptation within the hawthorn race mirror the host shift to apple (deeper initial diapause depth), whereas others are different (delayed diapause termination and eclosion for southern hawthorn flies compared to earlier diapause breakage and eclosion in the season for apple flies).

To date, all marker loci differentiating the apple and hawthorn host races of R. pomonella have been associated with diapause traits (Feder et al., 1993; Filchak et al., 2000). Six allozymes mapping to three different inverted regions of chromosomes 1-3 in the R. pomonella genome (haploid, n = 6) show consistent frequency differences between sympatric apple and hawthorn fly populations in the northeastern and midwestern USA (Feder et al., 1988, 1990; McPheron et al., 1988). Alleles at these six allozymes correlate with adult eclosion time (Feder et al., 1993). In addition, alleles at higher frequency in sympatric apple than in hawthorn fly populations have been associated with a reduced tendency for direct development, consistent with apple flies having deeper initial over-wintering diapauses (Feder et al., 1997a,b). However, because the allozyme loci are located in inversions it is not known whether it is the allozymes themselves or linked loci that affect diapause traits in R. pomonella. The six allozymes also show latitudinal clines that are particularly pronounced in the hawthorn race (Feder & Bush, 1989). Alleles at higher frequency in the South are those more prevalent in sympatric apple than hawthorn populations in the North, consistent with southern hawthorn flies having deeper initial pupal diapauses and later eclosion times. However, these 'southern alleles' also correlate with later eclosion time within the apple race, although not as strongly as they do for hawthorn flies (Feder et al., 1993). Because these alleles are present at higher frequency in apple than hawthorn fly populations at sympatric sites, either other genes in addition to the allozymes located outside of the inversions or different alleles at linked loci in the apple race associated with the same allozyme alleles within the inversions are likely responsible for early diapause termination and eclosion time in apple flies.

Here, we exposed hawthorn flies in a viability selection experiment on the pupal life stage to different combinations of pre- and over-wintering rearing conditions to examine in greater detail how allozyme genotypes affect diapause and to test for interactions between the allozymes and environmental conditions. The rearing treatments were varied to reflect both the differences in local fruiting time between sympatric apple vs. hawthorn trees in the Northeast, as well as differences experienced latitudinally within the hawthorn race across its geographic range in the USA. Previous studies have documented gene\*environment interactions between the allozymes and pre-winter and over-winter length, and pre-winter temperature (Feder et al., 1993, 1997a,b; Filchak et al., 2000). However, these studies tended to vary one environmental variable at a time. In the current study, we are interested in how pre- and over-winter conditions may interact to affect *R. pomonella* genetics. Our a priori prediction was that environmental conditions should mainly have additive effects. For example, warm and long prewinter treatments coupled with long over-wintering periods, conditions that individually favor apple race alleles at northern sites, should together result in an increased genetic response in the direction of the apple race.

## **Materials and methods**

## **Experimental design**

We used a three-way split design to examine the effects of host-related environmental factors on R. pomonella. Larvae collected from hawthorn fruit in the field were divided into 12 subsamples (minimum of 1 135 flies per subsample; Figure 1) and treated to different combinations of pre-winter temperature (18, 22, and 26 °C), pre-winter length (10 vs. 30 days), and over-winter length (8 vs. 30 weeks). We were particularly interested in combinations of treatments emulating differences faced by apple vs. hawthorn flies at sympatric sites in the northern USA [northern apple: warm (26 °C) and long (30 days) prewinter and long (30 weeks) winter vs. northern hawthorn: cold (18 °C) and short (10 days) pre-winter and long (30 weeks) winter] and northern vs. southern hawthorn flies [southern hawthorn: hot (26 °C) and long (30 days) pre-winter and short (8 weeks) winter]. We also explored the consequences of flies exposed to short pre-winter (10 days) and short winter (8 weeks) conditions not normally faced in nature.

## Sampling and details of experimental methods

Infested fruit were collected on 1 September 1999 from a hawthorn tree supporting a large fly population in the tens of thousands in East Lansing (MI, USA), in the overlap region between the apple and hawthorn host races in the Midwest. Previous allozyme studies have shown that the fly population from this tree is genetically highly variable and representative of the species R. pomonella, as a whole (Feder & Bush, 1989; Feder et al., 1990). Fruit were subdivided into three constant temperature incubators maintained at 18, 22, and 26 °C with an L14:D10 photocycle. Emerging larvae were collected daily from the incubators and counted. Approximately 15% of the larvae from each sample were immediately frozen at -80 °C for an untreated genetic control. The remaining individuals were divided into the two pre-winter length treatments. Larvae were placed into Petri dishes containing moist vermiculite to pupate and were then returned to their respective incubator of origin for an additional period of either 10 or 30 days. After this, pupae were subjected to 10 °C for 2 days and then moved to a refrigerator that cycled



**Figure 1** (A) Percentages of pupae entering different rearing treatments that eclosed as adults during the 150-day monitoring period following over-wintering. Significant levels for main effects, and two- and three-way interactions for pre-winter length and temperature, and winter length on fly survivorship, as determined by randomization ANOVA analyses, are given in Table 1. Sample size (n) = initial number of pupae in each sub-treatment for a given pre-winter temperature. Data points sharing letters in common do not statistically differ from one another. (B) Mean time to adult eclosion for flies in treatments. Significant levels for main effects, and two- and three-way interactions for pre-winter length and temperature, and winter length on mean adult eclosion time, as determined by randomization ANOVA analyses, are given in Table 1.

between 0 and 5 °C to simulate winter. Pupae were further subdivided into over-winter length treatments of 8 or 30 weeks. At the termination of the over-winter treatment, Petri dishes were removed from the refrigerator and kept in a 23 °C, L14:D10 incubator. Eclosing adults were collected and sexed over a 150-day period following heating and frozen for later genetic analysis.

## Genetic assay

Eclosed adults and control larvae were scored for three allozymes, NADH-diaphorase-2 (*Dia-2*), Malic enzyme (*Me*), and beta-Hydroxyacid dehydrogenase (*Had*), using standard horizontal starch gel electrophoresis methods (Feder et al., 1989; Berlocher et al., 1993). These markers were chosen because *Dia-2*, *Me*, and *Had* each maps to one of the three chromosomes (1–3, respectively) displaying host related differentiation associated with diapause (Feder et al., 1993). These markers reside in inversions which show latitudinal clines in *R. pomonella* (Feder et al., 2003). As a result, the three allozymes themselves may or may not directly affect diapause in *R. pomonella* but, at the least, they are linked to a gene, or genes, having direct effects on diapause.

## Statistical analysis

The effects of rearing treatments on eclosion time, survivorship, and the allozymes, as well as the effects of allozyme genotypes on percentages of adult eclosion and eclosion time were tested for significance using a randomization version of an ANOVA model written by the authors. Response or dependent variables (survivorship, eclosion time, or Dia-2, Me, or Had frequencies) were randomized with respect to independent treatment variables (rearing conditions or allozyme genotypes) for a particular test 10 000 times. The proportion of randomization results which failed to exceed the observed results was taken as a measure of statistical significance at the  $\alpha = 0.05$  level. We used a Monte Carlo approach because certain of the data were non-normally distributed (Manly, 1997). Allozyme frequency and survivorship differences between treatments were also compared using two-tailed Fisher exact tests.

## **Results**

### Percentages of flies eclosing from rearing treatments

In general, increasing pre-winter temperature from colder, more northern-like rearing conditions (18 °C) to higher, more apple or southern-like temperatures (26 °C) resulted in decreased percentages of adult eclosion (Figure 1A) (Note: adult eclosion refers to those individuals that emerged during the 150-day heating period after over-wintering. It reflects survivorship to a large degree. However, there may have been a portion of flies in deep diapause that were still alive after the 150-day heating period and required more than a 1-year cycle to eclose. These multi-year flies would have been counted as noneclosing in our estimates). The observed decrease in adult eclosion associated with higher pre-winter temperature was accentuated by lengthening the pre-wintering period from 10 to 30 days (Figure 1A), again reflecting a further

Table 1	Main and interaction effects of rearing treatments on survivorship, eclosion time, and the allozymes Dia-2, Me, and Had, as deter
mined t	y randomization version of an ANOVA model. Given are the proportions of randomization results which failed to exceed th
observed	results taken as a measure of statistical significance at the $\alpha = 0.05$ level

Effect	Survival	Eclosion	Dia-2	Me	Had
PW	0.0170	< 0.0001	0.1698	0.0212	0.0077
Temp	< 0.0001	< 0.0001	0.3310	0.0097	0.0152
OW	< 0.0001	< 0.0001	0.0002	< 0.0001	0.6624
PW*Temp	< 0.0001	< 0.0001	0.1533	0.1799	< 0.0001
PW*OW	< 0.0001	0.3888	0.4815	0.6085	0.8439
Temp*OW	< 0.0001	< 0.0001	0.3693	< 0.0001	0.0030
PW*OW*Temp	< 0.0001	< 0.0001	0.3903	0.4117	0.0151

PW, pre-winter length; Temp, pre-winter temperature; OW, over-winter length.

shift to more apple or southern-like environmental conditions. Thus, the northern hawthorn flies used for the study from East Lansing were sensitive to warm and long prewinter treatments emulating conditions experienced by apple and southern hawthorn populations in nature, as reflected by the metric of decreased adult eclosion. The primary exception to this general pattern was seen for short pre-winter (10 days) and short over-winter (8 weeks) conditions not normally experienced in the field (see the light stippled line in Figure 1A). Here, the percentage of adult eclosion was low in the colder 18 °C treatment and increased slightly in the 26 °C treatment, generating a significant overall three-way interaction between pre-winter length, pre-winter temperature, and winter length (P<0.0001; Table 1).

## **Eclosion time**

Mean eclosion time did not differ greatly among pupae experiencing apple, northern hawthorn, and southern hawthorn environmental conditions (Figure 1B). In contrast, mean eclosion time decreased dramatically with increasing pre-winter temperature for flies exposed to the combination of short pre-winter and short over-winter conditions (see the light stippled line in Figure 1B). In addition, the mean eclosion time for flies in the long, cold pre-winter and short over-winter treatment was high. As a result, a significant overall three-way interaction was observed among pre-winter length, pre-winter temperature, and over-winter length treatments (P<0.0001; Table 1).

## Genetic responses to environmental treatments

Allozyme frequency differences among eclosing adults in the apple, northern hawthorn, and southern hawthorn treatments were consistent with predictions of our working model for *R. pomonella* diapause. Shorter and colder pre-winter periods, regardless of over-winter length, tended to result in increased frequencies of Dia-2 100, Me 100, and Had 100 alleles in eclosing adults (see Figure 2A-C and summary Figure 3A and B). Dia-2 100, Me 100, and Had 100 are the most common alleles in northern hawthorn flies like the East Lansing source population. In contrast to the northern hawthorn treatments, the longer and hotter pre-winter treatments emulating apple and southern hawthorn conditions shifted the frequencies of Dia-2 100, Me 100, and Had 100 downward toward levels found in apple and southern hawthorn flies. Once again, the results for the combination of short pre-winter and short over-winter rearing conditions contrasted with those for the apple, northern hawthorn, and southern hawthorn treatments. Here, Dia-2 100, Me 100, and Had 100 allele frequencies all increased rather than decreased with increasing pre-winter temperature and length (see light stippled lines in Figures 2A-C and 3C). Indeed, Had displayed a significant three-way interaction effect with prewinter temperature and length, and over-winter length (Figure 2C; Table 1).

### Allozyme effects on adult eclosion time

The allozymes *Dia-2*, *Me*, and *Had* all showed significant relationships with adult eclosion time (Figure 4; Table 2). Consistent with the results from previous studies (Feder et al., 1993, 1997a,b; Filchak et al., 2000) and in accord with the geographic pattern and latitudinal allozyme clines in the host races, flies possessing increasing numbers of *Dia-2*, *Me*, or *Had 100* alleles generally eclosed earlier within the apple, northern hawthorn, and southern hawthorn treatments than flies possessing alternate alleles at these three loci (Figure 4A–C). The primary exception were *Dia-2 70/100* heterozygotes in the northern hawthorn 10-day pre-winter, 18 °C temperature, 30-week winter treatment, that as a group eclosed later than other genotypes in this treatment (Figure 4A). In comparison, the relative order of eclosion for *Dia-2*, *Me*, or *Had* geno-



**Figure 2** Effects of pre-winter length and temperature, and winter length on (A) *Dia-2*, (B) *Me*, and (C) *Had 100* allele frequencies in eclosing flies. Significance levels for three-way interaction effects are given in Table 1 as determined by randomization ANOVA analyses. Sample size (n) = number of alleles scored. Dashed line represents allele frequencies for untreated control flies.



**Figure 3** Genetic responses for allozymes in eclosing flies across rearing treatment emulating (A) the transition from sympatric northern hawthorn to apple environmental conditions, (B) the transition from northern hawthorn to southern hawthorn environmental conditions, and (C) non-natural short pre-winter (10 days) and short winter (8 weeks) conditions with increasing pre-winter temperature.



**Figure 4** Mean eclosion times for (A) *Dia-2*, (B) *Me*, and (C) *Had* genotypes for flies in indicated rearing treatments. Significance levels for differences among allozyme genotypes in eclosion time are given in Table 2, as determined by ANOVA.

**Table 2** Main effects of the allozymes *Dia-2*, *Me*, and *Had* on adult eclosion time for indicated rearing treatment combinations of pre-winter length (10 or 30 days), pre-winter temperature (18 or 26 °C), and over-winter length (8 or 30 weeks), as determined by randomization version of an ANOVA model. Given are the proportion of randomization results which failed to exceed the observed results taken as a measure of statistical significance at the  $\alpha = 0.05$  level

Effect	Dia-2	Me	Had
10 days, 18 °C, 8 weeks	0.0420	0.0440	0.0443
10 days, 18 °C, 30 weeks	0.0007	0.0335	0.0023
30 days, 26 °C, 8 weeks	0.0168	0.9251	0.0384
30 days, 26 °C, 30 weeks	0.0315	0.9601	0.2152

types was reversed for flies in the short 10-day pre-winter length, low -18 °C pre-winter temperature, and short 8-week over-winter treatment (Figure 4A–C). In this case, flies possessing increasing numbers of *Dia-2*, *Me*, and *Had 100* alleles eclosed increasingly later than flies possessing the alternative alleles at these three loci indicative of a gene\*environmental interaction.

Tests for interaction effects among the allozymes on eclosion time were generally not significant. The allozymes usually contributed additively to eclosion time. However, *Dia-2* and *Had* did display a significant interaction effect with eclosion time in the northern hawthorn 10-day prewinter, -18 °C pre-winter temperature, 30-week winter treatment (P = 0.022, randomization test), where within the *Had 100/122* heterozygote genotype class, *Dia-2 85/85* homozygotes eclosed the earliest rather than the latest.

## Discussion

The current study examined the interactive effects of the three major environmental factors (pre-winter temperature, pre-winter length, and over-winter length) known to affect eclosion patterns and the genetics of R. pomonella flies, generating ecologically based reproductive isolation. Although complex, the results are most easily understood when partitioned into experimental treatments emulating environmental conditions normally vs. not normally experienced in nature. In this regard, rearing treatments emulating natural northern sympatric hawthorn, northern sympatric apple, and southern hawthorn conditions supported our working model for R. pomonella diapause and our a priori prediction concerning largely additive interactions among environmental factors on fly genetics. In contrast, short pre-winter and winter treatments produced unanticipated findings suggesting that we have yet to identify all of the interacting elements contributing to host and latitude-related life history adaptation in the fly.

Under more natural conditions faced by *R. pomonella* flies infesting apples, northern hawthorns, and southern hawthorns in the field, percentages of eclosing flies, eclosion time, and genetic responses for indicator allozyme loci coincided well with predictions.

Our working model for R. pomonella is based on the supposition that the allozyme alleles Dia-2, Me, and Had 100 are associated with faster development rates and shallower initial entry into diapause. These alleles are favored in northern hawthorn populations because hawthorns are relatively late fruiting in the field season. As a result, hawthorn flies in the north are selected to rapidly complete larval feeding and quickly enter diapause before the onset of winter. In contrast, the alternate southern or apple race alleles are associated with slower development rates and an initially deeper pupal diapause due to flies having to withstand longer and hotter pre-wintering periods in the South or in the earlier fruiting apple. Consequently, in the current experiment, hotter and longer pre-winter conditions shifted allozyme frequencies in eclosing flies in the direction of apple or southern hawthorn populations, whereas colder, shorter pre-winter periods induced changes toward northern hawthorn frequencies. Moreover, the effects of pre-winter temperature displayed a gene\*environment interaction with over-winter length (Figure 5). The longer the winter, the more pronounced the genetic shift toward apple race allozyme frequencies when pre-winter conditions were hot and long. In comparison, the longer 30-week winter increased selected for northern hawthorn race genotypes when pre-winter conditions were cold and short. Filchak et al. (2000) argued that this gene\*environment interaction accentuates diapause related genetic differentiation between the apple and hawthorn host races at sympatric sites. Genetic correlations with eclosion time in the apple, northern hawthorn, and southern hawthorn treatments also coincided well with previous lab and field observations (Feder et al., 1993, 1997a). Flies possessing increasing numbers of Dia-2, Me, and Had 100 alleles (northern hawthorn genotypes) tended to eclose earlier than other flies, in accord with these genes being associated with faster development rates and shallower diapause. The genetic correlations of Dia-2, Me, and Had 100 with earlier adult eclosion also agree with the clinal pattern of latitudinal allozyme variation in the hawthorn race. Hawthorn flies from northern populations eclose earlier than flies from southern populations when reared under standardized conditions (Dambroski & Feder, 2007) and possess higher frequencies of Dia-2, Me, and Had 100 alleles (Feder & Bush, 1989; Feder et al., 1990). However, the current results clash with the observation that apple flies from



**Figure 5** Two-way interactions between pre-winter temperature and winter length for (A) *Dia-2*, (B) *Me*, and (C) *Had 100* allele frequencies in eclosing adults. Significance levels for two-way interaction effects are given in Table 1, as determined by randomization ANOVA analyses. Sample size (n) = number of alleles scored. Dashed line represents allele frequencies for untreated control flies.

sympatric northern sites eclose earlier than hawthorn flies under standardized conditions (Dambroski & Feder, 2007). Because the apple flies generally have lower frequencies of *Dia-2*, *Me*, and *Had 100* alleles than hawthorn flies in the North, given the results of the current study, apple flies should eclose later not earlier than hawthorn flies. Thus, the current results underscore that other genes in addition to the allozymes are likely responsible for early eclosion in apple flies.

The most significant result from the current study, however, was that eclosion patterns and allele frequency shifts were diametrically opposed between short pre-winter and short winter treatments vs. natural apple, northern hawthorn, and southern hawthorn treatments. A seminal question is therefore whether the observed differences in the short pre-winter and short winter treatment signify a fundamental misconception concerning diapause regulation in *R. pomonella* or reflect missing details about the mechanism of action underlying the gene\*environment effects. We contend that the latter is likely and hypothesize that the condition of pupae could represent an important missing mediator of diapause timing and over-wintering survivorship. We term this the pupal energy reserve hypothesis.

We are attracted to the potential significance of pupal energy reserves for diapause for several reasons. First, the timing of many types of life history transitions in a broad diversity of organisms has been shown to be dependent on body condition. For example, metamorphosis timing in both anurans and insects is dependent on reaching a minimum threshold body size (Denver et al., 2002; Nijhout, 2003) and reproductive timing in insects, birds, and mammals is directly affected by body condition, particularly fat and protein reserves (Frisch, 1989; Juliano et al., 2004; Schoech et al., 2004; Hatle et al., 2006). Second, it is important that insects exit diapause with enough reserves to support post-diapause functions such as tissue building and metamorphosis, dispersal, and reproduction. As a consequence, a number of studies have suggested that diapause timing is affected by body condition wherein larger individuals with more nutrient reserves diapause for longer, survive diapause better, and enjoy greater post-diapause reproduction (reviewed by Hahn & Denlinger, 2007). Third, faster growth, due to increased anabolism, is typically also associated with greater rates of catabolism, which can remain elevated even after the end of the growth phase (Metcalfe & Monaghan, 2001; Criscuolo et al., 2008). Fourth, individuals that experience less metabolic demand by over-wintering in cooler sites, and therefore deplete less of their reserves, also tend to diapause longer and survive diapause better (Hahn & Denlinger, 2007).

We suggest that similar energy reserve considerations may apply to *R. pomonella* diapause. In particular, we

hypothesize that the diapause status of *Rhagoletis* pupae is determined, in part, by the levels of stored energy reserves they possess (Figures 6 and 7). The central elements of the hypothesis are the following:

1. Pupae must be above a certain threshold level of stored energy to maintain diapause (see stippled line to left in Figure 6D). Pupae that fall below this level will terminate diapause and attempt to complete adult development (see checkered line in center in Figure 6D). Non-diapause, direct development is therefore due to pupae exhausting their energy reserves and falling below the diapause threshold during extended periods of pre-winter heating. Pupae that exhaust their energy reserves and fall below the threshold during winter die (see solid line to right in Figure 6D).

**2**. Levels of stored carbohydrates and lipids are determined by the balance between resource accumulation during larval feeding and resource use during the prewinter transition to pupation and the over-wintering period of diapause maintenance (Figure 6A).

**3.** Longer, hotter pre-winter periods and longer overwintering periods put increasing strain on the energy resources of pupae, eroding the reserves flies need to survive, complete adult development, eclose, and reproduce (Figure 6B).

4. The allozymes *Dia-2*, *Me*, and *Had* (or linked genes) interact with environmental conditions to affect the net energy reserves pupae possess entering winter (Figure 6B). In particular, we hypothesize that the northern hawthorn alleles Dia-2, Me, and Had 100 are associated with higher total metabolism (i.e., greater rates of both anabolic and catabolic reactions) and that the alternative apple/southern hawthorn alleles equate with reduced metabolic levels. These genetic differences may generate countergradient selection in nature (Levins, 1969; Berven et al., 1979; Conover & Schultz, 1995; Blanckenhorn & Demont, 2004). Northern genotypes will experience short, unfavorable growing seasons (conditions) in the field more often than apple or southern genotypes, resulting in flies having similar fat storage phenotypes despite underlying genetic differences in growth rates.

**5.** The higher metabolism rates for the northern hawthorn alleles *Dia-2*, *Me*, and *Had 100* carry over from larval/pupal stages to post-diapause adult development (Figure 6D). As a result, flies possessing *Dia-2*, *Me*, and *Had 100* alleles use their reserves faster following diapause termination and tend to eclose the earliest.

The pupal energy reserve hypothesis potentially accounts for the seemingly anomalous results observed in the short, cold pre-winter, short 8-week winter treatment. The key



Energy reserve model for gene\*environment diapause interactons in *Rhagoletis* A Fly life cycle and pupal energy reserves

Figure 6 Pupal energy reserve model for Rhagoletis pomonella diapause. (A) Overview of fly natural history depicting relationship of various life history stages to the procurement and usage (loss) of stored energy reserves related to larval feeding, pre-winter pupal development, over-wintering, and adult eclosion. Ovals represent idealized levels of stored energy (carbohydrates and lipids) for pupae at different stages of the season. (B) Gene\*environment (G\*E) interaction of differential allozyme efficiency with pre-winter temperature depicting how under colder conditions northern sympatric hawthorn genotypes (i.e., individuals possessing the alleles Dia-2 100, Me 100, and Had 100) tend to have higher pupal energy reserves entering winter than sympatric apple/southern hawthorn genotypes, whereas the reverse is the case under warmer conditions. The lengths of the dark arrows in the figure are proportional to the amount of energy obtained during larval feeding and the white arrows are proportional to the amount of energy expended to enter and maintain pupal diapause during the ensuing pre-winter period. The net difference between the arrows, schematically depicted by the stippled lines and the accompanying ovals, represent the stored energy level pupae have to survive winter, break diapause, and complete adult development. (C) Relationship between stored energy levels for pupae during winter and the diapause status and potential for adult development and eclosion of flies. Pupae with stored energy levels above the threshold for diapause breakage (checkered line) will tend to remain in diapause for a delayed period following heating until reserves fall below the threshold, sometimes requiring a second chilling:heating cycle. Pupae with reserves below the break threshold and above the minimum level needed for adult development (dark solid line) will immediately initiate the completion of adult development under conducive (warm) environmental conditions. Pupae falling below the minimal level will not survive and die due to having inadequate reserves to maintain core metabolic needs in the cold and/or complete adult development upon initiation. (D) Relationship of fly genotypes to adult eclosion time. The higher metabolic rate of flies possessing Dia-2, Me, and Had 100 alleles results in their completing post-diapause development faster than alternate genotypes.



**Figure 7** Predictions of the energy reserve model for allele frequency, survivorship, and eclosion time responses of flies to varying environmental rearing treatments. Shown are idealized energy reserve distributions for northern (N) vs. southern (S) genotype pupae at the end of the prescribed over-wintering period in relation to threshold levels for delayed diapause termination, diapause breakage, and fly death depicted in C. Bracketed lines indicate the proportions of pupae in the energy reserve distributions for N and S genotypes predicted to eclose following heating. Arrows translate the consequences of predicted differential eclosion patterns to allozyme allele frequency, survivorship, and emergence time variation among rearing treatments. Actual experimental values for survivorship (% eclosing flies), mean *Dia-2, Me*, and *Had 100* allele frequency (N freq.), mean eclosion time in days, and whether S or N genotypes eclosed first are also given for each treatment below the energy reserve distributions. Note the positive signs of the slopes for the genetic, survivorship, and eclosion order of genotypes responses to increased pre-winter heating in the 10-day pre-winter, 8-week winter treatment (A) compared to the opposite (negative) signs for the other treatments (B–D), indicative of G\*E interactions.

consideration is that this treatment may have stressed, but did not kill, pupae possessing apple/southern fly genotypes. In contrast, northern genotypes were not stressed and still maintained large energy reserves following 8 weeks of over-wintering. Consequently, apple/southern fly genotypes immediately terminated diapause and eclosed following the 8-week winter treatment, whereas northern genotypes did not (see 18 °C pre-winter temperature treatment in Figure 7A). As a result, the overall percentage of adult eclosion was low, a high frequency of eclosing flies possessed apple/southern alleles, and flies possessing apple/southern hawthorn genotypes eclosed earlier, not later, than individuals possessing *Dia-2*, *Me*, and *Had 100* alleles. After 30 weeks of winter treatment, however, many apple/southern fly genotypes exposed to short, cold pre-winter conditions had depleted their resources and died (see 18 °C pre-winter temperature treatment in Figure 7B). However, hawthorn/northern genotypes were now in good condition for emergence. As a result, the overall percentage of adult eclosion increased, a high frequency of eclosing flies possessed hawthorn/northern alleles, and flies possessing hawthorn/northern genotypes eclosed earlier, not later, than individuals possessing *Dia-2 70, Me 80*, and *Had 122* alleles.

Empirical support for the energy hypothesis can be found in a larval rearing study performed by Filchak et al. (1999). In this experiment, field-collected hawthorn fruits were placed in protected conditions that slowed the rate of fruit rot vs. exposed conditions that promoted rapid fruit rot. The rationale was that accelerating the rate of fruit rot should favor rapidly developing larvae. The results were consistent with this prediction; individuals surviving the rapid fruit rot treatment possessed higher frequencies of northern hawthorn race alleles, had a greater probability of successfully completing development, and greater overall body size, suggesting that these genotypes have higher growth rates.

If correct, the diapause energy hypothesis could have important implications for understanding insect/host plant interactions. Although many insect groups have radiated via shifting and adapting to novel host plants (Berlocher & Feder, 2002; Drès & Mallet, 2002), it is not always clear why certain plants have been successfully utilized, whereas others have not. Part of the answer certainly relates to plant defensive secondary compounds (Agrawal et al., 1999), part of the reason likely involves the presence of interspecific competitors and parasites (Denno et al., 1995; Stewart, 1996), and part of the cause may be due to limitations in the insect sensory system for recognizing alternative hosts (Bernavs & Wcislo, 1994; Bernavs, 1998). However, it is still puzzling why seeming well-suited potential hosts [e.g., serviceberry, Amelanchier spec. (Rosaceae) in the case of *R. pomonella*] are often not attacked. The interaction of host nutritional quality, host phenology, insect over-wintering energy demands, and genetics may play a key role here. It could well be that an insect could survive perfectly well on a novel host during favorable growing periods but suffers later because it is inadequately provisioned for winter or ecloses at an inopportune time during the subsequent field season.

In conclusion, R. pomonella provides a model to investigate the physiology and genetics of diapause in the context of geographic and host plant-related adaptation. In the case of the apple and hawthorn host races, this ecological adaptation has added significance in generating reproductive isolation contributing to incipient sympatric speciation. The current study revealed unexpected responses for flies in short pre-winter and over-winter treatments suggesting that additional physiological factors may influence R. pomonella diapause. Part of the mechanism of action of the allozymes (or linked genes) may involve energy storage and metabolic processes that secondarily affect diapause rather than directly setting the diapause timing phenotype. Regardless, anomalous results may sometimes be a blessing and not uncommon when conducting studies of natural selection, even when the genetics and physiology of a system seem straight-forward (e.g., see the Ectodysplasin locus for threespine stickleback fish; Barrett et al., 2008). Rather, these unanticipated findings can provide an opportunity for resolving important aspects of the biology of a system otherwise missed. We believe that this is the case

for *R. pomonella*. It will be interesting to determine whether and how lessons learned concerning diapause in *R. pomonella* translate to other holometabolous insects that over-winter as larvae or pupae. As we move into the genomics era for non-model genetic but model ecological systems, such comparisons will become increasingly possible. The results portend a rich database for discerning the physiological and genetic mechanisms for diapause and placing the process in the context of how insects respond to critical life history challenges, generating biodiversity.

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