

Selective maintenance of allozyme differences among sympatric host races of the apple maggot fly

(sympatric speciation/fitness trade-offs/host plant phenology/*Rhagoletis pomonella*/host x environment interactions)

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ABSTRACT Whether phytophagous insects can speciate in sympatry when they shift and adapt to new host plants is a controversial question. One essential requirement for sympatric speciation is that disruptive selection outweighs gene flow between insect populations using different host plants. Empirical support for host-related selection (i.e., fitness trade-offs) is scant, however. Here, we test for host-dependent selection acting on apple (*Malus pumila*)- and hawthorn (*Crataegus* spp.)-infesting races of *Rhagoletis pomonella* (Diptera: Tephritidae). In particular, we examine whether the earlier fruiting phenology of apple trees favors pupae in deeper states of diapause (or with slower metabolisms/development rates) in the apple fly race. By experimentally lengthening the time period preceding winter, we exposed hawthorn race pupae to environmental conditions typically faced by apple flies. This exposure induced a significant genetic response at six allozyme loci in surviving hawthorn fly adults toward allele frequencies found in the apple race. The sensitivity of hawthorn fly pupae to extended periods of warm weather therefore selects against hawthorn flies that infest apples and helps to maintain the genetic integrity of the apple race by counteracting gene flow from sympatric hawthorn populations. Our findings confirm that postzygotic reproductive isolation can evolve as a pleiotropic consequence of host-associated adaptation, a central tenet of nonallopatric speciation. They also suggest that one reason for the paucity of reported fitness trade-offs is a failure to consider adequately costs associated with coordinating an insect's life cycle with the phenology of its host plant.

Host plant-associated fitness trade-offs are critical for sympatric speciation in phytophagous insects (1). Fitness trade-offs refer to trait(s) or gene(s) that confer a selective advantage to an insect on one plant while incurring a cost on alternative plants. Such trade-offs are important because they can act as postzygotic barriers to gene flow between insect populations specialized on different host plants (1).

There is currently little empirical evidence for host plant-related fitness trade-offs in phytophagous insects (ref. 2, although see refs. 3–5 for some exceptions), which has raised questions concerning the likelihood of sympatric speciation (6). But many tests for fitness trade-offs have concentrated on larval feeding performance and the metabolic detoxification of plant secondary compounds while neglecting potential costs associated with coordinating an insect's life cycle with host plant phenology (7). Here, we test for fitness trade-offs in apple (*Malus pumila*)- and hawthorn (*Crataegus* spp.)-infesting populations of the apple maggot fly *Rhagoletis pomonella* (Diptera: Tephritidae) by examining whether and how differ-

ences in the fruiting phenologies (seasonalities) of apple and hawthorn trees affect the genetics of these flies.

Previous studies have confirmed the status of apple and hawthorn populations of *R. pomonella* as genetically differentiated and partially reproductively isolated host races (8–12), the initial stage in sympatric speciation. However, tests for host plant-associated fitness trade-offs in the fly have all failed. In particular, reciprocal egg transplant experiments performed by Prokopy *et al.* (13) gave no evidence for larval feeding specialization related to chemical or nutritional differences between apples and hawthorns. Apple and hawthorn race larvae survived equally well in hawthorn fruits. Both races fared equally poorly in apples. Although these results are in accord with hawthorns being the ancestral host of *R. pomonella*, they are discouraging with respect to the issue of fitness trade-offs.

Could the failure to document fitness trade-offs in *R. pomonella* be due to overlooking the interaction between fly development and host plant phenology? Apples and hawthorns represent different temporal resources. Fruits on apple varieties favored by *R. pomonella* generally ripen ≈ 3 weeks earlier in the season than hawthorns (12). One consequence of this difference is that larvae leave abscised apple fruits and pupate an average of 16 days earlier than they do from hawthorns (14). Many *R. pomonella* pupae exposed to elevated temperatures fail to enter a protracted diapause (15). In nature, such “nondiapausing” flies are doomed because they either eclose in the fall when host fruits are not present (16) or start, but do not complete, adult development before the onset of winter, deplete their energy reserves, and die. The exposure of apple-origin pupae to longer periods of warm weather may therefore favor flies with deeper diapauses or with lower metabolisms/slower development rates in the apple race. Selection pressures are likely to be different for hawthorn flies. The relatively late phenology of hawthorns means that slow developing hawthorn flies may not enter diapause quickly enough before the onset of winter to avoid freezing to death. We shall refer to this explanation as the “diapause trade-off hypothesis.”

In this communication, we report on a test of the diapause trade-off hypothesis in which we experimentally altered the prewintering period for hawthorn-origin pupae in an attempt to induce a genetic response at six allozyme loci displaying host-associated differentiation (referred to henceforth as the “prewinter experiment”). Our rationale was that lengthening the prewintering period would expose hawthorn pupae to extended periods of warm weather, conditions that they would face if they had infested a host plant with an earlier fruiting phenology, such as apples. Such treatments would be expected to cull pupae in shallow states of diapause from the hawthorn population and change allozyme frequencies in the direction of the apple race. Conversely, brief prewinter treatments should favor rapidly developing pupae that quickly enter diapause.

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MATERIALS AND METHODS

The Life History of *R. pomonella*. *R. pomonella* are univoltine (for reviews of the fly's biology, see refs. 1, 17, and 18). Adult females lay their eggs into the ripening fruit of the appropriate host plant, which they identify by specific visual, olfactory, and tactile cues. Males recognize the same cues and mating occurs exclusively on or near the fruit of the host plant. Larval feeding and development are confined to the fruit chosen by a larva's mother for oviposition. When a mature fruit ripens and falls to the ground, larvae leave the fruit and burrow into the soil, where they pupate. Flies over-winter in a facultative pupal diapause. A majority (92–98%) of pupae break diapause and eclose as adults the following summer (16, 19). But a few pupae take more than one cycle of chilling and heating to complete development (16, 19).

Genetic Differences Between the Host Races. Six allozyme loci [malic enzyme (*Me*), aconitase-2 (*Acon-2*), mannose phosphate isomerase (*Mpi*), NADH-diaphorase-2 (*Dia-2*), aspartate amino transferase-2 (*Aat-2*), and hydroxyacid dehydrogenase (*Had*)] display significant allele frequency differences between apple and hawthorn fly populations across eastern North America (8–12). These six allozyme loci map to just three different regions of the *R. pomonella* genome (20–22). *Aat-2* and *Dia-2* are located on linkage group I, separated by 0.6 centimorgans (22). *Me*, *Acon-2*, and *Mpi* are all tightly linked on group II; not a single recombinant was observed among these three loci in 162 F1 progeny scored from single-pair test crosses (21). Finally, *Had* maps to linkage group III. Significant levels of linkage disequilibrium exist among loci within, but not between, linkage groups I, II, and III in natural *R. pomonella* populations (8, 10). For example, correlation coefficients derived from Burrow's composite genotypic disequilibrium values between *Aat-2 50* and *Dia-2 100* and between *Me-100* and *Acon-2 95* are as large as -0.78 and 0.54 , respectively (10).

Evidence suggests that the six allozymes displaying inter-race differences are associated with fly development. Adults eclosing the earliest in both host races possess high frequencies of *Me 100*, *Acon-2 95*, *Mpi 37*, *Aat-2 +75*, *Dia-2 100*, and *Had 100* (12). These six alleles also tend to be more common to the hawthorn than the apple race (8, 10).

The Prewinter Experiment. Infested fruits were collected from beneath a hawthorn tree at a field site near the town of Grant, MI on September 15, 1989. Fruits were placed on wire screens above plastic trays, and the trays were stored at 26°C in a constant temperature room with a 15:9 h light:dark cycle. Puparia were collected from the plastic trays on a daily basis and divided into seven subsamples. One daily subsample was immediately frozen to serve as an untreated genetic control. Flies also were collected from an apple tree at the Grant site on August 15, 1989 to provide a genetic baseline for the apple race. Pupae from the remaining six hawthorn subsamples were put into Petri dishes that contained moist vermiculite, and the Petri dishes were held at 26°C in a constant temperature room for 2, 7, 14, 21, 28, or 32 days. After this time, the Petri dishes were placed in cold storage at 4°C in a refrigerator to simulate winter. Petri dishes were removed from the cold 30 weeks later and placed in an incubator at 21°C with a 14:10 h light:dark cycle. Adult flies were collected on a daily basis as they eclosed from within the Petri dishes during the periods preceding (nondiapausing flies) and after (diapausing flies) chilling. The Petri dishes were monitored for 5 months after their removal from the cold before being returned to the refrigerator to be exposed to a second yearly cycle of chilling and heating. We repeated the cycle to ensure that flies in very deep diapauses or with 2-year life cycles had adequate time to eclose in the prewinter experiment. However, no adults eclosed during the second year of the study. Flies were genetically scored using standard starch gel electrophoretic techniques (20, 21). In

addition to *Me*, *Acon-2*, *Mpi*, *Aat-2*, *Dia-2*, and *Had*, we also scored flies for isocitrate dehydrogenase (*Idh*). *Idh* was included as a control locus because it does not display inter-host differentiation at the Grant site (8, 10).

The diapause trade-off hypothesis leads to three predictions about the prewinter experiment. (i) Hawthorn flies that survive long prewinter treatments (i.e., 32 days) and eclose after the 30-week over-wintering period should be genetically similar to apple flies. Because such treatments expose hawthorn pupae to environmental conditions that we believe simulate those faced by apple flies, we should observe a genetic response in the direction of the apple race. (ii) Nondiapausing flies that eclose before winter should display high frequencies of *Me 100*, *Acon-2 95*, *Mpi 37*, *Aat-2 +75*, *Dia-2 100*, and *Had 100*, the allozymes associated with earlier eclosion times, and by implication faster development rates (12). (iii) Brief prewinter treatments (i.e., 2 days) should favor the alleles *Me 100*, *Acon-2 95*, *Mpi 37*, *Aat-2 +75*, *Dia-2 100*, and *Had 100* because of the need for flies to enter diapause quickly before being exposed to cold temperatures.

Originally, we had hoped that the prewinter experiment could test prediction (iii), that brief prewinter treatments favor allozymes associated with rapid fly development. However, after the prewinter experiment and a related study examining the effects of winter length were completed, we discovered that the 30-week chilling period at 4°C used in the prewinter experiment represented a relatively long and warm winter for hawthorn flies at the Grant site. These conditions acted as a selective counterbalance to the brief prewinter treatments and so were inappropriate for testing prediction (iii). The results we report here therefore address predictions (i) and (ii) of the diapause trade-off hypothesis.

We also must stress that the prewinter experiment is not designed to test whether the allozymes themselves are directly under selection. Rather, its purpose is to infer the relationship between host phenology and pupal development through an analysis of allele frequency changes induced at allozyme loci. As such, the allozymes should be viewed as genetic markers tagging three regions of the *R. pomonella* genome known to differ between the races and that have been associated with a diapause-related trait, the timing of adult eclosion. We assume that any genetic response observed for the allozymes will, at the very least, be indicative of selection at linked loci. Of course, if the allozymes are indirectly responding to selection via linkage, then the prewinter experiment will underestimate the true magnitude of the genetic response.

RESULTS

Survivorship (i.e., over-wintering success) decreased in the prewinter experiment when the prechilling period was increased beyond 7 days (Fig. 1). The increase in mortality was caused by both direct and indirect factors. Indirect mortality was due to prewinter treatments ≥ 28 days causing flies to eclose as nondiapausing adults (Fig. 1). This eclosion represented indirect mortality because, even though these flies were not immediately killed, they would have zero fitness in nature, eclosing in the fall when host fruit was no longer present. Direct mortality could be inferred from the decline of diapausing flies eclosing between the 7 and 32-day treatments (Fig. 1). This mortality was likely caused by increasing numbers of pupae breaking diapause in the 14- to 32-day treatments and having insufficient time to complete adult development before chilling. It is also conceivable that prolonged prewintering periods induced an extended pupal diapause in flies. If this was true, then flies did not actually die in the 14- to 32-day treatments but instead remained entrenched in diapause throughout the course of the study. However, we monitored adult eclosion for 150 days after the first over-wintering cycle (an adequate period of time for *R. pomonella* eclosion) and

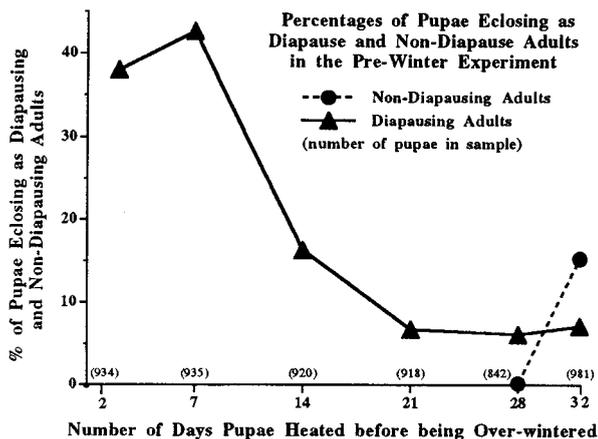


FIG. 1. Percentages of hawthorn-origin pupae that eclosed as diapausing adults (▲, flies eclosing after 30-week chilling period) and nondiapausing (●, flies eclosing before chilling) for the 2- to 32-day prewinter treatments. Total number of pupae in each prewinter treatment are given in parentheses.

also observed no adults eclosing after the second yearly cycle of chilling and heating. These two considerations argue against the extended diapause scenario.

Genetic analysis of the prewinter experiment supported prediction (i) of the diapause trade-off hypothesis. *Me 100*, *Acon-2 95*, *Mpi 37*, *Dia-2 100*, and *Aat-2 +75* frequencies all decreased (became more “apple-like”) in diapausing hawthorn flies surviving the longer prewinter treatments (Figs. 2 and 3). Allozyme frequencies did not decline in a stringently linear fashion with survivorship, however (Figs. 2 and 3). For example, a significant decrease in the percentage of diapausing flies was seen by the 21-day treatment (Fig. 1). But, several of the loci responded most dramatically to selection between the 28- and 32-day treatments (Figs. 2 and 3). Nevertheless, we came close to transforming the hawthorn race genetically into the apple race after only a single generation of mass selection by the 32-day treatment. The prewinter treatments did not significantly affect allele frequencies at the control locus *Idh* (data not shown).

Genotypes showed similar responses to selection as allele frequencies, at least for the loci *Me* and *Acon-2* (Note: We confined our analysis to *Me* and *Acon-2* because frequencies for certain genotypes of the other allozymes were too low to draw any meaningful conclusions.) Comparisons of genotype frequencies between diapausing flies surviving the 32-day treatment and the control sample were used to estimate relative fitnesses for *Me* and *Acon-2* genotypes. The results indicated that *Me 100* and *Acon-2 95* homozygotes suffered the highest mortality (mean relative fitness for *Me 100/Me 100* homozygotes = 0.343 and for *Acon-2 95/Acon-2 95* homozygotes = 0.407), followed by heterozygotes (mean fitnesses = 0.552 and 0.496, respectively), and then non-*Me 100* and *Acon-2 95* homozygotes (mean fitnesses standardized to 1.00). The mortality induced by prolonged heating was therefore most heavily inflicted on individuals with “hawthorn fly genotypes,” but hybrids between the races are also predicted to be adversely affected by selection.

The outcome of the prewinter experiment also was consistent with prediction (ii) of the diapause trade-off hypothesis. Nondiapausing flies in the 32-day treatment had higher *Me 100*, *Acon-2 95*, *Mpi 37*, *Dia-2 100*, *Aat-2 +75*, and *Had 100* frequencies than either 32-day diapausing flies or the untreated hawthorn fly control (Figs. 2 and 3).

DISCUSSION

The results from the prewinter experiment support the diapause trade-off hypothesis. Hawthorn-race flies, when exper-

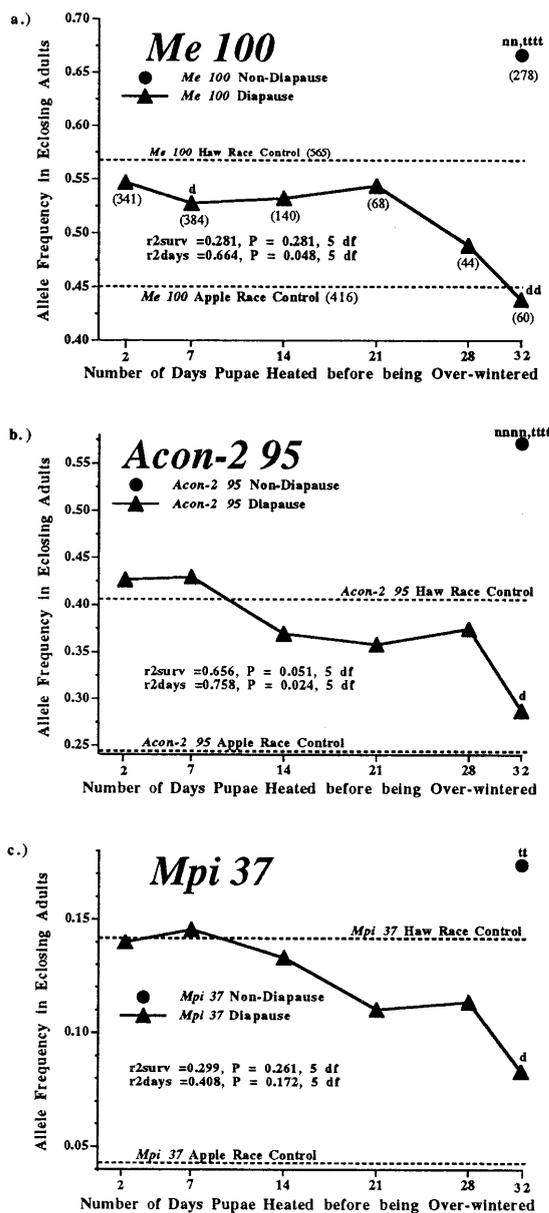


FIG. 2. (a–c) Allele frequencies for *Me 100*, *Acon-2 95*, and *Mpi 37* in diapausing (▲) and nondiapausing (●) hawthorn -adults in the 2- to 32-day prewinter treatments. Allele frequencies for baseline apple and hawthorn fly control samples are designated by dashed lines. Sample sizes (number of flies scored) are given in parentheses. d, significant allele frequency difference between diapausing flies and hawthorn control sample as determined by one-tailed, randomized Fisher exact tests; n, significant difference between nondiapausing flies and hawthorn control sample; t, significant difference between diapause and nondiapause flies in the 32-day treatment. Number of letters designates significance level (one letter, $P < 0.05$; two, $P < 0.01$; three, $P < 0.001$; and four, $P < 0.0001$). Linear regressions (r^2 and P values, 5 df) for arcsin-transformed allele frequencies vs. percentage survivorship (surv) and length of prewinter treatment (days) also are given.

imentally challenged with the apple tree phenology, evolved toward the naturally occurring allozyme marker frequencies of the apple race of flies. From this finding, we inferred that prolonged exposure to warm weather favors pupae in deeper states of diapause or with lower metabolic/development rates in the apple than the hawthorn race. Although further studies are needed to pinpoint the exact physiological basis for the adaptation, the basic messages are clear: Host plant-associated fitness trade-offs exist for *R. pomonella*, and they are related to host phenology.

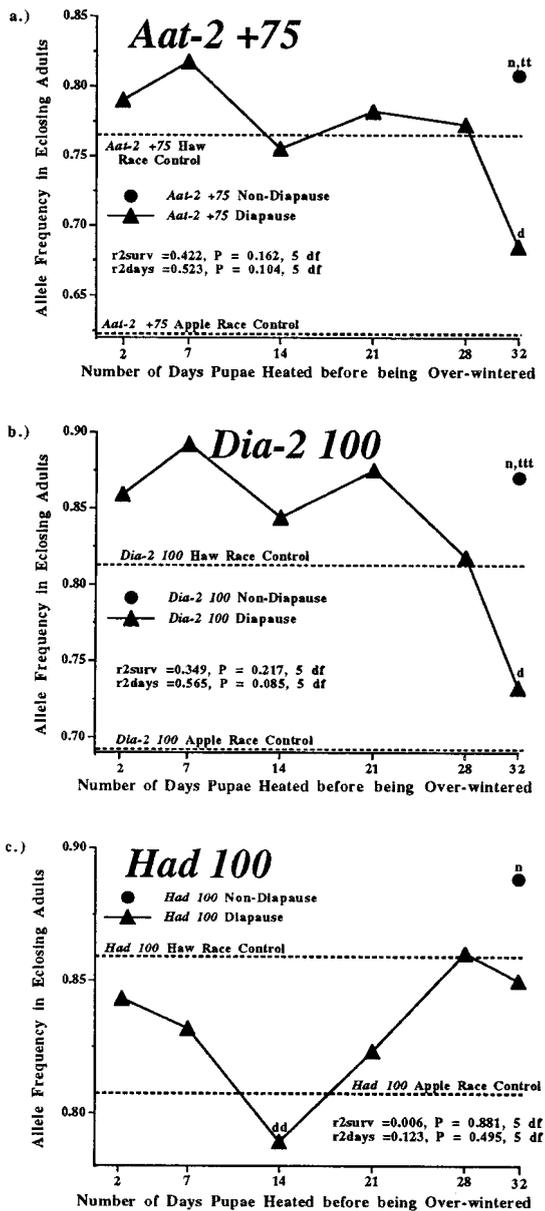


FIG. 3. (a–c) Allele frequencies for *Dia-2 100*, *Aat-2 +75*, and *Had 100* in diapausing (▲) and nondiapausing (●) hawthorn adults in the 2- to 32-day prewinter treatments. See Fig. 2 legend for complete details. +75 for *Aat-2* refers to the pooled frequency of all alleles of electrophoretic mobility ≈ 75 relative to the most common 100 allele. Previous studies have indicated that the +75 and –75 classes of *Aat-2* alleles are in very strong linkage disequilibrium with the 100 and 70 alleles, respectively, at the *Dia-2* locus (7, 9, 26).

How strong is host-associated selection in *R. pomonella*? Are selection pressures imposed by host phenology strong enough to account for the continued differentiation of the apple fly race in the face of gene flow from hawthorn populations? At least for the allozymes, the answer is yes. Mark–release–recapture studies indicate that host-specific mating limits genetic exchange between the host races at the Grant site to $\approx 6\%$ per generation (23). So, selection must counterbalance 6% gene flow each generation to maintain the genetic status quo for the races. If we assume that the control fly samples represent allozyme frequencies in the races after gene flow but before selection, then the following relationships result:

$$A_c = 0.94 A_o + 0.06 H_o \text{ and } H_c = 0.06 A_o + 0.94 H_o$$

where A_c and H_c are allozyme frequencies at a locus in the apple and hawthorn race controls, and A_o and H_o are allozyme frequencies in the apple and hawthorn races before gene flow.

By solving these equations for A_o and given A_c , we can estimate the magnitude of selection coefficients (s values) needed to maintain allozyme differences for the apple race. For *Me 100*, *Acon-2 95*, *Aat-2 +75*, and *Had 100*, these coefficients are 0.063, 0.136, 0.074, and 0.041, respectively, under an additive fitness model. We do not know which prewinter treatments would be most appropriate to use for a comparison of the conditions faced by the host races. However, we do know that apple flies pupate ≈ 16 days earlier than hawthorn flies at the Grant site (14). We could therefore get a relative gauge of the force of selection differentiating the races by comparing allele frequencies in the hawthorn control sample with those in the 14-day treatment, the 26°C, 15:9 h light:dark conditions used to rear pupae in the prewinter experiment being close to those experienced by apple flies at the height of the field season. Based on this criterion, selection coefficients against *Me 100*, *Acon-2 95*, *Aat-2 +75*, and *Had 100* homozygotes under an additive fitness model are 0.264, 0.254, 0.083, and 0.552, respectively. These coefficients are greater than those needed to counteract 6% gene flow from the hawthorn into the apple race.

An important implication of the diapause trade-off hypothesis that we have yet to discuss is that allozyme frequencies within the host races should track local ambient temperature conditions because temperature affects both the diapause status of flies and when host trees fruit (24, 25). Two lines of evidence support such a relationship. First, *Me*, *Acon-2*, *Mpi*, *Dia-2*, *Aat-2*, and *Had* display latitudinal allele frequency clines among both apple and hawthorn fly populations in the United States (10, 11, 26), with populations from southern latitudes having high frequencies of alleles more common to the apple race at the Grant site. Second, allozyme frequencies in the hawthorn race at the Grant site for all loci except *Had* were significantly related to ambient temperatures in the spring of the preceding year over an 11-year period (Fig. 4). Because a majority of pupae break diapause after their first winter (16, 19), flies sampled in year N are representative of pupae that survived the previous season (year N – 1). High spring temperatures in year N – 1 meant an early field season and were associated with increased frequencies in year N of alleles common to the apple race (or southern populations). Conversely, cold springs selected for alleles more common in northern populations. The apple race showed similar trends across years at the Grant site, but the regression coefficients were smaller than those in the hawthorn race and were not significant (Fig. 4). This pattern suggests that the early phenology of apples has reduced the apple race's capacity to respond genetically to thermal variation [Note: The apple race also displays less steep frequency clines than the hawthorn race (10, 11).] The diapause trade-off hypothesis therefore accounts not only for differentiation between the races but also for the relationships between climatic conditions and temporal and geographic genetic variation within the races. Consequently, our findings have a direct bearing on nature and suggest that the apple race was derived from the slowest developing portion of the hawthorn fly emergence distribution.

One question that remains is why changes in allozyme frequencies did not show a strict linear relationship with survivorship in the prewinter experiment. One possibility is that the allozymes have a complicated genetic relationship with diapause. In this regard, dominance, threshold effects, epistatic interactions, or loose linkage between the allozymes and target loci under selection (if the allozymes are not the foci of selection) could all cause a significant departure from linearity. Mortality factors unrelated to the allozymes, such as death due to disease, parasitism and desiccation, also could

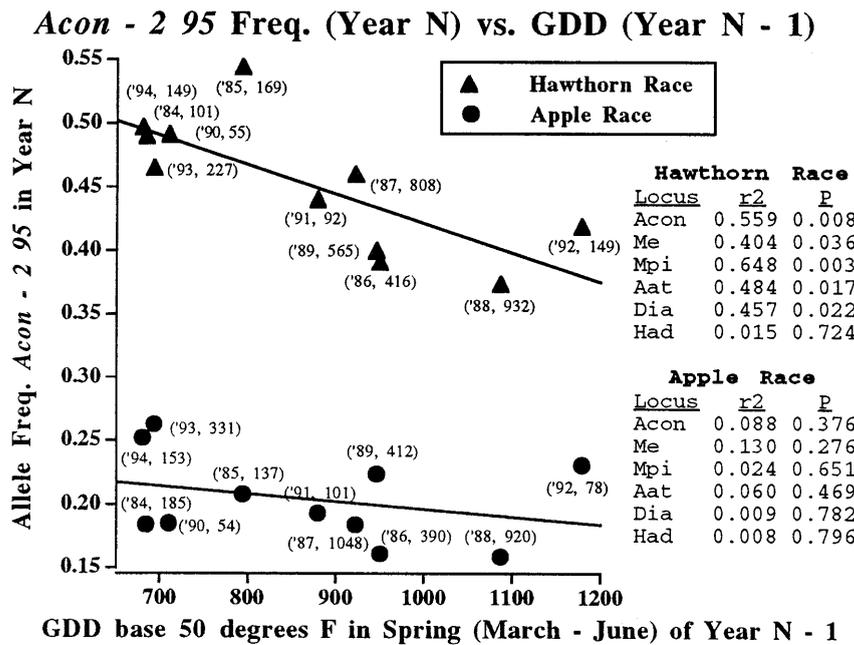


FIG. 4. Allele frequencies for *Acon-2 95* in apple (●) and hawthorn (▲) fly races at Grant, MI, from 1984–1994 (year N) plotted against growing degree days (GDD) base 50°F in the spring (March–June) of the preceding year (N – 1). Year numbers (N) and sample sizes (number of flies scored) for allozymes appear in parentheses. Linear regressions (r^2 and P values, 10 df) for arcsin-transformed allele frequencies vs. growing degree days for all of the allozymes are given in the table.

have contributed to the observed discrepancy. Finally, the binomial variance associated with estimates of gene frequencies, together with the division of hawthorn pupae into control and treatment subsamples, would further disrupt the relationship between survivorship and the allozymes. It is therefore not surprising that we did not find perfect declines in allele frequencies. The important point is that we found a genetic response to selection. Future studies can be designed to unravel the cause(s) for departure from strict linearity in the prewinter experiment.

In conclusion, we have shown that postzygotic reproductive isolation can evolve as a consequence of *R. pomonella* flies adapting to phenologically different host plants, a key tenet of sympatric speciation. This isolation is ecologically based: Genotype \times environment effects, not detrimental gene \times gene interactions due to the mixing of incompatible genomes, are causing postmating isolation. As such, apple and hawthorn fly races represent a special class of ecological polymorphism, one in which the polymorphism results in partial reproductive isolation by affecting patterns of mating and gene flow.

Finally, studies seeking fitness trade-offs must consider the ramifications of host phenology across the entire life history of an insect and not just focus on feeding stages (7). We have identified one aspect of host-dependent selection in *R. pomonella* related to the prewintering period. There are others; we have found that the duration of winter and the speed with which fruits rot also differentially select on allozyme loci in the host races (unpublished work). A panoply of host-associated fitness trade-offs may therefore await discovery in phytophagous insects, urging caution about extrapolating from the current paucity of examples.

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