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Environmental interactions during host race formation: host fruit environment moderates a seasonal shift in phenology in host races of *Rhagoletis pomonella*

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Summary

1. Host race formation is a common form of ecological speciation during which new populations that exploit a novel host (e.g. nutritional resource) experience divergent natural selection, causing adaptive divergence from the ancestral population. Typically, multiple selection pressures drive this divergence, suggesting that interactions among environmental effects may be critical during the speciation process.

2. Host-race-forming phytophagous insects often experience divergent natural selection imposed by seasonality and nutritional environment, two factors likely to interact through their effects on growth and life-history timing. We tested for the presence of such an interaction in the apple maggot fly, *Rhagoletis pomonella*, asking whether nutritional effects interact with seasonality to determine overwintering success.

3. We find evidence for such an interaction, wherein feeding on a novel host fruit actually mitigates the negative effects of novel seasonality. Feeding on apple (novel) compared to hawthorn (ancestral) fruit conferred greater fly lipid reserves the overwintering, diapause stage. Earlier, seasonal emergence characteristic of the apple host race imposes an overwintering survival cost. But feeding on apples offsets this cost, which confers greater pupal lipid reserves and consequently larger adult body size post-winter. Seasonality and host fruit interact to determine fly lipid reserves, and host race differences in lipid content are maximized under the most stressful conditions typically experienced by the apple host race. F1 rearing and genetic association tests revealed no evidence for genetically based divergence in lipid content, suggesting that differences in lipid storage among the fly host races are driven primarily by the host fruit environment.

4. Our results suggest that interactions between seasonality and host plant environment shape natural selection and therefore influence adaptive divergence during host race formation.

Key-words: diapause, life history, lipids, physiology, *Rhagoletis*, seasonality, speciation

Introduction

Host race formation is a common mechanism of ecological speciation, wherein a derived population (host race) that mates and feeds on a novel resource forms from an ancestral population that mates and feeds on a different resource (Bush 1969). Examples from diverging host races of phytophagous insects provide the bulk of the empirical evidence for this phenomenon, demonstrating that natural selection drives reproductive isolation (Berlocher & Feder 2002; Fordyce 2010).Insect host races typically diverge along three major ecological axes: ability to locate resources (Sheck & Gould 1995; Sezer & Butlin 1998; Dambroski *et al.* 2005), feeding-related performance (Ehrlich & Raven 1964; Ferrari, Via & Godfray 2008) and timing of life-history events (i.e. a change in seasonality) to coincide with seasonal resource availability (Feder & Filchak 1999; Dopman, Robbins & Seaman 2010; Ording *et al.* 2010). Divergence along each of these ecological axes has been explored

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in speciating populations, but interactions between these axes have rarely been considered in the context of host race formation.

Here, we explore an interaction between seasonality and nutritional resource environment in host races of a phytophagous insect. Interactions between the resource environment and selection for seasonal synchrony are likely to occur because resource quality can affect development time, body size and energy storage, all of which may affect the timing of critical life-history transitions. For example, the immature larva is the primary feeding stage in many insects, and selection can drive host race differences in reproductive/oviposition timing when tissues of the novel host plant on which the larvae feed (e.g. leaves or fruits) are available at different times of the year compared to the ancestral host plant (Feder & Filchak 1999). However, growth and development rates also affect time to sexual maturity, thus selection to synchronize reproductive timing with resource availability may depend upon the quality of the resource itself.

Growth and development rates may also affect seasonal timing through the diapause response. Many insects use seasonal dormancy, termed diapause, to synchronize their life cycles with favourable times of resource availability and to mitigate the stresses of inclement seasons (Tauber, Tauber & Masaki 1986).The timing of initiation and termination of diapause determines the seasonal cessation and the onset of growth and reproduction, and both transitions may be influenced by growth rate and body size prior to diapause (Tauber, Tauber & Masaki 1986; Gotthard, Nylin & Wiklund 1999; Hahn & Denlinger 2007).

Both seasonal timing and host resource effects appear to play critical roles in the divergence of the hawthorn and apple host races (hereafter referred to as 'apple flies' and 'hawthorn flies') of the apple maggot fly, *Rhagoletis pomonella* (Walsh), a model for sympatric speciation. A derived host race of *R. pomonella* that mates on and infests (i.e. oviposits into) recently introduced apple (*Malus domesticus*) has formed from an ancestral host race that mates on and infests North American native hawthorn (*Crataegus* spp.) fruit (Bush 1969). One key to resource-driven divergence is that each host fruit is only available for a short time window each year, and this time window differs between apple fruits and hawthorn fruits. At sympatric field sites, apples fruit about 3 weeks earlier than hawthorns. *Rhagoletis pomonella* has only one generation per year, adult flies are short-lived (*c.* 4 weeks), and the difference in fruiting time exerts strong differential selection to synchronize adult emergence with either hawthorn fruit or apple fruit availability. Apple flies have evolved earlier (*c.* 3 weeks) adult emergence in response to this selection, causing temporal reproductive isolation from hawthorn flies (Feder & Filchak 1999).Apple and hawthorn flies show clear genetic differentiation that is maintained despite moderate gene flow (*c.* 6% per generation), and some of these genetic differences are also associated with emergence time (Feder, Hunt & Bush 1993).

Differences in seasonal emergence timing among the fly host races are mediated by the diapause response, which has diverged among the host races in one additional way. *Rhagoletis pomonella* diapause as pupae and overwinter in the soil, terminating diapause and initiating adult development to synchronize mating and oviposition with host fruit availability. Apple flies terminate diapause earlier in the year, causing earlier adult emergence to coincide with apple fruit availability. Changes in seasonal timing can have additional selective consequences (Ragland & Kingsolver 2008); in this case, earlier adult emergence and larval feeding in apple flies cause apple-fly larvae to exit host fruits and enter pupal diapause earlier in the late summer/early fall when temperatures are relatively warm. Thus, apple flies must weather a longer, warmer pre-winter period before temperatures drop below the developmental minimum (Dambroski & Feder 2007). Experimentally imposing longer, warmer pre-winter periods selects for progressively decreasing frequencies of hawthorn-fly-like genotypes and increasing frequencies of apple-fly-like genotypes at five allozyme loci that show clear and consistent frequency differences among the host races (Feder *et al.* 1997a). This evidence strongly suggests that in addition to differences in seasonal timing, apple flies have evolved in response to warmer, longer pre-winter conditions.

In contrast to differentiation between the *R. pomonella* host races in seasonal responses, evidence for host race differentiation in performance on their respective host fruits is equivocal. Larval transplant experiments show that both host races survive to pupation at a higher rate in hawthorn fruits and at a slightly but equally reduced rate in apple fruits (Prokopy, Diehl & Cooley 1988). Moreover, mortality selection imposed through warm pre-winter conditions (as described above) does not depend on the larval host fruit environment (Feder *et al.* 1997a).

These previous studies provide no evidence for interactions between host fruit and seasonal effects, but survival overwinter is not the only fitness component that influences post-winter, reproductive success. Overwintering in the non-feeding pupal stage requires substantial nutritional stores that will influence post-winter performance. Metabolic rates of insects are highly temperature-sensitive, and higher environmental temperatures increase metabolic rate, even during diapause (Irwin & Lee 2003). Diapausing pupae must contain enough nutritional reserves to maintain themselves overwinter and to complete adult metamorphosis after winter, a major anabolic and catabolic undertaking (Hahn & Denlinger 2007). Further, emerging adults must have enough nutrients left after diapause and metamorphosis to dig themselves out of the ground and disperse to find food. The earlier phenology and longer, warmer pre-winters experienced by apple flies increases metabolic stress, and diapausing apple-fly pupae should burn more metabolic reserves compared to hawthorn-fly pupa. But nutritional reserves packed on prior to diapause will depend on the larval nutritional environment. Thus, host fruit environment may interact with seasonality to determine post-winter nutritional stores and post-diapause adult performance.

Here, we test the hypothesis that two selective factors, host fruit environment and seasonality, interact through their

effects on fuel storage and fuel depletion, respectively. Differences in fuel storage between the apple-fly and hawthorn-fly host races of *R. pomonella* could be based on genetic, environmental or gene-by-environment interaction effects. We performed several experiments to test for host race differences in nutrient storage under seasonal conditions typically experienced by apple flies and hawthorn flies, asking: (i) do pupae of the two fly host races differ in body size or lipid reserves when reared from their respective natal host fruits and when reared from a common host fruit, (ii) are differences in pre-winter pupal lipid stores related to survival, body size or post-emergence adult lipid stores under apple-fly- and hawthorn-fly-like pre-winter conditions, and (iii) are pupal lipid stores associated with genetic loci previously shown to respond to selection under apple-fly-like seasonal conditions?

Materials and methods

STUDY SITES AND FIELD COLLECTION

Rhagoletis pomonella ranges as far south as disjunct populations in central Mexico, but apple and hawthorn plants occur sympatrically primarily in the Midwestern United States. We sampled from four geographic sites that cover a large latitudinal portion of this sympatric distribution: (i) Urbana, IL (URB; 40·1°N), (ii) Dowagiac, MI (DOW; 42·0°N), (iii) Grant, MI (GRT; 43·3°N) and (iv) Fennville, MI (FEN; 42·6°N). All of these sites demonstrate genetic differentiation between apple and hawthorn flies (Feder & Bush 1989; Michel *et al*. 2010).

Infested hawthorn and apple fruits were collected from each field site in the late summer/early fall of 2007 and transported to laboratory facilities at the University of Florida. Fruits were immediately and haphazardly split into two groups placed in either a 21 or 25 °C incubator to simulate the warmer conditions experienced by apple flies vs. the cooler conditions experienced by hawthorn flies (both set to 14 : 10 light/dark cycle). Fruits were placed in wire mesh baskets above plastic tubs where larvae exiting the fruits pupated. Pupae were collected every day, placed in Petri dishes with moist vermiculite and held in the incubator of collection (e.g. pupae reared at 21 °C were held at 21 °C) for an experimentally manipulated long or short duration.

EXPERIMENTAL TREATMENTS AND SAMPLING

We manipulated the temperature and duration of the pupal, pre-winter period to simulate the combinations of apple-fly- and hawthorn-fly-like field conditions in an experiment performed on pupae reared from natal fruit. Longer and warmer pre-winter treatments are more apple-fly-like, and cooler, shorter pre-winter treatments are more hawthorn-fly-like. We reared both apple-fly and hawthorn-fly larvae through the pupal stage in each of four conditions: (i) a short and cool pre-winter period with pupae held at 21 °C until 10 days after pupariation (formation of the sclerotized and tanned puparium, followed several days later by the formation of the pupa;Dean & Chapman 1973), (ii) a long and cool pre-winter period with pupae held at 21 °C until 30 days after pupariation, (iii) a warm and short pre-winter period with pupae held at 25 °C until 10 days after pupariation, and (iv) a warm and long pre-winter period with pupae held at 25 °C until 30 days after pupariation. All pupae were weighed to ±0·001 mg at 10 days post-pupariation and a sample of *c*. 300 pupae were immediately frozen for genotyping and pre-winter lipid assays from both the 21 and 25 °C treatments. Preliminary data suggested that by 10 days post-pupariation, pupal weights were stable following massive water loss during larval–pupal metamorphosis (D.A Hahn, unpublished data). Following the pre-winter treatments, *c*. 300 pupae from each treatment were placed individually in wells of 96-well microtiter plates, and the plates were stacked in plastic boxes with reservoirs of saturated KCL solution to maintain 85% relative humidity (Winston & Bates 1960).The boxes were placed in a cold room at 5 °C for 25 weeks, simulating winter (Feder *et al*. 1997b). After 25 weeks, the boxes were moved to a 21 °C incubator and monitored for adult emergence daily for 5 months. Emerged flies were frozen for post-eclosion lipid assays (described below). Numbers of pupae collected from the Urbana site were limited, so Urbana larvae/pupae were only reared at 21 °C. Parasitism by parasitoid wasps is common in field-collected pupae (Forbes, Hood & Feder 2010), and all puparia from which parasitoids eclosed were excluded from the analysis (< 0·5% and *c*. 3% for apple and haw flies, respectively). We dissected pupae that did not eclose and it was often, but not always obvious whether the pupae were parasitized depending on the degree of decomposition. We excluded all post-mortem pupae that were not easily classified from further analysis.

An additional sample of *c*. 300 pupae from each fly host race from the Grant and Fennville sites were left in moist vermiculite and overwintered at 5 °C for 25 weeks to establish the F1 generation for common-garden rearing studies. Following the overwinter treatment, open Petri dishes with vermiculite containing pupae were set-up in four mating cages in a 24 °C, 15:9 L : D incubator, one each for Grant apple flies, Grant hawthorn flies, Fennville apple flies and Fennville hawthorn flies. Each cage contained sugar cubes and a slurry of 1 : 1 autolysed brewers yeast and brown sugar for adult nutrition that were regularly replenished (Dambroski & Feder 2007). Beginning 10 days after the first fly emerged, each cage was provided continuously with a red delicious apple from Washington State (to provide a uniform host fruit environment) and apples were removed and replaced every 3 days to limit crowding in the fruit. Hawthorn-fly cages were additionally provided with host fruit lures infused with hawthorn volatile blends to encourage oviposition. Apples removed from the cages were transferred to a 21 °C incubator, held in wire baskets and checked daily for pupation as described above. Pupae were then placed in moist vermiculite for 10 days at 21 °C, weighed and immediately frozen for genotyping and lipid assays.

LIPID ASSAYS

Lipid assays were performed on subsets of pupae from various host race/site/treatment combinations. For genetic associations, 96 pupae from each host race reared from its natal fruit at 21 °C and 96 pupae from each host race reared from apples (also at 21 °C) in the F1 common-garden experiment were first decapitated on a cooled platform (−25 °C; razor section through the second segment below the operculum) and the heads returned to the freezer for genotyping. The bodies were re-frozen then freeze-dried for 48 h (preliminary data showed that dry mass stabilizes within 48 h). Dried bodies were weighed and then homogenized in 1 mL of 4 : 1 chloroform/methanol plus 50 mg dehydrated silicic acid to bind phospholipids (Zera & Larsen 2001). After extraction on a shaking platform for 30 min, 100 μL of 0·9% NaCl was added and mixed to separate the extraction into polar and organic phases. The polar fraction and silicic acid were discarded, while the organic fraction was dried down with a nitrogen stream,

re-suspended in 1 mL of chloroform and lipid content was estimated using a vanillin assay with triolein standards ranging from zero to 100 μg (Vanhandel 1985).Our initial estimates showed that this neutral lipid fraction in *R. pomonella* pupae is *c*. 95% triglycerides when separated on TLC (DA Hahn, unpublished data). Each series of assays included two replicate triolein dilution series for standard curve estimation, and standard curves always fit with $r^2 > 98\%$. Each individual sample was technically replicated twice, replicate samples were fit to the standard curve and any replicates with estimates differing by > 0·05% were rerun. We applied the same methods for phenotypic comparisons among population and across temperatures except that pupae were cut in half prior to freeze drying (to reduce drying times) and both halves combined for lipid extraction/assays. We estimated the lean mass of individuals by subtracting the estimated neutral lipid mass from the total dry mass, and we use lean mass as a covariate in our lipid analyses.

GENOTYPING

We genotyped samples from the two Michigan populations reared from natal fruit and as F1s reared from red delicious apples at 21 °C. We genotyped 29 microsatellite loci that have previously been shown to have associations with clinal variation and with selection for apple-fly-like pre-winter conditions in *R. pomonella* (Michel *et al.* 2010). Most of the microsatellite loci have many low-frequency alleles (up to 23), and the genetic studies in Michel *et al.*(2010) were performed on pools of alleles used to score homozygotes (all alleles falling into the pool), heterozygotes (at least one allele not in the pool), or alternate homozygotes (no alleles in the pool). We used these same pools and scoring schemes to determine whether the same allele combinations that responded to selection in the previous experiment are also associated with lipid content.

DNA was isolated from pupal heads using Puregene extraction kits (Gentra systems). Purified genomic DNA was then used for microsatellite PCR amplification (38 cycles of 94 °C for 20 s, 55 °C for 15 s and 72 °C for 30 s, with a final incubation for 10 min at 72 °C; Michel *et al.* 2010) of 29 loci characterized from an enriched GT dinucleotide repeat *R. pomonella* library (GenBank AY734885–AY734965; (Velez *et al.* 2006). Genotyping was performed on a Beckman Coulter CEQ 8000, and we genotyped an average of 86 individuals per locus reared from both natal fruit and the F1 common-garden pupae for each fly host race (see Table S1, Supporting information for all sample sizes).These microsatellite loci are distributed across all five of the *R. pomonella* linkage groups (primer sequences and linkage group number are available in Table S1, Supporting information).

FRUIT SUGAR ANALYSIS

Fruits generally provide sugar-rich diets, and differences in sugar content between apples and haws may influence lipid synthesis and storage in each host race. We performed an HPLC-based assay of sugar content (AOAC 2006) on three replicate pools of fruit (2–3 apples and 15–20 haws per pool) from each of the following: apples collected in Grant and Fennville, MI and haws collected in Urbana, IL and South Bend, IN. We quantified and compared the proportional content (dry mass/total dry mass) of fructose, glucose and sucrose.

STATISTICAL ANALYSES

We constructed statistical models for six sets of data within our study: (i) To determine whether lipid content or body mass differs between apple-fly and hawthorn-fly pupae in the field, we reared larvae to pupation from natal fruit collected at each of our field sites at 21 °C and estimated their total dry mass, their lipid mass, and their lean mass (total dry mass minus lipid mass), (ii) to determine the effect of apple-fly-like and hawthorn-fly-like pre-winter periods on pupal lipid content and lean mass, we reared flies of both host races from natal fruit collected at a single field site (Fennville, MI) under warm (10 days at 25 °C) and cool (30 days at 21 °C) pre-winter regimes, (iii) to determine whether host fruit affects lipid content or lean mass of fly pupae, we reared the progeny of apple and hawthorn flies collected at two field sites, Grant and Fennville MI, in a common-garden environment in the laboratory all on red delicious apples from Washington State, (iv) to determine whether pupal lipid depletion differed between the two host races during overwintering, we compared post-emergence adult lipid content and lean mass between apple and hawthorn flies reared from their natal fruits collected from Fennville, MI and reared at 25 °C in the laboratory, (v) to determine the effects of temperature and duration of the pre-winter period on survival to adult emergence, we compared all temperature (21 and 25 °C) and pre-winter duration (10 or 30 days) treatments across both apple and hawthorn flies collected from their natal fruits across all populations except Urbana, IL (sample sizes from 25 °C were too limited), and (vi) to determine whether alleles at marker loci shown to respond to selection imposed by apple-fly-like pre-winter conditions (Michel *et al.* 2010) also associate with pupal lipid reserves, we tested for genetic associations between microsatellite loci and lipid content and body mass for pupae reared from natal fruit and as F1s on apple from the two Michigan sites.

For all phenotypic analyses of body mass and lipid content, we fit linear fixed effects models including fruit, collection site, temperature and pre-winter duration where appropriate. We also included eclosion time as a covariate for the analysis of post-eclosion traits. Response variables were either lipid mass or lean mass (calculated as dry mass minus lipid mass). All analyses of lipid content included lean mass as a covariate and a block effect to account for assay-to-assay variation in neutral lipid estimates (assays were performed in groups of 24 samples, and samples within a group were arranged in a randomized complete block design). We fit a mixed logistic regression model to the survival data with fixed effects as above and including a random grouping factor for each plate (housing up to 96 larvae per plate). Linear fixed effects and logistic mixed effects models were fit in R (R Development Core Team, 2009) using the lm and glmer packages, and the best models were selected using stepwise, backward selection on Akaike Information Criterion (AIC) scores. We started with a full model (including all interactions) and sequentially removed non-significant parameters (parameters whose inclusion increased the AIC), starting with the highest order interactions. For brevity, we report only tests for fixed effects of biological interest, although random and block effects were significant in all models. Post hoc comparisons were performed using linear contrasts.

We used Kruskal–Wallis tests to estimate associations between lipid content and genotype. To reiterate, alleles at each locus were pooled to score homozygotes, heterozygotes and alternate homozygotes as in Michel *et al.*(2010). These three genotypic classes served as three levels of the one-way nonparametric ANOVA, and the test was applied individually to each locus. Response variables were either lean mass or lipid mass. Because larger individuals typically also have greater lipid mass, we statistically removed the effects of the lean mass covariate using linear models prior to the association analysis with lipid mass (i.e. the genetic association analysis was performed on the residuals). We then applied an FDR correction to all *P*-values to account for multiple testing. Tests were performed on (i) the entire

pool of genotyped individuals including apple natal fruit pupae, hawthorn natal fruit pupae and apple-reared F1 pupae from both Grant and Fennville. We also performed separate analyses within each of these groups to identify potential genotype by fruit interactions.

Results

NATAL FRUIT COMPARISONS ACROSS SITES

Apple and hawthorn pupae did not differ in lean mass within any collection site (Fig. 1a; no significant post hoc comparisons), although the ANOVA model produced a significant collection site by fruit interaction. Initial analysis of lipid mass revealed a collection site by lean mass (covariate) interaction, so we statistically removed the effects of lean mass for lipid mass data from each individual collection site using separate ANCOVA models. The linear model fit to these mass-corrected lipid mass data revealed significant effects of collection site and host fruit, but no significant interaction (Fig. 1b). Thus, apple host race pupae reared from natal fruit had greater lipid content than hawthorn host race pupae across all collection sites. There was some variation among populations in lipid mass, but that variation does not appear to be strongly related to latitude.

NATAL FRUIT COMPARISONS ACROSS REARING TEMPERATURE

Both apple and hawthorn pupae from Fennville reared from natal fruit had greater lean mass at lower rearing temperature, following the temperature size rule (Fig. 2a). There were no significant temperature by fruit interactions, and hawthorn pupae from this collection site had greater lean mass than apple pupae at both temperatures (the same, non-significant trend is apparent in the analysis including all collection sites; Fig. 1a). Initial analysis of lipid mass revealed a significant fruit by lean mass interaction, suggesting that the lean mass vs. lipid mass scaling relationship differs between the host races when they are reared in natal fruit. We fit separate ANCOVA models that revealed a larger intercept for apple pupae and a steeper slope for hawthorn pupae (Fig. 3). Lean mass-corrected values from these analyses were used to fit the

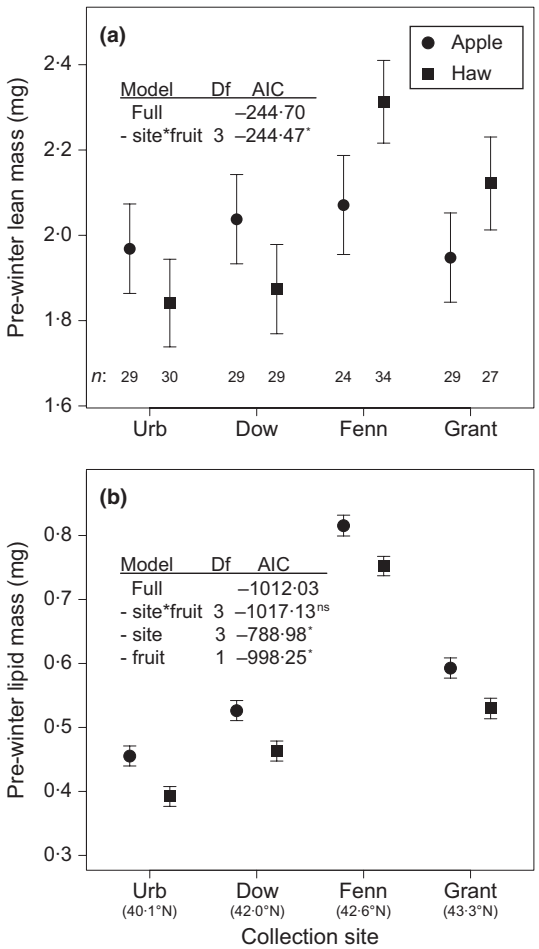

Fig. 1. Mean ± SE lean mass (a) and lipid mass (b) for apple and hawthorn pupae reared from natal fruit collected at four sympatric field sites (Latitudes in parentheses). Lipid mass values are body-size-corrected and model fits use Akaike Information Criterion backward selection with only starred terms significant.

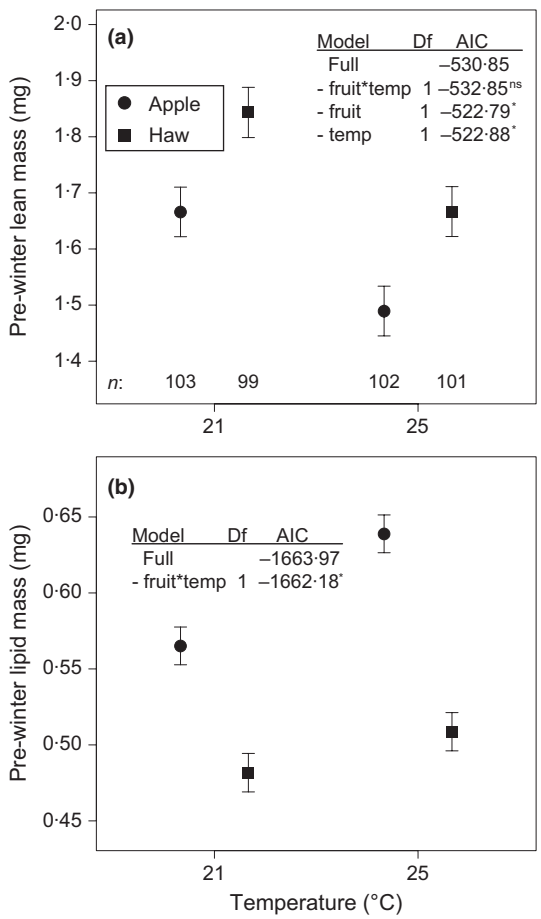

Fig. 2. Mean ± SE lean mass (a) and lipid mass (b) for apple and hawthorn pupae reared at either 21 or 25 °C from natal fruit collected at the Fennville field site. Lipid mass values are body-size-corrected and model fits use Akaike Information Criterion backward selection with only starred terms significant.

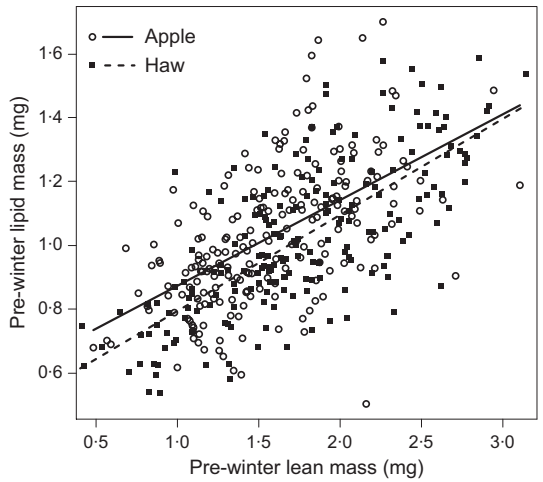

Fig. 3. Scaling relationship of lipid mass to lean mass in apple and hawthorn pupae from natal fruit collected at the Fennville field site. The fitted relationships for apple and hawthorn pupae differ in both intercept and slope (see Results).

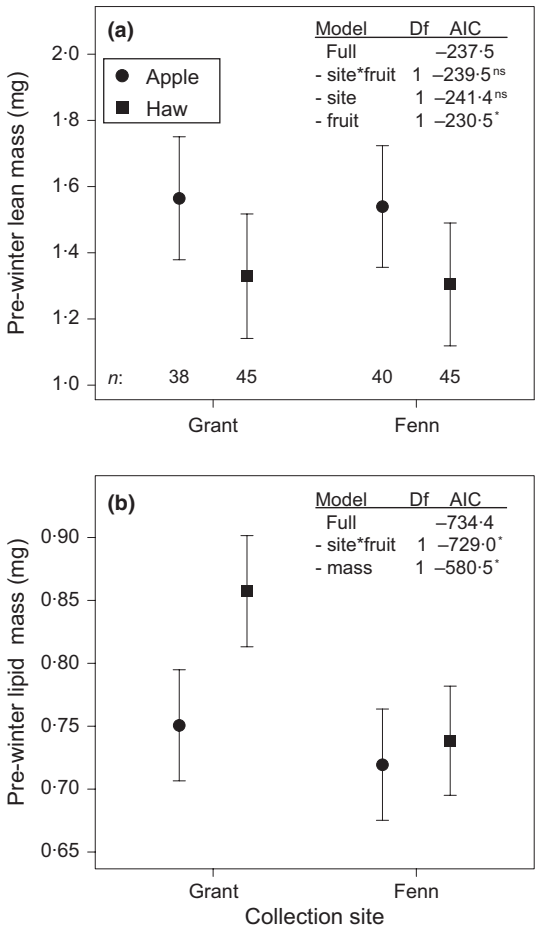

Fig. 4. Mean ± SE lean mass (a) and lipid mass (b) for apple and hawthorn pupae reared at 21 °C from the F1 generation of apple and hawthorn flies from the Grant and Fennville field sites. Lipid mass values are body-size-corrected, and model fits use Akaike Information Criterion backward selection with only starred terms significant.

linear model including fruit and temperature effects. There was a significant fruit by temperature interaction; the difference between the host races was more pronounced at the higher rearing temperature, although apple pupae were significantly fatter than hawthorn pupae at both temperatures (Fig. 2b). Thus, the differences in lipid content between the host races were consistent with the results of the previous analysis (population comparisons) and more pronounced under the hotter rearing conditions more typical of apple-fly-like pre-winter conditions in the field.

F1, COMMON-GARDEN HOST RACE COMPARISONS

When reared as F1s in a common host fruit (apple), apple-fly pupae had slightly greater lean mass than hawthorn-fly pupae (Fig. 4a), and the scaling relationship differences between apple and hawthorn pupae for lean mass vs. lipid mass disappeared (i.e. no significant host race by lean mass interaction). Moreover, apple pupae were not fatter than hawthorn pupae when both were reared on apples. Rather, there was no significant difference in lipid mass between host races collected at Fennville ($t = 0{\cdot}77$, $P \geq 0{\cdot}1$), while hawthorn pupae from the Grant were significantly fatter than apple pupae from the Grant ($t = 4{\cdot}6$, $P \leq 0{\cdot}001$; Fig. 4b). Contrasted with the natal fruit experiments, these results suggest that greater lipid content in apple pupae reared in natal fruit is caused by host fruit effects, not by genetic differences among the host races.

POST-EMERGENCE ADULT LIPID CONTENT

Fennville apple flies emerging as adults post-winter after a 25 °C pre-winter treatment had slightly but significantly greater lean mass than hawthorn flies (mean ± SE; apple = 0·83 mg ± 0·15, hawthorn = 0·72 mg ± 0·16; $t = 2{\cdot}19$, $P = 0{\cdot}03$; Table 1). However, there was no significant difference between the host races in lipid content

Table 1. ANCOVA model fits for lipid mass and lean mass of post-winter, eclosed adults from the Fennville field site reared at 25 °C pre-winter

Model	d.f.	AIC
Lipid mass		
Full		–851·7
Fruit × DP	1	$-853{\cdot}6^{NS}$
Fruit	1	$-854{\cdot}4^{NS}$
ECL (covariate)	1	$-856{\cdot}4^{NS}$
DP	1	$-856{\cdot}5^{NS}$
Lean mass (covariate)	1	$-838{\cdot}12^{*}$
Lean mass		
Full		–394·2
Fruit × DP	1	$-395{\cdot}0^{NS}$
DP	1	$-396{\cdot}3^{NS}$
Fruit	1	$-393{\cdot}5^{*}$
ECL (covariate)	1	$-393{\cdot}7^{*}$

NS, non-significant; DP, duration pre-winter; ECL, time to eclosion; AIC, Akaike Information Criterion.
Starred terms are significant at $P < 0{\cdot}05$.

(mean ± SE; apple = 0·45 ± 0·025, hawthorn = 0·43 ± 0·026; t = 1·06, P = 0·29; Table 1). The duration of the pre-winter period (10 or 30 days) had no significant effect on either lipid mass or lean mass. Time to eclosion had a significant effect only on lean mass; later eclosing flies were larger, but this covariate did not interact with any other factors in the model (tested with interaction terms in ANCOVA, data not shown). Apple pupae from Fennville reared pre-winter at 25 °C had lower lean mass and greater lipid mass prior to winter (see above). Thus, it appears that instead of maintaining greater lipid storage as newly emerged adults, apple pupae metabolized their greater reserves to produce a greater ratio of adult lean mass to pupal lean mass (0·56) compared to hawthorn pupae (0·43).

POST-WINTER SURVIVAL

Sampling lipid content is destructive, so we could not directly assess the relationship between pre-winter lipid content and overwinter survivorship. But, we compared survivorship between natal fruit-reared host races that our above analyses have shown to differ in lipid content. Larger individuals survived better (wet mass significant; Table 2), and although there was a significant effect of collection site, post hoc comparisons revealed no significant differences in survival among the sites (FEN vs. DOW z = −0·079, P = 0·99; GRT vs. DOW z = 1·642, P = 0·227; GRT vs. FEN z = 2·014, P = 0·108). There were significant temperature effects (Table 2), with lower survival at the higher rearing temperature. There was a single significant two-way interaction between duration of the pre-winter and host race (Table 2) in which apple pupae survived to eclosion at a higher rate than hawthorn pupae under shorter pre-winter conditions, but the two host races did not differ under the longer pre-winter (Fig. 5). Thus, apple pupae start with greater lipid reserves but survive no better than hawthorn pupae under the more apple-fly-like (longer) pre-winter conditions.

Table 2. Logit model fit for post-winter survival to eclosion. The Fennville, Grant and Dowagiac collection sites are included in the analysis

Model	d.f.	AIC
Full		1700
Site*Temp*DP*fruit	2	1700^{NS}
Site*Temp*DP	2	1698^{NS}
Site*Temp*fruit	2	1696^{NS}
Temp*DP*fruit	1	1695^{NS}
Site*DP*fruit	2	1693^{NS}
Site*Temp	2	1691^{NS}
Temp*fruit	1	1690^{NS}
Site*fruit	2	1690^{NS}
Temp*DP	1	1688^{NS}
Site*DP	2	1684^{NS}
DP*fruit	1	1688^{*}
Site	2	1685^{*}
Temp	1	1691^{*}
Mass	1	1709^{*}

NS, non-significant; DP, duration pre-winter; site, collection site; AIC, Akaike Information Criterion.
Starred terms are significant at P < 0·05.

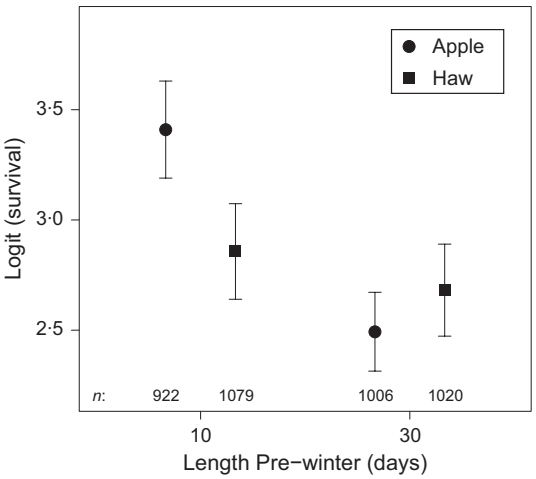

Fig. 5. Mean ± SE survival (logit) for apple and hawthorn pupae reared from natal fruit and exposed to either a short (10 days) or long (30 days) pre-winter. Values are averaged across populations and across pre-winter temperatures.

GENETIC ASSOCIATIONS

We tested for genetic association with lipid content at 29 loci, 19 of which have been previously shown to significantly change in frequency in response to rearing in long, warm, apple-fly-like pre-winter conditions (Michel *et al.* 2010). Kruskal–Wallis tests for data pooled across host races, sites and natal fruit/F1 common-garden rearing revealed no significant associations (Table S1, Supporting information). Moreover, analysis within each site, host race, host race within site and F1 common-garden host race revealed no significant interactions (Table S1, Supporting information). We also attempted to detect any locus-by-locus interactions by testing pairwise allelic combinations, but these tests also revealed no associations (data not shown). These results corroborate the results of the organismal F1 common-garden analysis, providing no evidence for a genetically based difference in lipid storage among the host races.

FRUIT SUGAR CONTENT

Apple fruits did not differ consistently from hawthorn fruits in total sugar content (Table 3). Apple fruits from the Grant had lower sugar content than hawthorn fruits, but apple fruits from Fennville had higher sugar content than hawthorn fruits. The two fruit types differed mainly in the ratios of the assayed sugars. Apple fruits had a much greater ratio of fructose to glucose, and the disaccharide sucrose was present in apple fruits but not detectable in hawthorn fruits.

Discussion

Host race formation in phytophagous insects often involves a simultaneous shift in seasonality (Feder & Filchak 1999;

Table 3. Mean ± SE Sugar content (% dry mass) of apples and haws from the Grant (GRT), Fennville (FEN), South Bend (SBE) and Urbana (URB)

	Fructose	Glucose	Sucrose	Total	Fructose/glucose
SBE hawthorn	4·72 ± 0·07	7·09 ± 0·09	< DL	11·82 ± 0·15	0·67 ± 0·003
URB hawthorn	3·65 ± 0·13	7·17 ± 0·38	< DL	10·81 ± 0·46	0·51 ± 0·02
GRT apple	4·86 ± 0·07	1·79 ± 0·16	2·04 ± 0·11	8·69 ± 0·23	2·76 ± 0·23
FEN apple	6·62 ± 0·18	1·66 ± 0·12	3·74 ± 0·16	12·02 ± 0·21	4·05 ± 0·35

DL (detectable limit) = 0·1%.

Whipple *et al*. 2009; Dopman, Robbins & Seaman 2010; Ording *et al*. 2010; Kerstes & de Jong 2011) and nutritional environment (Egan & Ott 2007; Ferrari, Via & Godfray 2008; de Jong *et al*. 2009), and each of these factors imposes potentially very strong selective pressures that ultimately lead to adaptive divergence. The combined effects of changes in both host plant and seasonality have not been explored, to our knowledge, but our data suggest that their interaction may have important ecological implications for host race formation. In a host race shift, both the nutritional resource and seasonal environment can be novel, and a simple hypothesis might predict that both have deleterious effects that select for improved performance. Our data suggest, however, that one factor (resource quality) may actually mitigate selection by the other (seasonality). Specifically, feeding on apple fruits promotes greater fattening in apple flies, which in turn mitigates some of the demands of a seasonal shift to more metabolically stressful conditions. (Fig. 6).

Genetic and phenotypic data from a series of studies show that the shift to an earlier phenology in apple flies causes correlated selection through the temperature and duration of the pre-winter period (Feder *et al*. 1997a; Filchak, Roethele & Feder 2000).Moreover, the apple-fly populations demonstrate an evolutionary response as evidenced by changing allele frequencies. Because apple flies emerge earlier than hawthorn flies, pupation also occurs earlier and diapausing pupae are exposed to a longer, warmer pre-winter period where temperatures are consistently above the developmental minimum. Feder *et al*. (1997a) stressed hawthorn flies by exposing them to various durations of warm, 26 °C pre-winter conditions before overwintering (at 4 °C), then followed post-winter eclosion and tracked frequencies of allozymes known to differ in frequency among the fly host races. The frequency of hawthorn-fly-like alleles (alleles with higher frequency in the hawthorn host race) declined in the surviving individuals, and the apple-fly-like, alternate alleles increased with increasing duration of the pre-winter period. A similar study showed that progressively warmer pre-winter conditions also selects for apple-fly-like alleles and against hawthorn-fly-like alleles (Filchak, Roethele & Feder 2000).

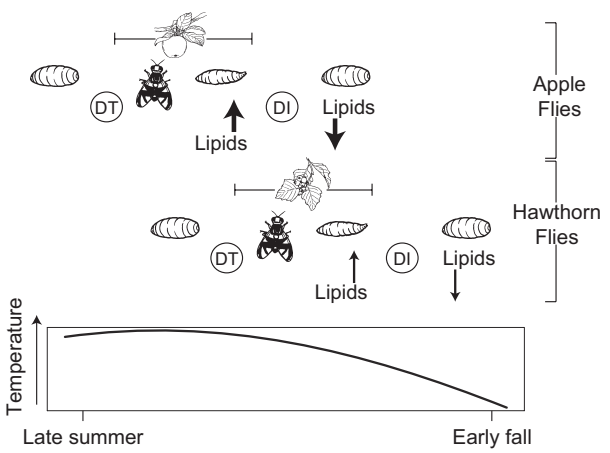

Fig. 6. Conceptual illustration of fly host race differences in seasonality and metabolic demand. Thicker arrows indicate greater lipid provisioning (up) and greater lipid depletion (down). The apple flies terminate pupal diapause (DT) earlier to synchronize adult mating and oviposition into earlier fruiting apples. Larval feeding on apples confers greater fatness compared to feeding on hawthorns. This offsets greater lipid depletion in apple flies caused by earlier initiation of pupal diapause (DI) when temperatures are warmer than those experienced by hawthorn flies.

While hawthorn-fly pupa generally experience longer, warmer post-winter conditions because they terminate diapause later, the length and temperature of the pre-winter period is most critical because it affects early diapause termination. Phenotypically, pre-winter conditions typically experienced by apple flies select against individuals that prematurely terminate diapause (Feder *et al*. 1997a,b). High temperature, long-duration pre-winters cause a proportion of the population to forego diapause or to terminate diapause just before the onset of winter or early during overwintering. Flies eclosing prior to winter will leave no viable offspring because no host plants will be available. Terminating diapause but not eclosing prior to winter typically leads to overwinter mortality (Feder *et al*. 1997b; Filchak, Roethele & Feder 2000).

Increased pupal lipid storage could affect pre-winter diapause termination in two ways. First, nutritional status may affect the developmental decision to either enter or to forgo diapause. Increased body size is associated with increased tendency to diapause in several insect species (reviewed in Hahn & Denlinger 2007). Although there is no direct evidence that lipid storage *per se* affects diapause status, many animals make life-history decisions based on internal condition (e.g.Wilbur & Collins 1973; Wessels *et al*. 2011). Lipid stores constitute a large portion of energy reserves in *R. pomonella* (all treatment combinations in all experiments averaged above 30% lipid), and energy reserves that are continuously depleted during diapause are critical for survival overwinter

and successful adult eclosion (Hahn & Denlinger 2007). Second, moderately cold winter temperatures (down to *c*. 4 °C in the laboratory) do not immediately kill non-diapausing pupae, rather they tend to die off at greater percentages compared to diapausing larvae with longer duration of winter (Feder *et al*. 1997b). Increased energy storage before winter could enhance survivorship by mitigating the energy expense of a non-diapause developmental trajectory and providing more energy for successful metamorphosis and early adult performance the following year.

Two lines of evidence from our study suggest that apple flies have not evolved increased lipid storage in response to longer, warmer pre-winters. Michel *et al*.(2010) identified 22 microsatellite loci that associated with tendency to prematurely terminate diapause when faced with apple-fly-like, long, warm pre-winter conditions. We found no significant associations between a subset of these same loci and lipid content in pupae reared from natal fruit or pupae from both host races reared through an F1 generation in a common host fruit environment (apples). We also found that although apple pupae reared from apple fruit had greater lipid content than hawthorn pupae reared from hawthorn fruit, there was no difference between the host races when both were reared through an F1 common-garden generation in apple fruits. Therefore, feeding in apple fruits confers greater fatness, but apple flies do not appear to have evolved towards greater lipid storage.

We predict that apple-fly pupae should have greater lipid content than hawthorn-fly pupae in the field based on the results of the natal fruit experiments, and that greater fatness enhances overwinter performance. Both the ancestral and novel host races of *R. pomonella* realize greater survival to pupation in the ancestral hawthorn fruit compared to apple fruit. In addition, both fly host races survive at similar rates when reared in either hawthorn or apple fruits (Prokopy, Diehl & Cooley 1988). Despite decreased survival rates in apple fruits, those flies that do successfully pupate from apples do so with greater lipid reserves compared to flies reared in hawthorn fruits. Under our most stressful experimental conditions (i.e. the most apple-fly-like), survival did not differ between the fly host races, but apple-fly pupae from the Fennville site produced adults with slightly but significantly greater lean mass compared to hawthorn-fly pupae. Fly host race differences in lean mass of adults were reversed in pupae; Fennville apple-fly pupae had lower lean mass than hawthorn-fly pupae. Thus, the differences in adult lean mass, although slight, are more substantial when viewed over the course of pupal to adult development. Although the mechanisms underlying greater post-winter lean mass in apple-reared flies are unknown, one possible explanation is that greater fat mass conferred by feeding on apples allows pupae to spare lean mass that hawthorn-reared flies must catabolize to support overwintering metabolism. For example, perhaps, hawthorn-reared flies catabolize proportionally more amino acids during diapause and are not able to maintain their lean mass as well as apple-reared flies. Fecundity increases with increasing female body size in *R. pomonella* (Averill & Prokopy 1987), thus large adult size in apple flies confers clear fitness benefits.

Our data show that the interaction between host fruit environment and seasonality has important evolutionary consequences. Specifically, feeding on apple fruit appears to mitigate selection imposed by earlier seasonal eclosion and reproductive phenology in the apple host race of *R. pomonella*. We found a significant host fruit by pre-winter temperature interaction, wherein fly host race differences in lipid content became more pronounced under warmer, apple-fly-like pre-winter conditions (Fig. 2). Greater lipid content translates to greater post-winter adult mass in apple compared to hawthorn flies reared under the most stressful (longer and warmer), apple-fly-like pre-winter conditions. Successful post-winter eclosion is reduced for flies reared under apple-fly-like pre-winters compared to less stressful, hawthorn-fly-like conditions. But the reduction is equivalent in both host races. Thus, the fitness costs of reduced post-winter eclosion success when reared under an apple-fly-like pre-winter are at least partially offset by the fitness benefit of larger adult size in flies reared from apples.

Host race shifts are thought to occur most often when the novel host plants are moderately different in 'resource space', that is, when plant traits that affect insect fitness are different enough to promote divergent selection but similar enough that fitness on the novel host is high enough to prevent extinction (Nyman 2010). Novel host plants must be nutritionally similar enough to be exploited before novel adaptations arise, so scenarios where the novel host plant actually yields a fitness benefit through diet quality may be relatively common (e.g. Blair *et al*. 2010). This may be particularly common when the novel host plant is an agricultural product such as apples bred for high nutritional density and low toxicity. Our biochemical analysis focusing solely on dietary sugars suggests that apples do not consistently contain more sugar than haws, although they do differ in relative composition of fructose, glucose and sucrose. Different ratios of sugars can affect adult insect performance (e.g. Wyckhuys *et al*. 2008), but to our knowledge, there are no comparable studies on larval performance in frugivorous insects.

Alternatively, the difference in *R. pomonella* performance on hawthorn and apple fruits may be driven by competition. Both inter- and intraspecific competition are stronger in hawthorn fruits compared to apple fruits (Feder *et al*. 1995), suggesting that reduced resource competition alone could cause increased lipid provisioning. Intraspecific competition is likely to be less intense on the novel host during the early stages of host race formation, thus competition may also be a common mechanism generating fitness benefits on a novel host.

To conclude, performance differences related to lipid storage between the host races of *R. pomonella* are driven by host fruit environments rather than genetic differentiation. A shift towards earlier seasonality in the derived host race imposes greater mortality, but this fitness cost is offset by greater lipid reserves and greater adult size conferred by feeding on apple rather than hawthorn fruit. These results illustrate how the

seasonal and resource environments combine to influence performance in the field, and how the effects of one environmental factor may mitigate natural selection imposed by another. On the other hand, less benign resource conditions in a derived host race could exacerbate environmental stress in other host-race-forming species, intensifying seasonally mediated selection. In either case, considering the combination of resource and seasonal environments will likely be critical for assessing natural selection and ecological differentiation in accumulating examples of host race formation and allochronic isolation in phytophagous insects.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1. Microsatellite marker summary information and sample sizes.
Table S2. All Kruskal Wallis H statistics, nominal p-values, and FDR values from the tests for association between genotype and lipid content.

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