# Genome-wide variation and transcriptional changes in diverse developmental processes underlie the rapid evolution of seasonal adaptation

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**Many organisms enter a dormant state in their life cycle to deal with predictable changes in environments over the course of a year. The timing of dormancy is therefore a key seasonal adaptation, and it evolves rapidly with changing environments. We tested the hypothesis that differences in the timing of seasonal activity are driven by differences in the rate of development during diapause in *Rhagoletis pomonella*, a fly specialized to feed on fruits of seasonally limited host plants. Transcriptomes from the central nervous system across a time series during diapause show consistent and progressive changes in transcripts participating in diverse developmental processes, despite a lack of gross morphological change. Moreover, population genomic analyses suggested that many genes of small effect enriched in developmental functional categories underlie variation in dormancy timing and overlap with gene sets associated with development rate in *Drosophila melanogaster*. Our transcriptional data also suggested that a recent evolutionary shift from a seasonally late to a seasonally early host plant drove more rapid development during diapause in the early fly population. Moreover, genetic variants that diverged during the evolutionary shift were also enriched in putative *cis* regulatory regions of genes differentially expressed during diapause development. Overall, our data suggest polygenic variation in the rate of developmental progression during diapause contributes to the evolution of seasonality in *R. pomonella*. We further discuss patterns that suggest hourglass-like developmental divergence early and late in diapause development and an important role for hub genes in the evolution of transcriptional divergence.**

diapause | development | phenology | ecological genomics | adaptation

Growth and development are two fundamental processes of life, and variation in these processes fuel adaptive evolution. Changes in the timing and/or patterns of growth and development can drive morphological diversification, as detailed by the rapidly expanding literature on the evolution of development, or "evo devo" (1). However, growth and development play equally important roles in determining key life-history traits and, therefore, life-history evolution (2). In particular, rates of growth and development may play a crucial role in determining phenology, or seasonal timing, a critical adaptation to synchronize growth and reproduction with favorable environmental conditions.

A key question in ecological and evolutionary physiology centers on the regulation of phenology: How do internal processes "mark time" to set the timing of key life-history events such as juvenile-to-adult transitions or the onset of reproduction (3, 4)? Also, how does variation in these processes contribute to adaptive evolution, which may often be exceptionally rapid to track changing environments, for example under climate change (5–7) or during human-mediated biological invasions (8, 9)? Much of the research in this area focuses on dormant states that suppress growth and development and serve as master regulators of phenology (10–14). Variation in one or a few key regulatory genes can serve as the genetic substrate for phenotypic innovations that promote local adaptation of phenology (15, 16). For example, loci functionally associated with the sensing of daylength, or photoperiodism, often provide evidence for seasonal adaptation via monogenic variation, or by just a few major effect loci (17, 18). In these examples, central regulatory roles for candidate genes that transduce environmental signals through developmental switches that curtail or activate development provide a plausible path from mono/oligogenic variation to physiological variation to variation in whole-organism phenology.

However, there is also evidence that more complex, polygenic variation may underlie adaptive variation in phenology. Here, we present a study of an alternative model of phenology variation mediated through general rates of development, rather than

## Significance

Organisms living in seasonal environments must synchronize their growth and reproduction to favorable times of the year. Our study highlights how the timing of dormancy can rapidly evolve to synchronize insects with changes in seasonal food sources. Dormancy is often conceptualized as suspended animation or arrested development, but our results suggest slow, progressive development during dormancy, with the rate of dormancy affected by many genes. Moreover, a population that recently shifted to a food source available earlier in the year has rapidly evolved through changes in many of those same genes. This shows how complex genetics can facilitate adaptation while also leveraging a rapid shift in phenology to understand developmental regulation of dormancy, a fundamental life-history trait in seasonal environments.

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variation in one or a few key developmental switches, as described above. The timing of insect diapause, or dormancy, has long served as a model for the evolution of phenology. In addition to the timing of entrance into diapause, which may be functionally obligate or environmentally cued, a key trait is diapause duration, which sets the timing of exit from diapause and the resumption of active growth and reproduction (12). Marked physiological changes occur over the course of diapause, for example changes in hormone titers, receptor abundance, and other, transcriptome-wide changes (19–23), while overt, morphogenic development is typically thought to be largely arrested (24, 25). However, several studies provide evidence for some amount of morphogenesis during diapause (26–28), and an alternative hypothesis is that diapause may often involve a deceleration rather than arrest of development. Moreover, the rate of morphogenesis during diapause may also influence diapause duration (29), and therefore seasonal timing. While variation in key regulatory genes could certainly influence developmental rates, the available evidence suggests that insect development rate variation is often polygenic (30). Thus, if the rate of overwintering diapause development dictates phenology during the growing season, then variation in many loci of small effect may influence standing variation in and evolution of the timing of important, downstream life-history events like adult emergence and reproductive maturity.

Our study applies an integrative approach, leveraging naturally segregating variation in seasonal timing to identify developmental mechanisms underlying an adaptive shift in diapause duration in a tephritid fly, *Rhagoletis pomonella*. The apple maggot fly *R. pomonella* is native to North America, where its larvae infest fruits of native *Crataegus* (hawthorn) species throughout its range. Derived, genetically diverged populations of the fly have evolved to infest domesticated apples (*Malus pumila*), introduced to North America ∼400 y ago (31). Strong natural selection on two primary traits, host-finding behavior and phenology, help to maintain genetic divergence between the hawthorn-infesting and apple-infesting fly populations, or host races (32, 33). Both populations (hereafter apple and haw flies) have one generation per year and overwinter as diapausing pupae. Adults must emerge coincident with host fruit availability (32), and the timing of adult emergence is determined by the duration of diapause, which ends under natural temperature conditions without any photoperiodic cue (34). Apple flies have evolved an earlier emergence timing (∼3-wk-shorter diapause duration) to synchronize with apples, which fruit about 3 wk earlier than hawthorn at typical field sites (e.g., Michigan or Illinois). Previous analyses of genome-wide single-nucleotide polymorphism (SNP) variation suggest that emergence timing segregates as a polygenic trait and contributes to both geographic variation within each host race and genetic divergence between the host races (35–37). Diapausing apple and haw flies differ in expression of many transcripts at a single time point toward the end of winter, suggesting that they differ in overwinter diapause progression, contributing to earlier emergence of apple flies (38).

In the current study, we evaluate evidence for the development rate hypothesis for adaptive phenological variation using RNA sequencing (RNAseq), phenotyping of brain morphology, and whole-genome pooled resequencing (Poolseq) mapped to a new assembly and annotation of a genome from *Rhagoletis zephyria*, a closely related sibling species in the genus *Rhagoletis* (see *SI Appendix*, Fig. S1 for a conceptual diagram of the experimental design). First, we tested for transcriptomic and brain morphological changes that would be consistent with morphogenic development during diapause. Second, we tested whether expression trajectories during diapause diverged between the host races, including an analysis of the contribution of hub-like genes. Although a polygenic model might predict expression divergence of many genes, it does not preclude a disproportionate role for well-connected, hub-like genes that may facilitate rapid adaptation (39). Third, we used genomic surveys of natural populations and a pooled sequencing variant of genome-wide association (GWA) analysis of diapause duration to 1) determine the genomic distribution of associated variants, 2) test for signatures of selective sweeps, 3) test for functional enrichment of genes associated with morphogenic development, and 4) test for signals of *cis* regulatory variation associated with differentially expressed transcripts. Finally, we predicted that if diverse, general developmental mechanisms govern diapause duration, then our GWA would identify variants similar to those identified in a GWA of egg-to-adult development rate in *Drosophila melanogaster* (30). To test this hypothesis, we applied our GWA pipeline to the data from ref. 30 and statistically compared overlap between variants.

## Results

**Genome Assembly and Annotation.** We assembled a draft genome of *R. zephyria* for sequence read mapping. We used *R. zephryia* rather than *R. pomonella* because while they are highly genetically similar *R. zephryia* has much lower polymorphism (40), facilitating a more contiguous genome assembly using short-read technology. Using a combination of mate-pair short-read sequencing and Illumina TruSeq synthetic long-read sequencing, we assembled the genome of *R. zephyria* into 86,670 scaffolds greater than 1 kb summing to 1,113,962,544 bp, with an average scaffold length of 12,853 bp, a scaffold N50 of 62 kb, maximum scaffold length of 2.47 Mb, and 139,583 contigs (archived at NCBI as LYWK00000000; *Materials and Methods* and *SI Appendix*, *Supplementary Methods*). Although fragmented, the assembly (annotated by the NCBI Eukaryotic Genome Annotation Pipeline v7.1) predicted 25,597 protein-coding genes, 34,587 coding messenger RNAs (mRNAs), and of those coding mRNAs 23,310 models with full transcriptomic support from available NCBI SRA *Rhagoletis pomonella* RNAseq datasets (NCBI *Rhagoletis zephyria* Annotation Release 100; full details at https://www.ncbi.nlm.nih.gov/genome/annotation_euk/Rhagoletis_zephyria/100/).

**Consistent, Progressive, and Transcriptome-Wide Dynamism during Diapause.** The central nervous system (CNS) and associated endocrine glands play an important regulatory role in insect diapause in general (20), and the CNS demonstrates pronounced morphogenic change throughout pupal development in flies (41). Thus, we sequenced mRNA (RNAseq) from the dissected CNS (including the brain hemispheres, the ring gland, and the fused, ventral nerve cord) of diapausing apple and haw fly pupae across an overwintering time series of five time points (2 to 6 mo) to characterize patterns of differential gene expression over the course of diapause development.

A large proportion of the transcriptome changed in relative abundance during overwintering in both apple and haw flies. Calculating false discovery rate (FDR) from generalized linear models fit to five replicate pools (20 brains per pool) taken across the five overwinter time points per population (apple or haw) identified 7,152 transcripts (24% of the 29,399 gene models) showing significant differential expression (DE) across at least one time interval during diapause (FDR < 0.05). Of these 7,152 DE transcripts, 5,133 were DE in apple flies, 4,597 were DE in haw flies and 2,578 were DE in both host races, five times more overlap than expected by chance alone ($P << 0.001$, Fisher's exact test; Fig. 1*A*). Directionality was highly similar between the host races, including similar percentages of up- vs. down-regulated genes over time (54% and 53% up-regulated in apple and haw flies, respectively), with 95% of the 2,578 common transcripts regulated in the same direction in both host races.

Transcriptomic changes were also highly progressive over time. We focused on the 2,578 transcripts DE over time in both host races as the most robustly supported candidates for diapause progression. Cluster analysis of these transcripts identified six different clusters

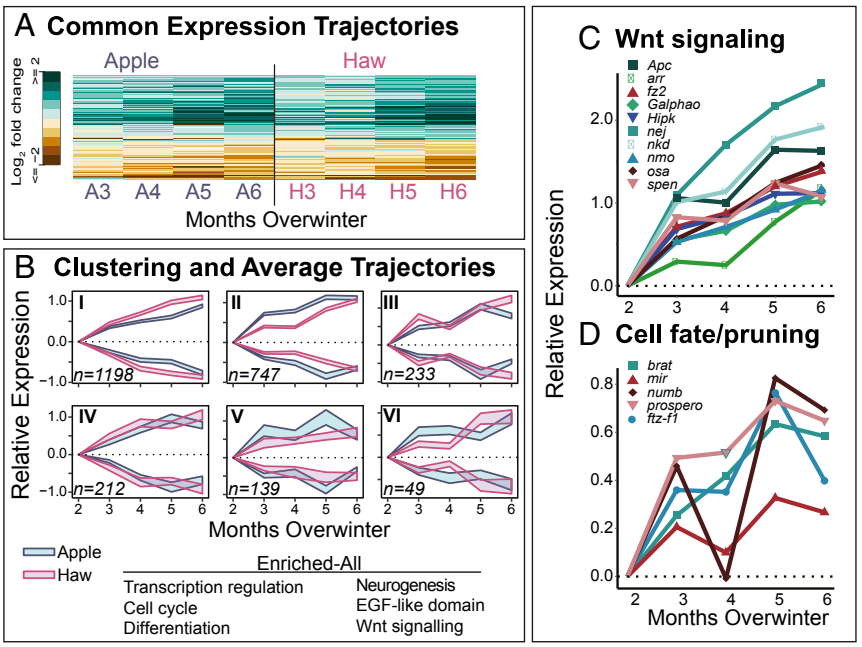



**Fig. 1.** Progressive trajectories of transcript expression across time common to diapausing pupae of both the apple and hawthorn host race. Relative expression values are log2 fold changes relative to the 2-mo time point of each host race. (*A*) Heat map of all 2,578 transcripts significantly differentially expressed across time in both host races; columns A3–A6 and H3–H6 represent values of apple and haw at 3 to 6 mo relative to 2 mo. (*B*) Trajectories of transcript expression within each of the six modules identified by clustering of coexpression analysis of 2,578 transcripts differentially expressed across time, including number of transcripts (*n*) associated with each module. Select functional categories identified through enrichment analyses of all transcripts combined are listed. Trajectories represent the approximate 95% confidence intervals (± 2 SE) of all genes up-regulated and down-regulated (average expression values above and below zero, respectively) over time. (*C*) Transcripts associated with the Wnt signaling pathway (subset with a maximum absolute log2 fold change >1) and (*D*) associated with neuron differentiation (cell fate genes) and pruning (*ftz-f1*; displayed values are averages of apple and haw flies).

of transcripts, each with mean "template" trajectory shapes that were also highly similar between the host races (Fig. 1*B*), all demonstrating mostly monotonically increasing or decreasing expression over the course of diapause development (Fig. 1*B*). The majority of transcripts fit the relatively linear trajectories in cluster I. Pupae moved from cold to warm temperatures at all sampled overwinter lengths remain in diapause for days to months (42), reinforcing that these transcriptional changes reflect only diapause development and not postdiapause development.

All of the observed changes in transcription took place over a time interval during which no large-scale changes in brain development occurred. The brains of higher flies change their shape drastically during pupal development, and *R. pomonella* appears to diapause in midpupal development, prior to the clearing of the eyes at a stage corresponding to p5 in *D. melanogaster* (43, 44). Using a simple metric of brain hemisphere width and height, we found no detectable changes in brain shape of haw fly pupae from 2 to 8 mo of their overwintering period as pupae (*SI Appendix*, Fig. S2, Table S1, and *Supplementary Methods*). From this, we infer that any development of the CNS during diapause would represent only incremental changes that happen over the equivalent time scale of hours in *D. melanogaster* pupae. However, these do not preclude substantial cellular differentiation and axon/dendrite pruning (e.g., ref. 45) that occur over time intervals much shorter than the duration of the established *D. melanogaster* pupal developmental landmarks (43).

Functional enrichment analysis of the 2,578 transcripts DE in both host races identified several functional categories of genes that are typically associated with cell/tissue development, including transcripts with epidermal growth factor (EGF) domains, and those affecting neurogenesis, cell differentiation, and the cell cycle (Fig. 1*B*; enrichment results within clusters yielded largely the same functional categories; Dataset S1). In particular, transcripts associated with the Wnt (wingless) signaling pathway were enriched, demonstrating progressive up-regulation over time (Fig. 1*C*). Although most CNS cell division occurs during larval development in *D. melanogaster*, there is additional asymmetrical cell division and neuronal differentiation up to 12 h postpupariation (41, 46, 47), corresponding to stage p4, and possibly p5 (43). In addition, extensive remodeling in the CNS via axon/dendrite pruning continues through much of pupal development in *D. melanogaster* (48). Thus, we also examined trajectories of major cell-fate-determining factors (49) and the transcription factor *ftz-f1*, whose up-regulation is associated with pruning in mushroom body neurons (47); all demonstrated progressive increases in expression over time in overwintering apple and haw fly pupae (Fig. 1*D*).

**CNS Transcriptome Trajectories Show Widespread Evolutionary Divergence.** There was extensive DE between the host races over the course of diapause. A total of 2,902 transcripts were significantly DE between the host races within at least one sampled time point and 74% of these were also significantly DE over time in at least one host race. DE was already extensive by 2 mo, but expression trajectories converged midwinter from 3 to 4 mo (Fig. 2*A*; also see convergence of mean expression trajectories for modules in Fig. 2*B*), followed by maximum divergence at 5 mo, with lower divergence at 6 mo. Note that convergence at 3 and 4 mo does not coincide with any general cessation of transcriptional dynamics that might be expected during developmental arrest; trajectories of expression for transcripts changing in expression level over time (Fig. 1), including those divergent between the host races (Fig. 2), continued to change over time early, mid, and late into winter.

Clustering of overwinter gene expression identified seven groups of transcript trajectories describing patterns of transcript change over time that differed between the host races (Fig. 2*B*). As with the results for transcripts whose trajectories changed

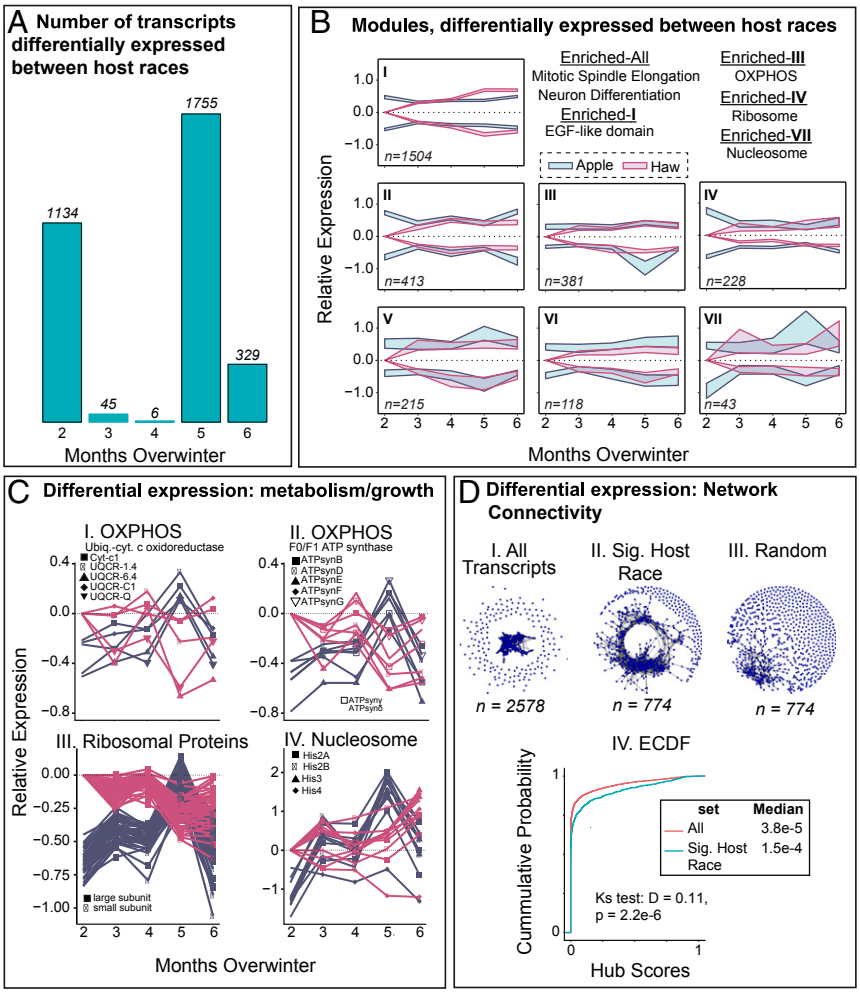



**Fig. 2.** CNS gene expression varies between host races. (*A*) DE was most pronounced (number of transcripts significantly DE) early and late in the overwintering trajectory, with relatively little DE at 3 and 4 mo. (*B*) Trajectories of transcript expression within each of seven modules identified by clustering analysis of 2,902 transcripts DE between apple and haw host races, including number of transcripts (*n*) associated with each module, and select functional categories identified through enrichment analyses of transcripts in select modules and of all transcripts combined (full enrichment results in Dataset S2). Expression values are log2 fold changes relative to the 2 mo time point in hawthorn pupae, and shaded trajectories represent the approximate 95% confidence intervals (± 2 SE) of all genes up-regulated and down-regulated (expression values above and below zero, respectively) over time. (*C*) Transcripts involved in the OXPHOS pathway (I, ubiquinol-cytochrome *c* oxidoreductase and II, F0/F1 ATP synthase families), ribosomal proteins (III), and the core nucleosome proteins (IV) were DE in apple relative to haw flies. (*D*) Transcripts significantly DE between host races had greater connectivity. Panels I through III illustrate connectivity as network plots where circles are transcripts and connecting lines are edges depicting correlations of time series expression exceeding $r = 0.94$. Panel IV illustrates that transcripts DE between host races have significantly higher median hub scores; the plot compares empirical cumulative distribution functions (ECDFs).

through time in both host races (Fig. 1*B*), functional enrichment analysis of all transcripts DE between the host races and of each module separately identified functional groups involved in developmental processes, including signals of cell division (mitotic spindle elongation) and differentiation (neuron differentiation; Fig. 2*B* and Dataset S2). We more closely examined three functional groups that were enriched: 1) OXPHOS proteins (here limited to members of the cytochrome *c*-oxidoreductase and F0/F1 ATP synthase families), to make inferences about changes in energy use and metabolism, 2) ribosomal proteins that provide a signature of active translation, and 3) core nucleosome proteins, whose transcripts are generally only abundant during active cell division [s phase (50)]. We also chose these groups because they include multiple members of gene families that tend to be coordinately expressed (51–53) and thus provide more robust evidence for biological processes than analyses of individual transcripts. For apple flies, transcripts in all of these functional groups showed a clear and common pattern of increase in abundance until 5 mo overwinter, followed by a decrease at 6 mo (Fig. 2*C*, blue lines). Compared to haw fly trajectories, apple fly transcripts in these functional groups started at generally lower abundance at 2 mo, peaked at higher abundance at 5 mo, then dropped to similar values at 6 mo. Dynamics were more variable among transcripts for haw flies across the three functional groups, although as expected ribosomal protein trajectories were highly concordant (Fig. 2*C*, pink lines). Overall, transcriptional signals of metabolism, translation, and cell division were consistent with elevated diapausing apple fly CNS development compared to haw flies by 5 mo overwinter, followed by an apparent cessation or suppression of development at month 6. Moreover, hundreds of other transcripts showed similar expression trajectories, wherein transcription was markedly up- or down-regulated at 5 mo in apple but not haw flies, but by 6 mo the trajectories had converged (e.g., Fig. 2*B*, modules III, V, and VII). Pupae of both host races remain in diapause when initially exposed to warmer temperatures (42).

Thus, this signal is unlikely to reflect a transition to a postdiapause, quiescent state of development in apple files. Instead, it may reflect earlier "potentiation" in apple flies, wherein chilling requirements necessary for diapause completion (25) are surpassed in a proportion of apple but not haw flies contributing to the CNS pools used to generate the RNAseq libraries. Note that this potentiation does not coincide with the end of diapause. Rather, it is a physiological landmark that occurs during diapause development, and more rapid potentiation may hasten the end of diapause.

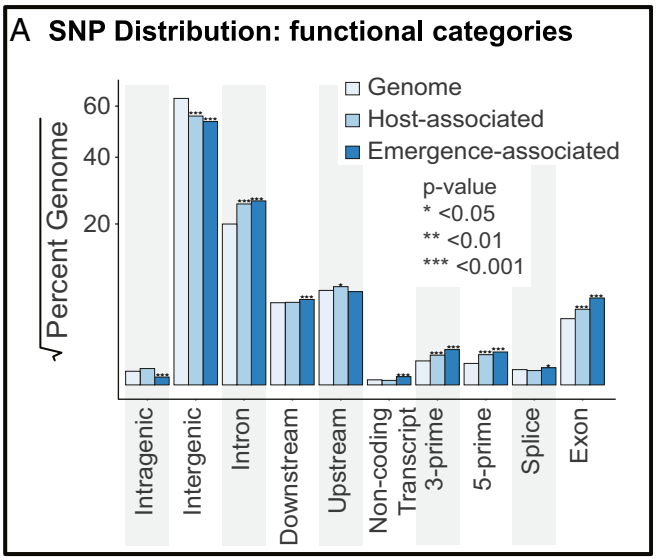
Rapid evolution of the diapause transcriptome could be facilitated by the evolution of genes that serve as hubs in a transcriptional network, wherein changes in hub transcription are correlated with changes in the transcriptional pattern of many genes connected to the hub, for example protein–protein or transcription factor–target interactions (39). We tested whether transcripts DE between the host races tended to exhibit high centrality (i.e., act as hubs) in a network with transcripts as nodes and edges assigned based on the strength of the correlation in the expression time series between nodes (*SI Appendix*, *Supplementary Methods*). We found that transcripts significantly DE across time in both host races and differentially expressed between host races had a significantly higher median hub score (metric of centrality; *Methods*) compared to transcripts differentially expressed across time but not between the host races ($P << 0.0001$, Kolmogorov–Smirnov test; Fig. 2*D*: network of the transcripts DE between host races is shown alongside a network of a random subset of transcripts for visual comparison).

**Genetic Associations with Emergence Timing Are Repeatable and Genome-Wide.** Adult emergence timing is determined by the postwinter duration of pupal diapause in *R. pomonella* (42) and thus serves as an easily measured phenotype on which divergent selection may be acting. Although emergence time depends on overwinter length, apple flies emerged earlier than haw flies at all measured winter lengths (*SI Appendix*, *Supplementary Methods* and Fig. S3) (54). We performed Poolseq analysis on pools of the earliest and latest emerging flies (5-mo overwinter length) from both host races to assess the genomic distribution and functional annotation of loci genetically associated with emergence timing. In total 28,133,855 SNPs and 1,667,189 indels passed our filters (a summary of read mapping statistics and quality control is available in *SI Appendix*, *Supplementary Methods*). Comparing allele frequencies between early- and late-emerging flies revealed 459,563 SNPs significantly associated with emergence timing in apple flies and another 586,460 associated SNPs in haw flies (FDR < 0.05). Of these, 53,335 SNPs were associated with emergence timing in both host races, substantially more than expected by chance (95% CI of the odds ratio: 6.62091 to 6.74868; $P << 0.001$, Fisher's exact test). Moreover, allele frequency differences between late- vs. early-emergence timing pools were extremely highly correlated between the host races when considering loci independently and significantly associated with emergence timing in each host race ($r = 0.96$, $P < 0.0001$, $n = 53{,}335$ SNPs; *SI Appendix*, *Supplementary Methods*), yielding a well-supported, repeatable set of emergence timing-associated loci. These results were robust to different coverage depth filters (*SI Appendix*, *Supplementary Methods*), and a similarly high correlation was estimated in a previous study using independent samples and the same experimental design except that individuals rather than pools were genotyped (37). Likewise, a 500-bp sliding window analysis recapitulated the high repeatability of association strength between the host races that we observed for single locus data (*SI Appendix*, *Supplementary Methods*). We also identified 69,720 (haw) and 85,523 (apple) indels associated with emergence timing (FDR < 0.05; 9,382 associated in both host races). We restricted most of the statistical tests discussed below to SNPs because of the relative ease of estimating allele frequencies but included indels in select functional enrichment analyses. Note that these analyses do not account for linkage disequilibrium (LD) and that the goal was not to determine individual causal variants. Rather, the goal was to determine the genomic distribution of associated variants and their potential functional consequences.

The 53,335 SNPs associated with emergence timing in both host races were nonrandomly dispersed across the genome, being underrepresented in intergenic regions and overrepresented in 5′ and 3′ untranslated regions, introns, and exons (Fig. 3*A*). However, the majority (75%) of emergence-associated SNPs in exons (2,853 total) coded for synonymous changes. These variants are not candidates for structural variation in translated proteins but could affect, for example, rate of transcription/translation (55). Associated SNPs were also nonrandomly dispersed across chromosomes, with excesses of associated SNPs (compared to chance expectations) on chromosomes 1 and 3 that fell mainly within regions previously identified as demonstrating elevated LD (Fig. 3*B*). This result is concordant with an earlier genetic association analysis of a more northern geographic site that identified an excess of emergence-associated markers for apple and haw flies in high-LD regions of chromosomes 1 and 3 representing putative inversions (37). The set of emergence-associated loci from ref. 37 was significantly enriched within the set of emergence-associated SNPs identified in this study, reinforcing the repeatability of our associations across studies done across different years and geographic sites (odds ratio = 5.3 and 8.1 for apple and haw flies, respectively, $P << 0.001$; *SI Appendix*, Table S2). We could not accurately estimate the number of genomic regions contributing to emergence timing without LD estimates in this study. However, previous work using individual sequencing of genome-wide markers robustly supports a polygenic basis for emergence timing (37) and the sheer number of markers repeatedly associated with the trait in this study is also consistent with polygenic inheritance.

We predicted that if general developmental processes underlie diapause duration, then emergence-associated variants should 1) be enriched for functional categories associated with active morphogenesis and 2) occur in genomic regions consistent with *cis* regulation of transcripts DE over the course of diapause. Functional enrichment analysis of a conservative set of loci associated with emergence timing (9,822 SNPs and indels significantly associated at FDR < 0.01 in both apple and haw flies, annotating to 1,335 Flybase gene models) identified enrichment of many functional categories generally associated with vigorous morphogenesis (Dataset S3). Many of these same categories, including those explicitly associated with neural development, were enriched in the sets of transcripts independently identified as DE over the course of diapause and between the host races (Figs. 1*B*, 2*B*, and 3*B* and Datasets S1–S3). The full list of emergence-associated genes included key hormone signaling genes (e.g., the juvenile hormone receptor *Met* and its interaction partner *ftz-f1*), the ecdysone receptor partner *usp*, the ecdysone biosynthesis protein *ecd*, cell cycling genes (e.g., *Myb* and *rbf*), five genes coding for proteins in the *Mediator* complex, a master regulator of general transcription (*MED1*, *MED18*, *MED24*, *skd*, and *kto*), and multiple genes in the Wnt signaling pathway (*fz*, *fz3*, *nkd*, *fry*, *sgg*, *shf*, *CkIIbeta*, and *CkIalpha*). Note that although LD among loci causes some nonindependence, these results are not entirely driven by enrichment of the same functional categories in the high and medium LD regions of chromosomes 1 and 3 (*SI Appendix*, *Supplementary Methods*). Regardless of LD, the results suggest that emergence-associated loci map to genomic regions enriched for genes associated with development, and specifically neurogenesis.

We tested for signatures of potential *cis* regulatory variation by asking whether SNPs associated with emergence timing in both host races were more proximal than expected by chance to the transcription start site (TSS) of genes DE during diapause in both host races (FDR < 0.05). Using a 5-kb interval, there were significantly more SNPs in proximity to a TSS of a DE gene (1,880) than expected by chance (null median = 1,685; $P < 0.0001$; *SI Appendix*, *Supplementary Methods*). Moreover, an additional category enriched in the SNP/Indel data, "chromatin organization," contained a



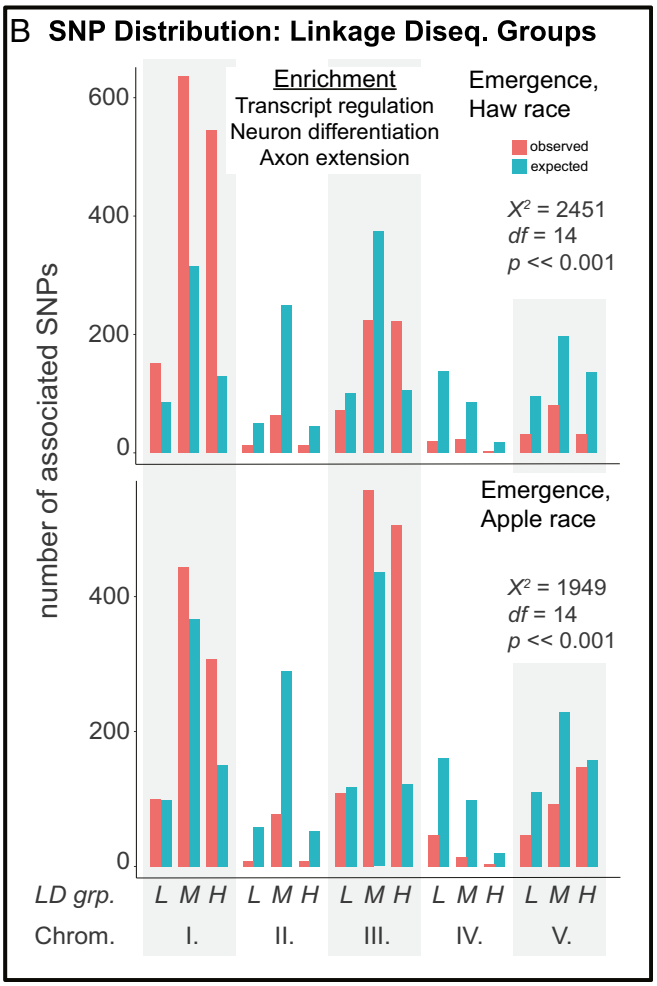




**Fig. 3.** Genome-wide distributions of SNPs. (*A*) Distribution of all host-associated (FDR < 0.05), and emergence-associated (FDR < 0.05 in apple and haw flies) SNPs within annotation categories. Fisher exact tests comparing counts in the genome vs. associated SNPs were performed within each category to test for higher- or lower-than-expected prevalence of associated SNPs. (*B*) Distribution of emergence-associated SNPs across chromosomes and LD classes (L = low, M = medium, H = high) in haw flies (*Upper*) and apple flies (*Lower*). $\chi^2$ tests were performed to test whether associated SNPs were nonuniformly distributed across chromosomes and LD classes. Several key functional categories enriched in the set of SNPs associated with emergence in both host races are listed.

number of core histone proteins, while "wnt signaling pathway" was marginally enriched (FDR = 0.1); elements of both of these lists were also DE across time and/or between the host races (Figs. 1 and 2).

**Emergence-Associated Variants Also Associate with *D. melanogaster* Development Rate.** If general developmental processes affect diapause duration in *R. pomonella*, we predict that similar sets of genes may affect other phenotypes related to development rate. We compared our GWA results to those from Burke et al. (30), the only GWA study of developmental rate in an insect that we could identify. That study focused on a very different phenotype in a different species: the rate of egg-to-adult development under nondiapausing conditions in *D. melanogaster*, applying a Poolseq comparison of control lines vs. lines selected for rapid development for over 600 generations (30). We applied the same analysis pipeline used in the current study of *R. pomonella* to the archived data from Burke et al. (30) (who report only results for nonsynonymous SNPs). Despite the clear difference in the phenotype, we found that the list of genes harboring SNPs associated with development time in *D. melanogaster* was significantly enriched in the list of genes with SNPs associated with adult emergence time in both the apple and haw host races (odds ratio [OR] = 1.5, $P << 0.001$; *SI Appendix*, Table S3). Thus, variation in the duration of diapause development in *R. pomonella* seems to be determined by similar sets of genes that influence the general rate of embryonic, larval, and or pupal development in *D. melanogaster*.

**Loci Associated with Emergence Timing Show Evolutionary Divergence.** If loci associated with emergence timing within fly populations contribute to evolved differences between the host races, we predict that emergence-associated loci should tend to diverge in allele frequency between the host races in a pattern consistent with soft selective sweeps on polygenic variation (56). We identified 14,650 SNPs and indels associated with host race differences (FDR < 0.05). Of these, 698 and 596 were also associated with emergence timing (FDR < 0.05) in apple- and haw-infesting flies, respectively, significantly more than expected by chance (OR > 2.3, $P << 0.001$ in both cases; *SI Appendix*, Table S4). Although strongly differentiated between the host races, allelic differences rarely approached 1.0 (median absolute value = 0.56 for SNPs with FDR < 0.05). This is stronger divergence than observed between sympatric *R. pomonella* host races sampled from a site near Grant, MI [maximum allele frequency difference of 0.231 (37)], consistent with the greater observed temporal separation at the Urbana, IL field site sampled in this study compared to Grant, MI (57). Genomic intervals containing these variants did not display signatures of hard selective sweeps as measured by Tajima's D (*SI Appendix*, Fig. S4), as would be expected for loci of major effect experiencing strong natural selection on new mutations (58). Thus, the genetic evidence is most consistent with soft selective sweeps on standing genetic variation, a result agreeing with previous work implying an ancestral origin in Mexico for much of the variation affecting diapause in *R. pomonella*, predating the formation of the host races (59).

The set of SNPs associated with emergence timing and host race differences (456 SNPs emergence-associated at FDR < 0.05 in apple or haw flies and associated with host race divergence at FDR < 0.05) were enriched for similar functional categories identified above for emergence-associated SNPs (Dataset S4). There was also a signal of between host race *cis* regulatory variation; 476 SNPs were associated with host race differences and emergence timing (FDR < 0.05) and were also localized within 5 kb of the TSS of genes DE between host races in the transcriptomic experiments above (FDR < 0.05), significantly more than expected by chance (null median = 386, $P < 0.0001$).

## Discussion

**General Developmental Processes Influence Diapause-Mediated Phenology.** Our results suggest that polygenic variation in diverse developmental pathways contributes to phenological variation within and between the ancestral hawthorn- and derived apple-infesting populations of *R. pomonella* that demonstrate evolved differences in seasonal life-history timing. Here, phenology is mediated by diapause duration, and this inference builds on the hypothesis that morphogenic, developmental progression (albeit drastically slowed) may be common during diapause. Further, the rate of diapause development may be controlled by the same processes that control general (i.e., not diapause-specific) developmental processes (29). Four key results support this hypothesis for *R. pomonella*.

First, there is clear evidence of progressive changes in the expression of a variety of developmental pathways in the CNS over the course of diapause, including up- and down-regulated transcripts as expected during morphogenesis (60). We did not detect pulses, or waves of gene expression at the whole-CNS level that are often associated with metamorphosis (61) and neurogenesis (60), although such patterns may be present in specific neuron subtypes or may have been obscured by interindividual variation within our sampling pools. Gross CNS morphology did not change during 6 mo of diapause, a pattern also observed based on fine-resolution imaging of brain substructures over time in diapausing butterfly pupae (62). However, the overall signal of development in the transcriptomic data, for example up-regulation of cell fate proteins associated with cell differentiation, and many transcripts associated with neurogenesis, is most consistent with some sort of developmental progression. There is continuing cell proliferation (41), differentiation (46, 47), and marked neural remodeling (48) during the early stages of pupal development in *D. melanogaster* that would overlap with the stage of pupal development at which diapause occurs in *R. pomonella*.

Second, the apple host race, with shorter postwinter diapause duration, demonstrated signatures of more rapid development based on progressive and more rapid up-regulation of growth- and development-related transcripts relative to the haw host race, with longer diapause postwinter duration.

Third, thousands of genetic variants were associated with variation in diapause duration. These variants were dispersed across the genome but concentrated in high-LD regions previously associated with emergence timing and host race divergence (37). The set of genes with emergence-associated variants, identified independently from differentially expressed transcripts, was also highly enriched for gene sets typically associated with morphogenic development, and specifically neuron development, during embryogenesis and metamorphosis in *D. melanogaster* (63). Also, associated variants tended to be located in genomic regions consistent with *cis* regulatory variation of genes differentially expressed across time during diapause.

Fourth, there was clear, statistically significant similarity between the sets of genes harboring variants influencing diapause duration in *R. pomonella* and the rate of egg-to-adult development in *D. melanogaster*, extremely disparate phenotypes united only by their connection to rate of development.

We expand on the diapause developmental rate hypothesis (29) and suggest that diapause duration in *R. pomonella* is affected by the rate of development through a pupal developmental stage that has evolved an exaggerated, expanded duration relative to an ancestral state of nondiapause pupal development common across higher flies (64). Such an evolutionary innovation would represent an extreme analog of the well-documented phenomenon of substantial variation in the duration of all individual life cycle stages across Insecta (65). Indeed, the developmental steps during CNS metamorphosis are indistinguishable between direct developing and diapausing pupae of the butterfly *Pieris napi* (62). Moreover, measurable morphogenesis seems to occur during diapause in other insects, particularly gonadal development during adult reproductive diapause (26, 28). Pupal diapause in *R. pomonella* occurs somewhere between the homologous stage p4 and p5 in *D. melanogaster* (43, 64), a developmental interval that takes hours in *D. melanogaster* and likely multiple days in *R. pomonella* artificially induced to develop without diapause [total duration of nondiapause pupal and pharate adult development is ~30 d (66)]. The end of diapause in *R. pomonella*, an event that takes place over a 48-h window (at 24 °C), is followed by developing clear spots (loss of fat body adhesion) at the site of the eyes and antennal discs (44), late events in stage p5 in *D. melanogaster* (43). There is marked brain development (and clearly transcriptional dynamism) in stages p4 and p5 in *D. melanogaster*, and equivalent steps may play out over extended periods of months in diapausing *R. pomonella*. For example, although most cells derived from brain tissue appear to be in a G0/G1 arrest during pupal diapause in *Sarcophaga crassipalpis* (flesh fly), there is also moderate expression of cell cycle-related transcripts during diapause (67), suggesting a small amount of developmental activity.

**Rapid Evolution of Seasonality via Diapause Development.**

***Transcriptomic divergence.*** Although divergence of gene expression during diapause was widespread across the transcriptome, two key patterns emerged in the distribution of regulatory differences across gene networks and across time. First, the network structure of the divergent diapause transcriptome supports a key role for hub-like genes with high network centrality. Specifically, well-connected, more hub-like genes were overrepresented in the set of transcripts divergently expressed between the host races during diapause. Thus, although evolved differences are polygenic, with no particular gene having a major effect, the rapid evolution of phenology in *R. pomonella* appears to have favored changes in transcription at loci with higher network conductivity than expected by chance. Recent work indeed predicts that the evolution of hub-like genes can facilitate rapid evolution by facilitating larger "jumps" in phenotypic space (39, 68). However, the generality of this result may depend on the strength of natural selection. For example, Yang and Wittkopp (69) found that transcription factors with higher connectivity demonstrated reduced expression divergence between *D. melanogaster* laboratory strains and no relationship with expression divergence between *Drosophila* species. However, the relative strength of drift vs. selection is likely very high between laboratory strains and fully divergent species, whereas divergence between *R. pomonella* sympatric host races is driven primarily by strong selection, with neutral variants rapidly homogenized by gene flow (70). With relatively few empirical examples available, more work will be needed to test the general relationship between strength of selection and the network structure of regulatory divergence.

Second, there was a clear, hourglass-like pattern of expression divergence between the apple and haw host races across the diapause developmental period, with high divergence early and late in diapause, but nearly undetectable divergence in the middle. The duration of diapause may thus be highly sensitive to perturbations in gene expression during the termination process, which is temporally proximal to adult emergence and ultimately affects seasonal timing in nature (71). Diapause initiation, the earliest phase of diapause development, may also play a key role in diapause duration (72). In particular, diapause intensity, or recalcitrance to premature diapause termination, is established during diapause initiation and varies within and among *R. pomonella* host races (57, 73, 74), driving earlier emergence times for some individuals capable of emerging with short winter lengths (see *SI Appendix*, *Supplementary Methods* for detailed discussion).

A remarkably similar pattern has been noted for the evolution of expression divergence between *Drosophila* species during embryogenesis (75), consistent with the hourglass model of body plan evolution, positing that critical events midembryogenesis are evolutionarily constrained (76). It is possible that key developmental events occur midway through diapause, often referred to as the "maintenance" phase (77), and tend to be similarly constrained for successful diapause development. However, because phenotypes that diverge between *R. pomonella* host races have evolved rapidly under strong selection, the hourglass pattern of divergence may simply reflect strong selection on diapause intensity and diapause termination that is idiosyncratic to this system. Accumulating data on diapause transcriptomes from diverse insect species (e.g., ref. 78) may provide a more general test for hourglass-like divergence across diapause, particularly as developmental time series are investigated at higher resolution (21).

***Genomic architecture.*** Polygenic trait architecture is common (79) and polygenic traits can rapidly evolve from standing genetic variation in nature (56). It has been difficult to make developmental and physiological inferences for such traits, let alone to identify the individual genes that "matter" for evolution (80). Previous studies in *R. pomonella* revealed large reservoirs of polygenic, standing genetic variation in phenology within and across geographic populations that contribute to the rapidly evolved phenological differences between host races (35–37, 70, 81). This mirrors results in many other studies and may help to explain why diapause-mediated phenology is so evolutionarily pliable (8, 9, 82–84). Building on these results, the extreme phenotyping design of the current study yielded high statistical power (85), leading to high technical repeatability (independent GWA in each host race) of genotype-to-phenotype relationships for many loci, each with comparatively small effect. Moreover, signatures of genome-wide allelic divergence but not of hard selective sweeps were consistent with polygenic adaptation through soft sweeps (58). Emergence-associated and host-divergent variants were overrepresented in putative regulatory regions and near TSS of differentially expressed, developmental genes, consistent with the *cis* regulatory hypothesis for morphological evolution (86). Thus, the integration of the genomic and transcriptomic data suggests that many developmental genes and pathways affect phenology through their individual effects on development rate. This is not only consistent with the general conclusions of a study of development rate in *D. melanogaster* (30) but also with the specific sets of genes identified in that study. There may be many genetic paths to the evolution of development rate, but many genes may also act pleiotropically on rates of multiple processes across ontogeny.

## Conclusions

Our study provides a high-resolution overview of physiological and developmental dynamics during diapause at the transcriptional level and evidence that variation in those processes underlies rapid life-history evolution. Our data support the hypothesis that development may be severely decelerated but not arrested during diapause. As a result, rates of developmental processes may ultimately be a major determinant of diapause duration and therefore phenology. Variation across many developmental processes appears to contribute to the rapid evolution of phenology in *R. pomonella* and mirrors genetic variation underlying development rate in *D. melanogaster*. This suggests that although there are many evolutionary routes to faster/slower development, common physiological mechanisms may underlie many, or even most, developmental intervals that contribute to life-history timing, whether or not they are mediated by diapause.

## Methods

**Genome Sequencing and Assembly.** *R. zephyria*, infesting snowberries (*Symphoricarpos*), and *R. pomonella* are sibling species in the *R. pomonella* species group (87). Here, we sequenced the *R. zephyria* genome to leverage *R. zephyria*'s lower rates of polymorphisms compared to *R. pomonella* (40). The genome of *R. zephyria* was sequenced using both individuals and pools of flies collected from East Lansing, MI, with a combination of short-read shotgun and Tru-seq synthetic long-read sequencing. Details on genome sequencing and assembly are available in *SI Appendix, Supplementary Methods*. Gene annotations are available from NCBI, but we also annotated all genes to FlyBase v 6.13 using BLASTX with a minimum e-value cutoff $< 1 \times 10^{-5}$; 21,163 out of 29,399 genes returned BLAST hits.

**RNAseq Experiment.** Infested apple and hawthorn fruits were collected from Urbana, IL, in the late summer and fall of 2014 and transported to the University of Florida. Emerging larvae were collected and held at 24±1 °C with 14 h:10 h light:dark for 10 d to complete pupation and enter diapause, then pupae were transferred to 3.5±1 °C at 95% relative humidity (RH); all pupae failing a screen for low metabolic rate (nondiapause pupae have high metabolic rates) were excluded from the study. At monthly intervals from 2 to 6 mo we dissected out the CNS, forming pools of 20 CNS per host race and time point. Additional details of rearing, subject selection, and tissue collection appear in *SI Appendix, Supplementary Methods*. Purification of RNA was performed as described in Meyers et al. (38). RNAseq libraries were prepared using Illumina's tru-seq stranded mRNA kit and sequenced using 100-bp single-end Illumina HiSeq 2500. Cleaned reads were mapped to the *R. zephyria* genome using STAR (88), and read counts were generated using RSEM and featureCounts (89). Multidimensional scaling plots revealed obvious outliers also with disproportionate library sizes; these were removed, leaving three samples in the 4-mo haw treatment (*SI Appendix, Supplementary Methods*). We fit read count data from a filtered set of 17,215 genes to generalized linear models using edgeR, including normalization steps (90). The full model included fixed effects of host race and time. Total DE (including all splice variants) was tested using likelihood ratio tests (glmLRT function). Additional details of the statistical models are available in *SI Appendix, Supplementary Methods*. Clustering of transcript trajectories over time was performed using weighted gene coexpression network analysis (91), calculating the distance matrix from the absolute correlation between expression trajectories over time, and excluding soft thresholding (*SI Appendix, Supplementary Methods*). Finally, we performed enrichment analysis by submitting Flybase-annotated gene lists to the online David functional enrichment tool (92), identifying categories with a Benjamini and Hochberg FDR less than 0.05.

**CNS Phenotyping.** We measured the width and length of brain hemispheres from dissected CNS under the same rearing conditions/time points using a hawthorn-infesting *R. pomonella* population collected from East Lansing, MI (*SI Appendix, Supplementary Methods*).

**Poolseq Experiment.** A large additional subset of pupae from the 2014 Urbana, IL, field collections were reared through 5 mo overwinter as above, moved to 24 °C at 95% RH, then observed for time to adult emergence. A total of 5,101 (range 22 to 127 d) haw and 791 (range 14 to 118 d) apple flies emerged, respectively (the sample size from field collections was much larger for haw flies). Random samples of 100 apple and haw flies from the entire emergence sample, the earliest-emerging 48 flies (0.11 and 0.07 quantiles for haw and apple), and the latest-emerging 48 flies (0.99 and 0.95 quantiles for haw and apple) were sampled from each distribution. We extracted DNA (Qiagen DNeasy kit) from the head of each sample, quantified DNA concentration using a PicoGreen assay (Invitrogen), and created six equimolar pools: apple fly random, early, and late pools and haw fly random, early, and late pools. Libraries were prepared at the University of Colorado Genomic and Microarray Core using the Nugen Ovation Ultralow System V2 and sequenced on an Illumina HiSeq 3000 (100-bp paired-end reads). Pools had an average of 150 million paired-end reads (range 140 to 170 million). Cleaned reads were mapped to the *R. zephyria* reference genome, SNP and indel variants called, and reads counts per sample at each variant generated (*SI Appendix, Supplementary Methods*). Allele frequencies were estimated from allelic proportions, and all variants were annotated against the *R. zephyria* genome using SnpEff (93). Previous studies have mapped a total of 5,079 RAD loci to the five main *R. pomonella* chromosomes, 4,908 of which have been mapped to regions within chromosomes that display high, intermediate, and low levels of LD (35–37). We assigned SNP variants that mapped to within 200 bp of mapped RAD loci to chromosomes and LD groups. Statistical associations with host race or emergence time were inferred using both the likelihood method of Lynch et al. (94) and Fisher's exact tests on allele count data; here we report the Fisher's exact test results, although both yielded similar results. Functional

enrichment analyses were performed as described above, and Tajima's D was estimated with a sliding window (step 500 bp and window size 1,000 bp) for each of the six pools using PoPoolation (95) (*SI Appendix, Supplementary Methods*).

**Data Availability.** RNA raw reads and raw counts are available from NCBI GEO (accession no. GSE140473 [samples GSM4162629–GSM4162678]). Sequence and transcriptomic data are available through NCBI (accession no. LYWK00000000). Pooled sequencing raw reads are available from SRA bioproject PRJNA590789 (biosamples: SAMN13338971–SAMN13338976). Raw data used in genome assembly are available through SRA bioprojects PRJNA331175 and PRJNA321204. All data analysis scripts are posted at GitHub, https://github.com/gjragland/Diverse-develomental-processes-influence-seasonal-phenology.

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