Title: Rapid brain development and reduced neuromodulator titers correlate with host shifts in *Rhagoletis pomonella*

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Abstract:

Host shifts are considered a key generator of insect biodiversity. For insects, adaptation to new host plants often requires changes in larval/pupal development and behavioural preference towards new hosts. Neurochemicals play key roles in both development and behaviour, and therefore provide a potential source for such synchronization. Here, we correlated life history timing, brain development, and corresponding levels of 14 neurochemicals in *Rhagoletis pomonella* (Diptera: Tephritidae), a species undergoing ecological speciation through an ongoing host shift from hawthorn to apple fruit. These races exhibit differences in pupal diapause timing as well as adult behavioural preference with respect to their hosts. This difference in behavioral preference is coupled with differences in neurophysiological response to host volatiles. We found that apple race pupae exhibited adult brain development three weeks faster after an identical simulated winter than the hawthorn race, which correlated with significantly lower titers of several neurochemicals. In some cases, particularly for biogenic amines, these differences in titers persisted through eclosion and sexual maturation of the adult, the stage at which host preference is exhibited. In summary, life history timing, neurochemical titre, and brain development rate are associated and could link differences in life history and host preference in this speciating system.

Introduction:

Adaptation to environmental and ecological factors has been shown to play an important role in population divergence and speciation in a large number of systems (1–3). For phytophagous insects shifting to new host plants, populations potentially need to adapt their growth and development to the new host, and at the same time modulate their behavioural preference to locate that host. Furthermore, populations on novel hosts must regulate their life-history timing to coincide with the new host phenology (4–11). How these multiple events are synchronized between ancestral and novel hosts with vastly different phenologies and characteristics is an area of intense study for understanding the genesis of insect biodiversity (7,12).

Rhagoletis pomonella (Diptera: Tephrtidae) provides a unique opportunity to identify associations between pupal development, life history timing, and adult host choice. This species of flies originally infested fruits of the native downy hawthorn (*Crataegus mollis*) until apples (*Malus pumila*) were introduced in Eastern USA around 180 years ago (12–14). This introduction of a new host facilitated a shift in host preference from their native host, downy hawthorn, to domesticated apples, eventually leading to two genetically diverged host races specific to each fruit (12,13). This host shift from hawthorn to apple fruit was facilitated by two factors. First, apple and hawthorn flies are univoltine and the adults are short lived, thus each host race must emerge synchronized with the fruiting time of their host plant. Due to the earlier fruiting time of apples, apple flies initiate their overwintering dormancy, termed diapause, earlier than hawthorn flies, and emerge as adults about one month earlier as well (13). Previous work shows that the earlier seasonal adult emergence of apple host-race flies is driven solely by the timing of the termination of pupal diapause (15). The difference in adult emergence timing that drives synchronization with host fruits results in a degree of mating isolation between the apple and hawthorn races (16). Second, adults of the two host races also exhibit distinct preferences for the volatiles of their respective host fruits, which serves as an important reproductive barrier because the flies mate directly on or near the ripe host fruit (17–19). Previous studies forcing hybridisation between the host races in the laboratory showed that F1 hybrids of these two populations have altered peripheral olfactory physiology suggesting developmental abnormalities regarding their response to the host volatiles. This prompted our study of neurochemicals during pupal and adult development between the host races (20). Recent studies have shown that the change in host fruit olfactory preference between the two host races does not occur at the chemoreception stage in the antenna (21–23), but rather at the first synapse of the olfactory system in the brain, the antennal lobe (24). The coupled difference in pupal diapause timing, adult preference behaviour, and adult brain physiology in *R. pomonella* provides a unique opportunity to examine if there are

corresponding differences in brain development between the races that could simultaneously impact both developmental rate and adult olfactory host choice.

Species from temperate regions like *R. pomonella* frequently use the timing of diapause to avoid the stresses of winter and also to synchronize themselves with the phenology of their hosts the next growing season (25). While diapause is often conceived of as a state of developmental arrest, it is in fact a dynamic, physiologically regulated process with defined phases of development including the diapause preparatory stage, diapause induction, diapause maintenance, and the resumption of rapid development at the end of diapause (26,27). In insects, the central nervous system (CNS) and associated endocrine glands produce neurochemicals like neurotransmitters, neurohormones, and neuropeptides that regulate diverse physiological events including the induction and termination of diapause (28). Both the brain and ring gland play important roles in regulation of pupal diapause in dipterans (29,30). Diapause can be regulated via changes in hormone/neurotransmitter titres, receptor abundance, or regulation of specific neurochemical pathways across the stages of diapause, such as has been found with the dopamine and serotonin pathways (31–33). Apart from life history timing, many of these same neurochemicals also play important roles in insect behaviour. Biogenic amines like dopamine, octopamine, and serotonin are known to have profound impacts on adult insect behaviour across many taxa (34–37).

The dual role of neuromodulators in regulating both insect development and host-seeking behavior suggests that changes in neuromodulator titres or production at specific life stages could impact both life history timing and preference of a phytophagous insect for its host plant through changes in brain development or differentiation. For univoltine insects undergoing diapause like *R. pomonella*, the impacts of neuromodulation on development and subsequent behavior could occur at several stages. First, changes in neuromodulator levels during the diapause preparatory or induction phase could impact diapause timing and subsequent development. Second, changes in neuromodulator levels during diapause maintenance could impact the onset of adult brain neurogenesis as the insect terminates diapause and undergoes metamorphosis. Third, changes in neuromodulation after diapause termination and during post-diapause metamorphosis could impact adult brain development and differentiation. Finally, changes in neuromodulation after ecolsion could impact adult host preference on its own. Given the multifaceted role of neuromodulators in regulating development and behavior, these impacts could occur at any or all of these stages of development. To assess this, one would need to track and compare neuromodulator titres over the course of insect diapause and brain development between closely related populations that differ in either life history timing, adult host preference behaviour, or both.

In this study, we use a variety of chemoanalytical, morphological, and immunohistochemical techniques to examine pupal-adult brain development, life history timing, and corresponding neurotransmitter levels in two closely related populations of *R. pomonella* that differ both in diapause timing and adult preference for their respective hosts. While a large number of studies have separately shown the impacts of neuromodulators on life history timing, diapause, or adult behaviour, there is a lack of knowledge as to whether the same chemicals can connect these different aspects of insect-host adaptation together to provide a single source for selection to synchronize life history timing and preference for a new host plant. The quantitative examination of the neurochemical underpinnings of development between two closely related host races provides the foundation to assess whether changes in levels of specific neurochemicals can link life history timing and host preference behaviour to facilitate host shifts and resultant speciation events.

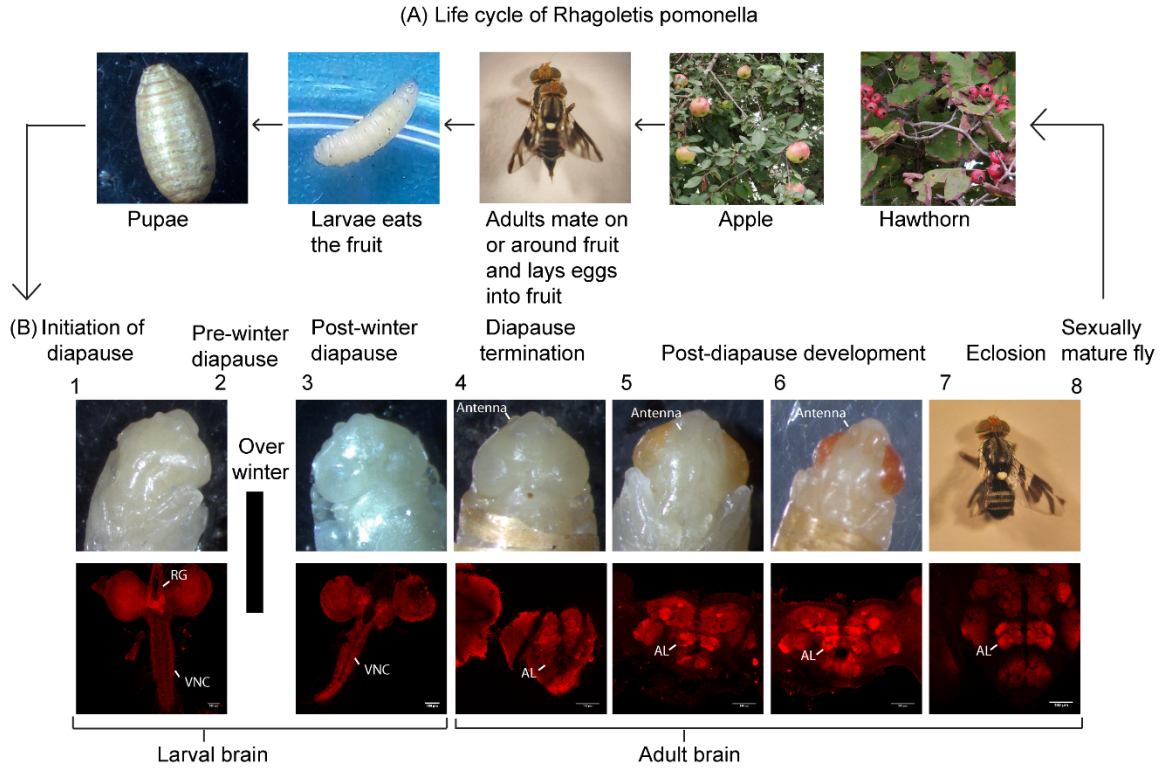

Figure 1: The life cycle of *Rhagoletis pomonella.* (A) Life stages of the fly from mating to oviposition from right to left. (B) Micrographic images of pupal, pharate-adult, and adult developmental stages from left to right. The upper stereomicrographs in the bottom panels show the head morphology while the lower confocal micrographs indicate the corresponding brain morphology of those stages using immunohistochemical nc82 staining across the different developmental stages (stages 1-8). The developmental stages are classified based on distinct head morphology, metabolic rate, or CNS development. Our imaging data showed that there are no morphological differences between stages 1 and 2 or between stages 7 and 8. The stages are as follows: **1**, Pre-winter diapause induction stage

pupae still have high metabolic rates (Methods & Figure s1, s2 and Movie 1, apple race, stage 1); **2**, pre-winter diapause induction stage when pupae have entered metabolic depression (Figure s1, s2 Figure 1 and Movie 2, hawthorn race, stage 2); **3**, post-winter diapause maintenance phase (Figure s2 and Movie 3, apple race, stage 3); **4**, end of diapause (Figure s2. Movie 4, hawthorn race, stage 4); **5**, midway through pharate-adult development (Figure s2, Movie 5, apple race, stage 5); **6**, late pharate-adult development ((Figure s2, Movie 6, apple race, stage 6); **7**, sexually immature adult fly, less than 7 days old ((Figure s2, Movie 7, hawthorn race, stage 7); **8**, sexually mature fly, more than 12 days old;

Methods:

Insect collection and maintenance

Apple and hawthorn fruits naturally infested with larvae were collected from four different sites in Michigan, USA (Grant, Fennville, Cassopolis, Lansing) in August and September 2016, and flies were reared from larvae to adulthood following previously established *Rhagoletis* husbandry methods (14). In May to August 2017, after leaving the pupae at room temperature for 15 days, they were shipped to India (with permit). This set of pupae were used to study brain development and quantification of neurotransmitters from adult flies. Eclosed adults were maintained on a diet of sugar and yeast on a 14L:10D light cycle at 25° C and 65% humidity. Post-eclosion, young flies 1-6 days old were classified as sexually immature whereas flies that were 12-14 days old were classified as sexually mature (39–41).

To study the pre-winter and post-winter brain development as well as quantify neurotransmitters, a second set of pupae were collected in summer 2018. After collecting infested fruits from the above field sites, fruit were transferred to a tray with a wire mesh attached and kept in an insect-rearing room at 25±1°C, 14L:10D light cycle. Every day newly emerged pupae were collected and transferred to petri dishes with damp vermiculite, and maintained within a chamber containing a saturated potassium chloride solution to maintain ~85% relative humidity. To differentiate diapausing and non-diapausing pupae during the diapause initiation stage, four different cohorts of pupae were set aside and subjected to metabolic rate measurements once they reached either 7 or 19 days after pupariation. Other cohorts of pupae at 10 days after pupariation were transferred to a dark refrigerator at 4°C with saturated KCL solution to stimulate over-wintering diapause for six months and study post winter development until they were hand-carried to India in November 2018.

Chemicals and Reagents

16% Paraformaldehyde EM grade (15710) was obtained from Electron Microscopy Sciences, Hatfield, PA, USA. Triton x and Bovine serum albumin were purchased from Sigma-Aldrich (Bangalore, India). All standards, ammonium acetate, Acetone, hydrochloric acid (HCl), boric acid, and reagents required for 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC)

synthesis, were obtained from Sigma-Aldrich (Bangalore, India). Deuterated internal standards 14 were supplied by CDN isotope (Quebec, Canada). Ascorbic acid was obtained from Himedia (Bangalore, India), and Formic acid (FA) was obtained from Fisher Scientific (Bangalore, India). Reverse-phase solid phase extraction (RP-SPE) cartridges (Strata-X, 8B-S100-TAK) were obtained from Phenomenex, Inc. (Hyderabad, India). High-purity MS grade solvents (methanol, acetonitrile, and water) were obtained from Merck Millipore (Merck Millipore India Pvt. Ltd., Bangalore).

Pre-diapause metabolic rate measurement

Even though pupal diapause is ecologically obligate in *R. pomonella*, a small number of pupae avert diapause under laboratory conditions and directly develop into pharate adults in the prewinter period (11). To eliminate these non-diapausing individuals from our sampling before overwintering, we used a protocol adapted from Ragland *et* al 2009 & Powell *et* al 2021 to phenotype pupae as diapausing or non-diapausing by measuring metabolic rates in the pre-winter period.

First we collected 7-day and 19-day-old pupae to measure their weight on an analytical balance with 5 μg precision (Mettler XP6, Toledo, OH, USA). Pupae were transferred to a 5 ml syringe used as a respirometry chamber for checking metabolic rate as an indicator of diapause or non-diapause status, held for 24h so adequate CO_2 could build up in the chamber, and CO_2 was measured on the 8th and 20th day respectively. Syringes were sealed with the plunger drawn back to produce a chamber of 1 ml internal volume. *R. pomonella* pupae were small enough to fit into one arm of the luer valve, allowing the full volume of the syringe to be injected. We also purged multiple empty syringes to serve as controls/blanks. Every 24 h, the full volume of each syringe was injected into a flow-through respirometry system consisting of a Li-Cor 7000 infrared CO_2 analyser (Lincoln, NE, USA) with a resolution of 5 parts per million (ppm) CO_2 interfaced to a Sable Systems International UI-2, recorded by Expedata data logging software (Las Vegas, NV, USA). The flow rate was fixed at 150 ml/min using a Sierra Instruments mass flow controller (Monterey, CA, USA). CO_2-free air, scrubbed with a drierite-ascarite-drierite column, served as the baseline for measurements, and the system was routinely calibrated with CO_2-free air and a certified standard mixture of 500 ppm CO_2 in nitrogen (Airgas, Jacksonville, FL, USA). For one replicate a total of 30-40 pupae were used to calculate the metabolic rate and after that, the brain samples were dissected on cold PBS(1x) and flash frozen with liquid nitrogen. For further details, see Supplementary Materials, Extended Methods.

Brain morphology and immunohistochemistry

In early November 2018, diapausing pupae were hand-carried with ice packs from Gainesville, FL, USA to Bangalore, India. After keeping pupae 6-months in the refrigerator (October 2018 - March 2019), in March 2019 all four different cohorts of pupae of the apple race were pulled

out at their respective 6-month time points and left at room temperature 25°C, 14L:10D light cycle, and 65% humidity as described in Insect collection and maintenance above. Every 5 days after removal from artificial overwintering, a few pupal caps (n=5-35) were removed to observe development externally and brains were also dissected from each individual to assess brain development (Figs. 1-2). Further, pupae were individually photographed using an infinity HD camera (lumenera, model number N9033210) attached to a stereomicroscope. After that, the brain was individually dissected using 50ul of 1X phosphate buffered saline solution, PBS (PH: 7.4) on ice with fine forceps, and stored at -80^{o}C. Four different cohorts of pupae of the Hawthorn race were pulled out in April 2019 at their respective 6 months of overwintering time points (November 2018 - April 2019) and were photographed and then dissected using the above protocol.

To characterize brain morphology, brain samples were dissected as mentioned above and then underwent immunohistochemical staining using a protocol adapted from those used in *Drosophila and Rhagoletis* (24,42,43). Further details can be found in Supplementary Materials, Extended Methods.

Quantification of neurotransmitters from the R. *pomonella* brain

After morphologically determining developmental stage, brain samples were removed from -80^{o}C storage and five brains of the same stage were pooled for each sample replicate. The sample processing was performed as in Ramesh *et* al, 2019 (44–46). After the brain samples were pulled out, samples were immediately transferred to a vial containing 190 µl of Acetone (with 0.1% Formic acid, FA) and 10 µl of 1% of ascorbic acid (1.76mg/ml), followed by derivatization with 6-aminoquinolyl –N-hydroxysuccinimidyl carbamate (AQC) as in Ramesh et al 2019. (44–46). Further details can be found in Supplementary Materials, Extended Methods. Adult fly brains were dissected individually, pooled into a group of five, and stored at -80^{o}C with190 µl of Acetone and 10 µl of 1% of ascorbic acid. After that 10 µl of internal standard (ISTD), a mixture of all 14 neurotransmitters, (0.5ug/ml i.e. 1ng on column) was added to brain samples in screw cap vials. This mixture was sonicated for 1 min, and then homogenised using a plastic pestle. It was then immediately centrifuged (13500 rpm 4^{o}C, 5 minutes), and the supernatant was transferred to a new tube. Simultaneously, the vials for standard solutions for calibration curves were also prepared (supplementary table s11). Serial dilutions of standard stocks were prepared with highest concentration considered on column 100% to five points including 50%,25%,12.5% and 6,25% of the maximum quantity for each of the targeted compounds, for pre-winter and post-winter brain samples. For sexually immature and sexually mature adults the standard stock was diluted to prepare 200%, 160%, 80%, 40%, 20% of the targeted compounds. After that 10 µl internal standards with 190 µl

acetone (0.1% FA) and 10 µl of ascorbic acid were added. All samples and standard tubes were dried in a speed-vac for 1hr. Apart from these, one more vial containing 16 amino acids (1 µl of 10 µg/ml) was added as an additional standard. These 16 amino acid standards helped to validate the method and retention time used for mass spectrometry (MS) every time we ran a sample. Once everything was dried in the speed-vac, 80 µl of borate buffer with 10 µl of ascorbic acid (1.76 mg/ml) was added to all tubes and vortexed. Before analysis, 10 µl of 10 mg/ml AQC (prepared in 100% Acetonitrile, ACN) was added and kept for 10 min at 55^{o}C. After that 3ul of 100% formic acid FA was added and the tube was vortexed to stop the reaction. Tubes were subsequently kept at room temperature until all SPE columns were rinsed and cleaned with 100% methanol and 0.1% FA. After that, 500 µl of water was added to all the tubes, then vortexed and the solution was loaded on SPE columns. Columns were washed twice with 0.1% formic acid prepared with LC grade water (Squeeze out all water). After that 1ml of ACN: MeOH 4:1 in 0.1% formic acid was added to the column and eluted in a new vial. All tubes were dried in a speed vac for 3hrs. Dried samples were stored at -20^{o}C until they were run on LC-MS. Each sample was thawed and reconstituted in 50 µl of 2% ACN prepared in 0.5% FA. The LC-MS instrument method and setup are described in detail in tables s9, s10, s11, s12 figure s6 and s7. The final quantification and analyses can be found in detail in Supplementary Materials, Extended Methods.

Statistical Analysis:

1) To compare the relative differences in timing of the proportion of apple vs. haw flies transitioning from pupal diapausing brain morphology to adult brain development post winter, we used a generalised linear model with a binomial, log-link function in R (V4.0.2) with ggplot, dplyr, and tidyr packed. In this logistic GLM host days after winter was used to explain the proportion of individuals transitioning from stage 3 to stage 4 of brain development between apple and hawthorn flies, as well as to estimate 95% CIs around the logistic estimats. 2) Respiration and neuromodulator data were analysed using linear mixed effects models in R (V4.0.2) using the lmer function from the lme4 package. In the models, age and host race were used as a fixed effects whereas cohort / batch number was used as a random effect. Further, Tukey's HSD tests with correction for multiple comparisons were performed with pairwise multiple comparisons using the lmerTest package. Satterthwaite approximations on the lmerTest package were used to test the significance of the effects. Linear Discriminant Analysis (LDA) was performed on the same transformed data using the "MASS" package in R, with "leave-one-out cross-validation" to estimate the classification accuracy of the models. 3) The interaction between host race and days on amount of precursor neurochemicals at brain development stage 4 was done using, two-way ANOVA, generalised linear model, in SPSS V.26. In the model host race and days were used as fixed factors and quantities of

each neurochemical was used as the dependent variable. Further, Tukey's LSD tests were performed for pairwise comparisons with significance cut offs of 0.0001,0.001, and 0.05.

Results:

***Rhagoletis pomonella* host races exhibit distinct brain development stages during pupation**

We observed external morphological and brain development in *Rhagoletis pomonella* pupae during diapause initiation, pre-winter diapause, post-winter diapause, diapause termination, pharate-adult, and eclosed adult developmental phases (Figure 1). The entire set of experiments were performed over a period of two seasons 2017 to 2019 with total of 2,091 individuals examined across both the host races. These phases were divided into 8 different stages according to changes in external head morphology, brain development, or metabolic rate (Figure 1 and s1). The pre-winter diapause initiation stage (stage 1, 8 days after puparium formation, n=130 total dissections, Methods & Figure s1, s2 and Movie 1, staining, n=15, apple n=7,hawthorn n=8), and the pre-winter diapause maintenance stage (stage 2, 20 days after puparium formation, n=150, Figure s1, s2 Figure 1 and Movie 2, staining, n=6, apple n=7,hawthorn n=8) were differentiated using respiration rates wherein pupae entering diapause exhibited higher metabolic rates 8 days after pupariation that stabilized at low levels of metabolic depression by 20 days after pupariation, when pupae were clearly in the diapause maintenance phase [Figure s1,(15,38)]. During these two pre-winter stages, brain morphology reflected that of the larval brain with a clearly identifiable ring gland and sub-oesophageal ganglion (Figure s2, A and Movie 1, 2). After a six-month artificial overwintering period at 4-5^{o}C, we observed substantial neural differentiation and remodelling had occurred compared to pre-winter diapausing brains (stage 3, n=297, Figure s2, B and Movie 3, staining, n=5, apple n=2, hawthorn n=3) in that the ring gland became disassociated and the place between the hemispheres containing the dorsal vessel became thin (c.f. stages 1 & 2; Figure s2, A, B & movie 2, 3).

Subsequent assessment of later stages also showed that adult brain (CNS) development and differentiation occurred after overwintering, at a stage during pharate-adult development where early antennal development and changes in head morphology were also observable (stage 4, n=125, Figure s2, C Movie 4, staining, n=5, apple n=4, hawthorn n=1). At this stage (4), brain region boundaries were apparent, but the regions themselves remained undefined (Figure s2, C & movie 4). By stage 5, orange pigmentation had accumulated in the eyes and a transparent antenna became well developed. At this stage, the central nervous system was more defined with structures including the antennal lobe, mushroom body, and suboesophageal ganglion (n=70, Figure s2, Movie 5, staining, n=3, apple n=1, hawthorn n=2).

The last three stages (including the sexually immature and mature eclosed adult stages) were similar in both brain and external head morphology, just with greater progression of bristle development and pigmentation of the pharate-adult cuticle (Stage 6, n=60, Movie 6, staining, n=2, apple n=1, hawthorn n=1; stage 7, n=85, Movie 7, staining, n=1, hawthorn n=1 and stage 8 n=85, Figure s2, D).

Apple race pupae exhibit more rapid brain development than the hawthorn race

To compare post-winter brain development between the two races of *R. pomonella*, both apple and hawthorn pupae were brought to room temperature (25^{o}C) after 6 months of simulated overwintering (4-5^{o}C) synchronized to their respective diapause initiation timing (Figure 2 A, B and Tables s1 and s2). By sampling a subset of pupae every 5 days from day 0 to day 70 after removal from artificial overwintering conditions, we observed that both host races exhibited onset of adult neurogenesis during the post-winter phases, as shown in Figure 1 and previously implied by Dowle et al. (2020). In the apple race, a substantial number of pupae exhibited adult brain morphology (Stage 4-6 in Figure 1) starting from day 0 after removal from simulated winter and until day 25 when 42% of the pupae sampled exhibited adult brain morphology (57/135). Conversely, in the hawthorn race only 4/152 (3%) total pupae exhibited adult brain morphology even until day 40 (Figure 2 A, B and Tables s1, s2). Logistic regression analysis (Figure 2 C) showed that apple race individuals began initiating adult brain development significantly earlier than hawthorn race individuals (x^2day = 265.79, p<0.001; x^2host = 88.04, p<0.001; x^2day*host = 34.2, p<0.001). Diapausing apple race pupae began adult brain development approximately 24 days earlier than diapausing hawthorn race pupae, with 18.34 ($\pm$0.56-0.42,95%CI) days for 50% pupae exhibiting adult brains in apple vs. 42.10 ($\pm$0.61-0.42,95%CI) days for hawthorn (Figure 2). Therefore, even though the artificial overwintering period was of the same duration for both the races, the transition from pupal to adult brain development began occurring significantly earlier in the apple race vs. the hawthorn race. In other words, not only has the apple race shifted its overall life cycle to coincide with host phenology, but the rate of development of the pupal brain from the onset of diapause to diapause termination and the initiation of adult brain morphogeneis has become more rapid.

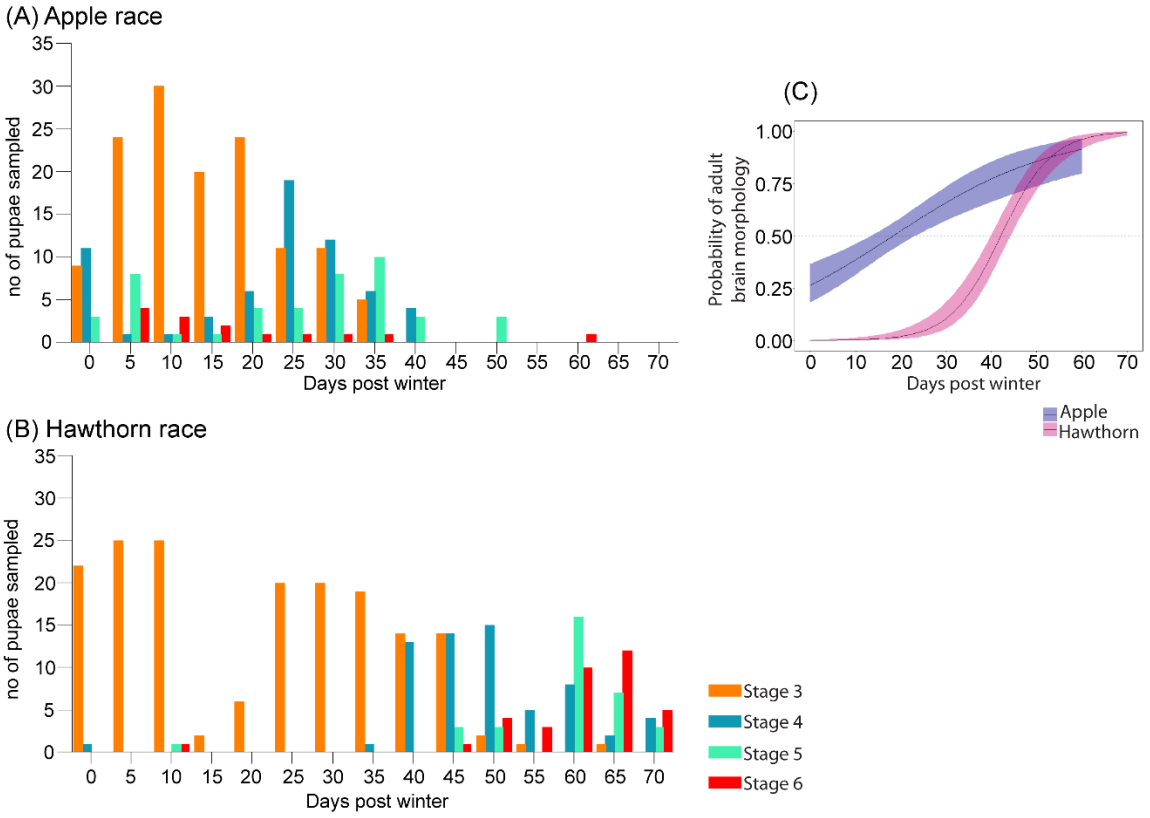

Figure 2: Post-winter brain development over time between the two host races. Brains were dissected from pupae and assessed every 5 days after simulated overwintering. A) Apple race, n=138 larval brain morphology (Stages 3 in Figure 1) vs. n=142 adult brain morphology (Stage 4-6 in Figure 1); B) Hawthorn race, n=201 larval brain morphology vs. n=157 adult brain morphology). C) Logistic regression curves with 95% confidence intervals for the proportion of adult brain morphology vs. larval brain morphology observed in *R. pomonella* apple and hawthorn race pupae over time after artificial overwintering.

Neuromodulator levels in developing Hawthorn race pupal brains are generally higher than in the apple race.

We next examined a total of 14 neurochemicals across 6 biochemical pathways in the developing brains of both hawthorn and apple race *R. pomonella.* These neurochemicals included both precursor molecules and their products (Figures, 3, s3, s4 & s5). We used linear mixed models to assess both within and between host race comparisons. All statistical results have been corrected for multiple comparisons using Turkey's HSD. In general, the titres of precursor chemicals increased first in the post-diapause stages as pharate-adult brain development began (transition from stage 3 to 4), whereas product molecule levels did not change until later stages, even as late as post-eclosion adult fly sexual maturation. Of the 14 neurochemicals examined, 13 showed significant differences in titre between the host races at one or more developmental stage. Of these, only three chemicals showed higher levels in the apple race, (dopamine, stage 5, p= 0.0001, serotonin, stage 6, p= 0.0001, and serine, stage 6, p= 0.009). For all other molecules, the hawthorn race exhibited higher levels than the

apple race. These chemicals further increased in titre as *R. pomonella* development progressed, until the point of adult fly sexual maturity, when host preference first emerges. At the stage of fly sexual maturity (Stage 8, Fig. 1), product neurochemicals from two major pathways showed a difference between the host races involving histidine to histamine and tyramine to octopamine, respectively (stage 7, apple race n=10, total of 50 brains; hawthorn race n=10, total of 50 brains; histidine, p = 0.005; stage 8, apple race n=10, total of 50 brains; hawthorn race n=10, total of 50 brains; tyramine, p= 0.011; octopamine, p= 0.011; histamine, p= 0.020). This indicates that the differences in neurochemical titre witnessed during pupal development persist even into fly sexual maturity, particularly the octopamine and histamine pathways.

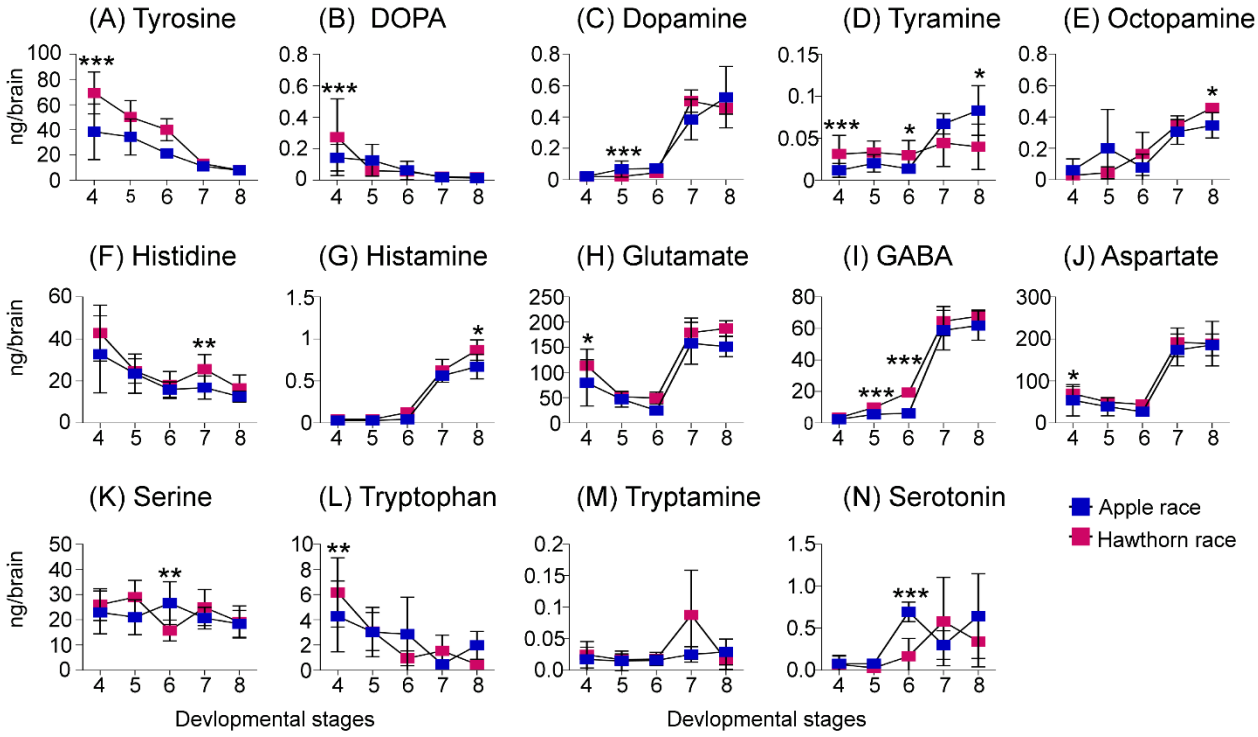

Figure 3: Quantification of biogenic amines and their precursors from the diapause termination stage of brain development (stage) 4 all the way up to sexually mature fly (stage 8) as defined in Figure 1 (A-N). Line graph of neurochemical titres for both host races at different developmental stages with 4-15 samples per stage, containing a pool of five brains in each sample, symbols represent mean with SD: A) tyrosine; (B) DOPA; (C) dopamine; (D) tyramine; (E) octopamine; (F) histidine; (G) histamine; (H) glutamate; (I) GABA; (J) aspartate; (K) serine; (L) tryptophan; (M) tryptamine; and (N) serotonin; Asterisks above indicate differences between host races at the equivalent stage of brain development. P-values represented are < 0.05 *, < 0.01 **, and < 0.001 ***, linear mixed effect model, followed by Tukey's HSD correction for multiple comparisons.

Rapid development of the apple race to adult brain morphogenesis corresponds to lower levels of neuromodulators

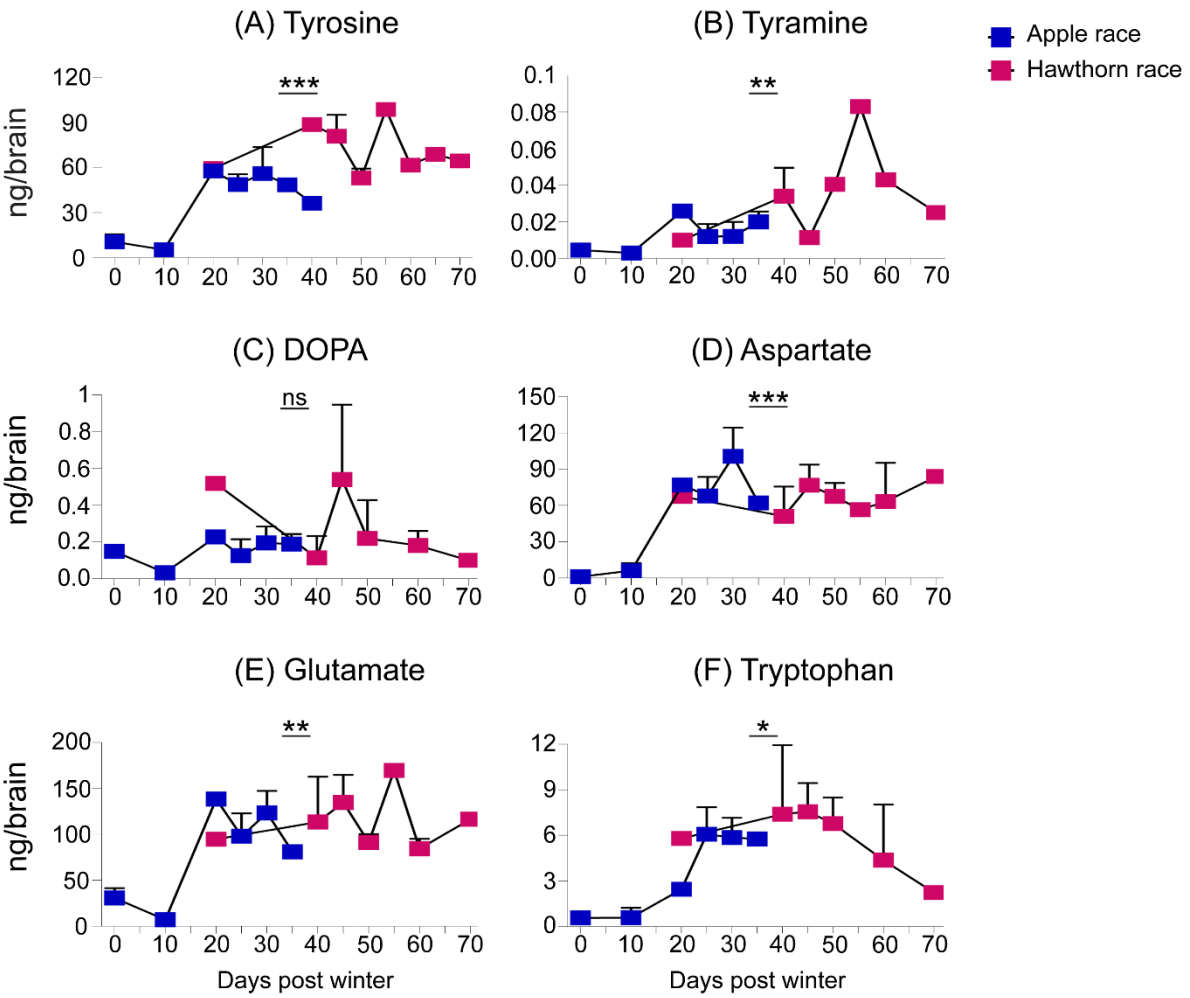

Figure 4: Quantification of precursor neurochemicals during the diapause termination stage of brain development (stage 4 only) collected between day 0 to day 70 after simulated overwintering. (A-F) Plots of neurochemical titres for both host races at developmental stage 4 with five brains per sample, A) tyrosine; B) tyramine; C) DOPA; D) aspartate; E) glutamate; F) tryptophan; Asterisks above indicate significant interactions between effects of host race*days for neurotransmitter titre. P values represented are < 0.05 *, < 0.01 **, and < 0.001 ***, analysis using Two-way ANOVA, post hoc Tukey's LSD test.

Given that in the apple race, the rate of adult brain development is significantly more rapid than the hawthorn race (Figure 2), and also exhibits lower titres of several neuromodulators, we next assessed whether the rate of development corresponded to lower levels of neuromodulators, particularly at the first stage of adult brain development (Stage 4). Figures 3, s3, s4 and s5 show that six precursor molecules including tyrosine, tyramine, DOPA, aspartate, glutamate, and tryptophan from four different biosynthetic pathways showed

significant differences between the host races at the first appearance of the adult brain during pupal-pharate adult metamorphic development, stage 4 in Figure 1 (stage 4, apple race n=14, total of 70 brains; hawthorn race n=11, total of 55 brains; tyrosine, p= 0001; DOPA, p= 0.0004; tyramine, p= 0.0001; aspartate, p= 0.022; glutamate, p= 0.002; tryptophan, p= 0.002;). For each chemical, the titres were higher in the hawthorn race compared to the apple race. To compare how these precursor neurochemicals titres change over the development time point between the hawthorn and apple races, we matched the Stage 4 brains sampled in Figures 3, s3 and s4 to the day they were sampled after winter, as referred to in Figure 2.

A two-way ANOVA was conducted to examine the effects of host race and days after winter on the titres of these eight precursor neurochemicals. Except for the neurochemical DOPA, there was a significant interaction between host race and day for each neurochemical titre (table s7, tyrosine, $F(12,10) = 10.9$, $P = 0.0001$; tyramine, $F(12, 10) = 4.95$, $p = 0.008$; DOPA, $F(12, 7) = 0.82$, $p = 0.632$; aspartate, $F(12, 11) = 10.1$, $p = 0.0001$; glutamate, $F(12, 11) = 6.47$, $p = 0.002$; tryptophan, $F(12, 10) = 4.35$, $p = 0.013$). Therefore, neurochemical titres were significantly lower in rapidly developing apple race pupae than at later time points, when hawthorn race pupae were beginning to develop adult brain morphology. This was true for precursor chemicals even when accounting for pupae that might have been impacted by the temperature effects of development during winter itself (i.e. until day 20) (table s8, tyrosine, $F(8,9) = 38.14$, $P = 0.0001$; tyramine, $F(7, 10) = 4.46$, $p = 0.017$; DOPA, $F(7, 8) = 0.84$, $p = 0.581$; aspartate, $F(8, 10) = 14.25$, $p = 0.0001$; glutamate, $F(8, 9) = 25.36$, $p = 0.0001$; tryptophan, $F(7, 10) = 7.19$, $p = 0.003$). These differences in titres between the races then carried through further developmental stages particularly during the sexual maturation of the adult fly in some cases (note stages 7 and 8 in Figures 3, s3, s4 and s5). As a result, more rapidly developing brains in apple race pupae exhibited significantly lower titres of neuromodulators than later developing hawthorn race brains, especially biogenic amines, and these differences were also reflected in the adult fly stage at which host preference is exhibited (39–41).

Discussion:

In this study, we have identified differences in rates of adult brain morphogenesis and levels of several neurochemicals between the apple and hawthorn host races of *Rhagoletis pomonella* across the transition from pupal diapause to post-diapause, pharate-adult development, and adult fly host preference stages. These two closely related populations differ in both life history timing and adult host preference behaviours, and are generally considered a model for incipient ecological speciation. The divergence of these two populations towards hosts with such different phenologies serves as an excellent system to examine how multiple life history events are synchronized between ancestral and novel hosts.

First, we found that while some neurogenesis occurred during the diapause maintenance phase in both host races, substantial adult brain development and differentiation was initiated only after winter and brain development progressed through several morphological stages to adult emergence. When we dissected brains from apple and hawthorn race pupae at regular intervals after they were removed from overwintering, almost no hawthorn pupae showed brain development beyond stage 3 for at least a month after overwintering, and it took up to 50 days for the majority of hawthorn pupae to terminate diapause and transition from stage 3 to stage 4 where pharate adult neural development was apparent (Figure 2B). In contrast, some apple race pupae were already progressing to stage 4 pharate adult development as soon as they were removed from overwintering (Figure 2B). This finding indicates not only has apple race shifted its entire life cycle to coincide with fruit phenology but its brain developmental rate during diapause has become more rapid. This result is also in agreement with recent transcriptomic work showing rapid up-regulation of growth and development-related transcript genes in the apple race with shorter post-winter diapause duration as compared to the longer post-winter diapause duration of hawthorn race (47).

To better understand how neurochemical signaling could be associated with the different stages of development in *R. pomonella*, we examined six major biosynthetic pathways known to impact brain development as well as behavior in insects (44,48). Our results indicate that the titers of several neurochemicals were significantly reduced in the apple race pupae across multiple developmental stages as compared to the hawthorn race, (Figures 3, 4, s3, s4 and s5). Further analysis showed that apple race adult brains contained lower titres of several neurochemicals at the onset of adult brain development, particularly those in the dopamine and octopamine pathways, than hawthorn race adult brains, and this difference is also apparent in adulthood, when host preference is exhibited. Given the importance of these neurochemicals to both development and behaviour, we hypothesize that this difference in neurochemical production between the host races could connect diapause termination timing and subsequent host preference in the adult flies, a key characteristic for host-associated divergence and ecological speciation in the *R. pomonella* system.

In agreement with our hypothesis, a recent study comparing olfactory neurophysiology between the apple and hawthorn races of *R. pomonella* identified a neuronal switch in the chemosensory system in the adult brain associated with differential host choice behaviour towards apple or hawthorn fruit (24). Such a switch in neurophysiology has similarly been observed between the Z and E strains of the European Corn Borer, *Ostrinia nubilalis*, where male preference for a particular isomer of the sex pheromone is controlled by cis-acting variation in a sex-linked transcription factor (bric à brac; bab) expressed in the developing male antenna (49). A recent study of the *Rhagoletis* brain transcriptome also shows variation

in cis-regulatory elements associated with differentially expressed transcripts during diapause development and an important role of hub genes in transcriptional networks that differ during diapause development between the two host races (47). In our current study, we show that the onset of adult brain differentiation (Stage 4) also corresponds with morphogenesis and emergence of the adult antenna, and this stage is accompanied by several significant differences in neuromodulator levels between the host races, specifically members of the dopamine and octopamine pathways (Figures 3 and 4). Interestingly, in *Drosophila* cis-regulatory variation in two genes involved in these pathways, tyrosine hydroxylase and dopa decarboxylase (Figure 3A), have been shown to impact neurogenesis (48). This provides a new hypothesis that differences in titres of these neurochemicals expressed during the transition from pupal diapause to early pharate-adult brain development might impact, or be impacted by, the expression of developmental genes in *Rhagoletis* and ultimately lead to changes in host-fruit preference in reproductively mature adults. Future studies that measure the expression of enzymes involved in the production of dopamine and octopamine and selective pharmacological treatments that act as agonists and antagonists of these biogenic amines at early developmental stages in these two host races could indicate if these pathways are involved in the differentiation of these two host races.

To conclude, we have characterized the progression of neurogenesis from diapause onset to adult reproductive maturation in both the apple and hawthorn host races of *R. pomonella*, an important model for ecological speciation and diversification. We identified significantly lower neurochemical levels, particularly biogenic amines in the dopamine and octopamine pathways, in the apple race of *R. pomonella* that correspond to more rapid adult brain development in this new host race. These differences in neurochemical titre between the races that are initially higher at the initiation of adult brain development are also apparent in the adult brains at stages when flies are showing clear host preference. Genetic and behavioural analyses of hybrids between these two host races suggest that their olfactory divergence is based on differences at only a few loci (50). Furthermore, a recent study of a similar neurological change in host preference in the European corn borer has implicated a cis-regularly element (49), several of which also influence biogenic amine pathways (48). Because biogenic amines have been implicated to impact both pupal diapause and adult behaviour, this study offers a new hypothesis correlating life history timing and adult host preference through developmental differences in neuromodulation. This hypothesis must now be tested in further studies assessing enzymatic expression and pharmacological manipulation of neuromodulator levels in developing pupae. As previously suggested, connecting host preference and survival through relatively simple changes could be a widespread mechanism

for generating biodiversity across phytophagous insects, contributing to the origin of the large number of species observed (24).

Data accessibility: All data supporting this manuscript will be uploaded to Dryad upon submission.

Authors' contribution: HK, SBO, and DAH conceived the study and designed experiments; JLF provided the adult flies and reagents for the experiment. HK conducted experiments; HK, DAH and SBO analyzed data; HK and SBO wrote the manuscript; all authors revised and approved the manuscript.

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