Title: Connecting neuromodulation, brain development, and life history timing associated with ecological speciation in *Rhagoletis pomonella*

Authors: Hinal Kharva[1,2], Jeffrey L. Feder[3], Daniel A Hahn[4], Shannon B. Olsson[1]

Affiliations:

[1]Naturalist-Inspired Chemical Ecology lab, National Centre for Biological Sciences, Tata Institute of Fundamental Research, GKVK Campus, Bellary Road, Bangalore 560065, India.

[2]School of Life Sciences, The University of Trans-Disciplinary Health Sciences and Technology, 74/2, Jarakabande Kaval, Post Attur via Yelahanka, Bangalore 560064, India.

[3] Department of Biological Sciences, University of Notre Dame, Notre Dame, Indiana 46556, USA.

[4]Department of Entomology and Nematology, University of Florida, Gainesville, FL 32611, USA.

Correspondence to: hinal@nice.ncbs.res.in, shannon@nice.ncbs.res.in

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Abstract:

Host shifts are considered a key generator of insect biodiversity. In insects, adaption to new host plants often requires synchronization of growth and development during larval and pupal stages with the new host as well as changes in adult preference for that host after metamorphosis. Neurochemicals such as biogenic amines play key roles in both development and behavior, 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 are known to exhibit differences in both diapause timing as well as adult neurophysiological response with respect to their hosts. In turn, we found that the apple host race exhibited adult brain differentiation during metamorphosis three weeks earlier than the hawthorn host race, which correlated with significantly lower titers of several neurochemicals. These differences in neurotransmitter titres between the races are carried through further developmental stages. In some cases, particularly for biogenic amines, the differences persisted through the eclosion and sexual maturation in the adult fly, the stage at which host preference is exhibited. We thus propose that changes in neuromodulation during specific stages of adult brain development could link differences in life history timing and host preference in these races, providing a new hypothesis regarding the genesis of our planet's most specious multicellular taxon.

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 (Berlocher and Feder, 2002; Schluter, 2001; Via, 2001). 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, these populations must regulate their life-history timing to coincide with the new host phenology (Craig et al., 1993; Dambroski and Feder, 2007; Feder et al., 1993; Groman and Pellmyr, 2000; Itami et al., 1998; Pratt, 1994; Smith, 1988; Wood and Keese, 1990). 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 (Bush, 1969; Itami et al., 1998).

Rhagoletis pomonella (Diptera: Tephrtidae) provides a unique opportunity to identify linkages between pupal development, life history timing, and adult host choice. This species of flies originally infested the native downy hawthorn (*Crataegus mollis*) fruit until apple (*Malus pumila*) was introduced in Eastern USA around 180 years ago (Bush, 1969; Feder et al., 1988; Walsh, 1867). 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 host races specific to each fruit (Bush, 1969; Feder et al., 1988). This host shift from hawthorn to apple fruit was facilitated by two factors. First, in nature, apple trees fruit roughly one month earlier than hawthorn trees. Apple and Hawthorn flies are univoltine and the adults are short lived, thus each host race must emerge according to 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 earlier as well (Feder et al., 1988). Previous work shows that the earlier seasonal adult emergence of apple host-race flies compared to hawthorn host-race flies is driven solely by the timing of the termination of pupal diapause (Powell et al., 2020). 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 (Feder and Filchak, 1999). 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 (Linn et al., 2003, 2004; Prokopy et al., 1971). 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 (Olsson et al., 2006b, 2006a; Tait et al., 2016), but rather at the first synapse of the olfactory system in the brain, the antennal lobe (Tait et al.,

2021). The coupled difference in pupal diapause timing and adult preference behaviour in *R. pomonella* provides a unique opportunity to examine if there are corresponding differences between the races that could simultaneously impact both developmental timing 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 (Denlinger et al., 2017). 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 (Koštál, 2006; Koštál et al., 2017). 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 (Denlinger, 2002). Both the brain and ring gland play important roles in regulation of pupal diapause in case of Dipterans (Giebultowicz and Denlinger, 1986; Judy, 1972). Diapause can be regulated via changes in hormone/neurotransmitters titres, receptor abundance, or regulation of specific neurochemical pathways across the stages of diapause. For example, elevated levels of dopamine induce onset of pupal diapause in the cabbage armyworm *Mamestra brassicae* (Noguchi and Hayakawa, 1997). Consistent with this observation of dopamine promoting diapause maintenance, in the Chinese oak silkmoth, *Antheraea pernyi,* pharmacological injection with agonists of D2-like dopamine receptors prolonged pupal diapause and delayed adult emergence, whereas the antagonist of the receptor triggered early pupal diapause termination and earlier adult emergence (Wang et al., 2015). Finally, in *Drosophila melanogaster*, down regulation of dopamine and serotonin pathways enhanced ovarian dormancy, whereas up regulation of octopamine signalling inhibited dormancy (Andreatta et al., 2018).

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. For example, elevated levels of octopamine lead to increased foraging activity in bees (Schulz et al., 2002). In *Drosophila melanogaster*, mutants with blocked dopamine and octopamine pathways showed changes in the formation of appetitive and aversive olfactory memories (Schwaerzel et al., 2003). Studies in other systems have shown that neuromodulators also impact sensory processing. For example, in female *Spodoptera littoralis*, dopamine levels increased

in the antennal lobe after mating, which corresponded to a switch in preference from searching for floral nectar for adult feeding and oogenesis to preferring vegetative cues that can be used for egg laying (Félix et al., 2008; Kromann, 2012).

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 impact 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. Our main objectives for this study are first to identify the developmental stages of diapause in both apple and hawthorn individuals and assess brain development at each stage. Second, we quantify levels of precursor amino acids and neurotransmitters across pupal diapause, post-diapause, pharate-adult development, and post-eclosion adult developmental stages of both host races. 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.

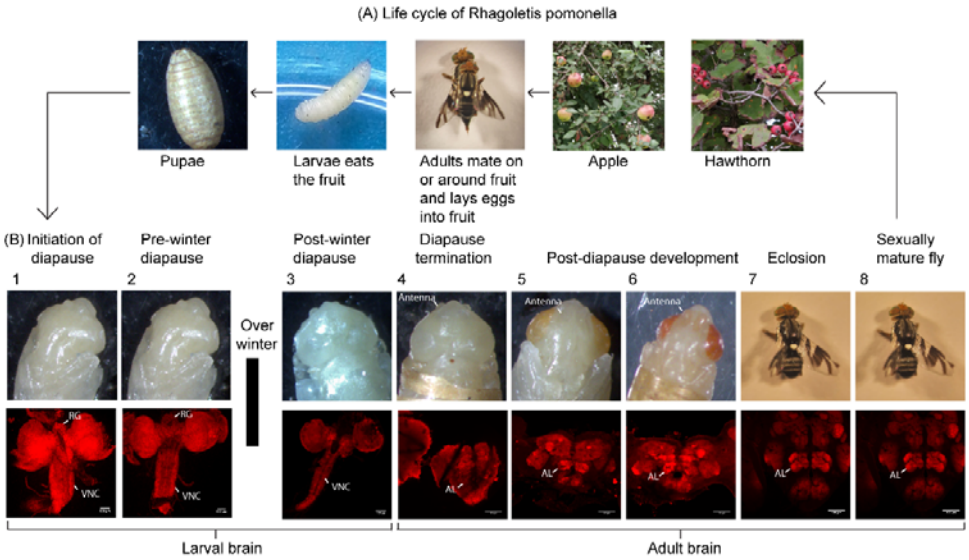

Figure 1: The life cycle of *Rhagoletis pomonella.* (A) Life stages of the fly from mating to oviposition from right to left. (B) Micrographic images of pupal, pharate-adult, and adult developmental stages from left to right. The upper stereomicrographs in the bottom panels show the head morphology while 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 or CNS morphology characteristics, including eye pigmentation and antennal development as previously described in (Ragland et al., 2009). The stages are as follows: **1**, Pre-winter diapause induction stage (n=130 total dissections), white head with undefined antenna, larval brain morphology and higher metabolic rates during larval-pupal metamorphosis (Methods & Figure 7, Figure s1 and Movie 1, staining, n=15); **2**, Pre-winter diapause induction stage (n=150), white head with undefined antenna, larval brain morphology and with metabolic rates lower than during the initiation phase (Figure 7, Figure 1 and Movie 2, staining, n=6); **3**, Post-winter diapause maintenance phase (n=297), white head with undefined antenna, some initial peripheral neurogenesis is visible (Figure s1 and Movie 3, staining, n=5); **4**, End of diapause (n=125), white head with initial antenna formation, adult brain development and differentiation with initial adult brain region boundaries forming and undefined internal region segmentation showing that pharate-adult morphogenesis has begun (Figure s1. Movie 4, staining, n=5); **5**, midway through pharate-adult development shown by an orange eye with transparent antenna (n=70), adult brain differentiation with defined region boundaries and segmentation (Figure s1, Movie 5, staining, n=3); **6**, late pharate-adult development shown by dark orange eyes and discernible antennal sensilla (n=60), adult brain with clear region segmentation as in adult fly (Movie 6, staining, n=2); **7**, sexually immature adult fly, less than 7 days old (n=85); **8**, sexually mature fly, more than 12 days old (n=85, Movie 7, staining n=1);.

Results:

Brain development across diapause and post-diapause developmental stages

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 7). The pre-winter diapause initiation stage (stage 1, 8 days after puparium formation), and the pre-winter diapause maintenance stage (stage 2, 20 days after puparium formation) 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 7,(Powell et al., 2020; Ragland et al., 2009)]. 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 s1 and Movie 1, 2). After a six-month artificial overwintering period at 4-5°C, we observed substantial neural differentiation and remodelling had occurred compared to pre-winter diapausing brains (Figure s1). At this post-winter diapause maintenance stage 3, we observed that the larval stage ring gland disappeared, and the two hemispheres of the brain developed into three distinct parts with the formation of optic lobe, that was not apparent in the brains sampled from pre-winter pupae (c.f. stages 1 & 2; Figure s1, A, B & movie 2, 3). Despite the fact that we observed clear differences in morphology and development between the brains of pre-winter and post-winter diapausing pupae, the external pupal head morphology was not apparently different or visible through naked eye. This result reinforces the concept that while slow, some brain development does occur over the course of diapause (Dowle et al., 2020).

Subsequent assessment of later stages also showed that adult brain (CNS) development and differentiation occurred after diapause termination, at a stage during pharate-adult development where early antennal development and changes in head morphology were also observable (stage 4). This stage has previously been correlated with a significant change in respiration rate signalling the end of diapause (Ragland et al., 2009). At this stage (4), brain region boundaries were apparent, but the regions themselves remained undefined (Figure s1, 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 (Figure s1, Movie 5). 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 (Figure s1, d & movies 5, 6, 7).

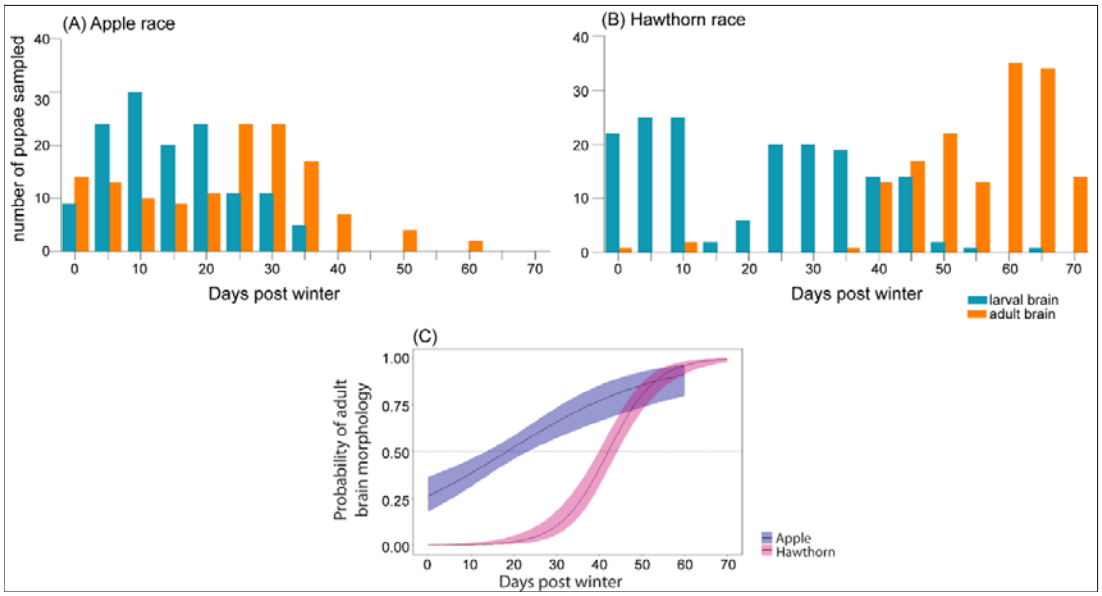

Figure 2: Post-winter brain development over time between the two host races. Brains were dissected from pupae and assessed every 5 days after simulated overwintering. A) Apple race, n=138 larval brain morphology (Stages 1-3 in Figure 1) vs. n=142 adult brain morphology (Stage 4-6 in Figure 1); B) Hawthorn 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. The estimated timing of 50% pupae exhibiting adult brain morphology for apple flies was 18.34 (±0.56-0.42,95%CI) days after removal from artificial overwintering versus 42.10 (±0.61-0.42,95%CI) days after removal from artificial overwintering for hawthorn flies.

To compare post-winter brain development between the two races of *R. pomonella*, both apple and hawthorn pupae were brought to room temperature (25°C) after 6 months of simulated overwintering (4-5°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 (±0.56-0.42,95%CI) days for 50% pupae exhibiting adult brains in apple vs. 42.10 (±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, brain development began occurring significantly earlier in the apple race vs. the hawthorn race.

Neurotransmitter levels across developmental stages

We next examined a total of 14 neurochemicals across 6 biochemical pathways in the developing brains of both hawthorn and apple races of *R. pomonella.* These neurochemicals included both precursor molecules and their products (Figures, 3, 4, & 5). 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. Quantification of neurotransmitters revealed that none of the neurochemicals showed any change in titre at pre-winter or during early or late diapause maintenance stages in either of the host races (stages 1-3 in Figure 1). Any changes in neurotransmitter levels therefore occurred only after diapause was terminated and pharate-adult brain differentiation and development began (stages 4-8 in Figure 1). In general, the titres of precursor chemicals increased first in the post-diapause stages as pharate-adult brain development began, whereas product molecule levels did not change until later stages, even as late as post-eclosion adult fly sexual maturation. There are three developmental stages where significant changes in neurochemical titres were found: first, during the transition between the larval brain to the pharate adult brain (stage 3 to 4 in Figure 1); second, between the transition from the pharate adult stage to the eclosed adult fly (stage 6 to 7 in Figure 1), and third, between sexually immature and mature adult fly stages (stage 7 to 8 in Figure 1). In general, six precursor molecules including tyrosine, tyramine, DOPA, aspartate, glutamate, and tryptophan from four different biosynthetic pathways also 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. For each chemical, the titres were higher in the hawthorn race compared to the apple race. At emerged adult fly stages 7 and 8, two

major pathways showed a difference between the host races involving histidine to histamine and tyramine to octopamine, respectively.

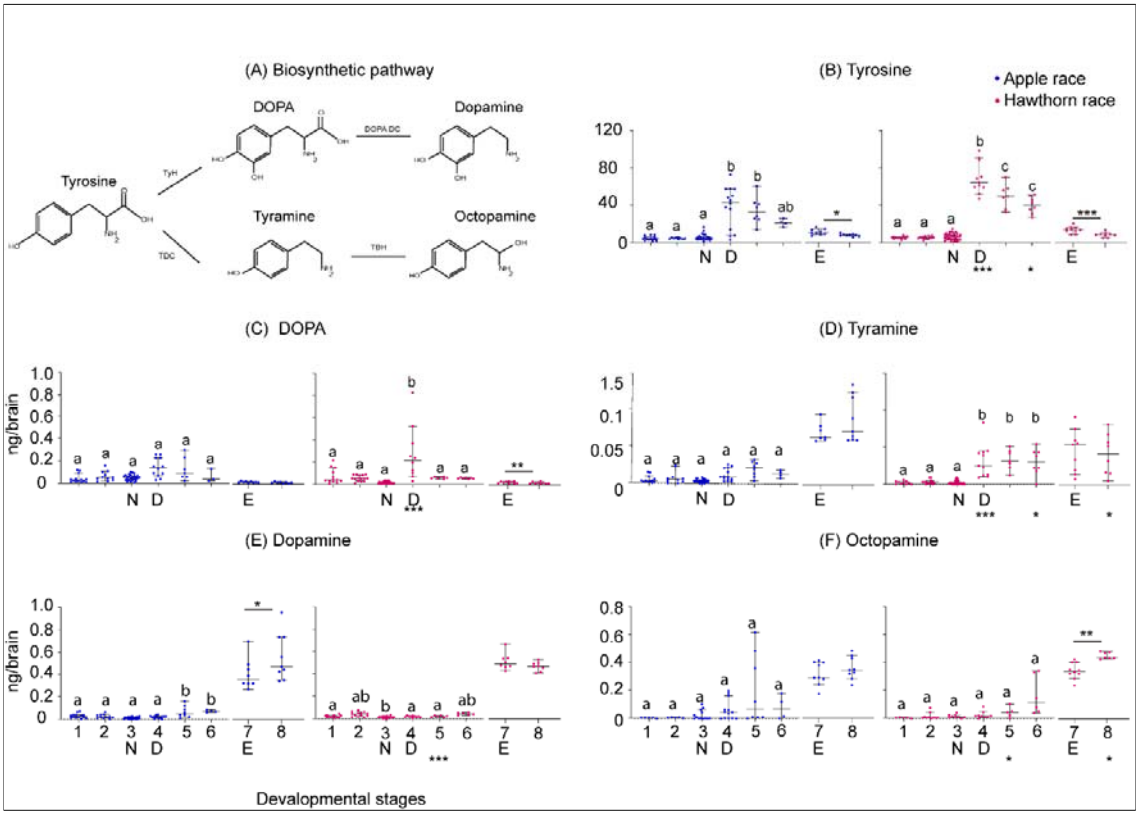

Figure 3: Quantification of biogenic amines and their precursors across the different stages of development as defined in Figure 1. (A) Biosynthetic pathways for octopamine and dopamine; (B-F) Scatter plots of neurochemical titres for both host races at different developmental stages with 4-15 samples per stage containing a pool of five brains in each sample, symbols represent median with 95% CI: B) tyrosine; (C) DOPA; (D) tyramine; (E) dopamine; (F) octopamine. Letters below x-axis highlight specific developmental stages: N, onset of adult brain neurogenesis, D, development and differentiation of adult brain associated with entering the pharate-adult phase, and E, adult eclosion. Letters above each stage indicate similar titres of mentioned neurochemicals between pupal and pharate-adult stages within each host race (P > 0,05), and asterisks indicate differences in neurochemical titre between the two adult fly stages – pre-maturation and after sexual maturation. Asterisks below the x-axis 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.

Specifically, quantification of dopamine, octopamine, and their precursors showed that the level of the precursor amino acid tyrosine significantly increased in both races between the

larval brain stage 3 and initial pharate-adult brain stage 4, then decreased progressively until adult fly sexual maturity approximately 10-12 days after eclosion (stage 8; apple race n=14, total of 70 brains, p = 0.0001; hawthorn race, n= 11, total of 55 brains, p = 0.0001). However, intermediate precursor molecules DOPA and tyramine increased detectably at stage 4 only in the hawthorn race (tyramine, apple race, n=14, total of 70 brains, p = 0.9359; hawthorn race, n=11, total of 55 brains, p = 0.001; DOPA, apple race, n=12, total of 70 brains, p = 0.0683; hawthorn race, n=10, total of 50 brains, p = 0.001). Finally, between sexually immature (stage 7) and sexually mature flies (stage 8), the levels of dopamine increased in the apple race but not the hawthorn race (apple race, n=10, total of 50 brains, p = 0.0371; hawthorn race, n=10, total of 50 brains, p = 0.861). In contrast, levels of octopamine significantly increased between young emerged adults and sexually mature adults in the hawthorn race, but not the apple race (apple race, n=10, total of 50 brains, p = 0.594; hawthorn race, n=10, total of 50 brains, p = 0.007).

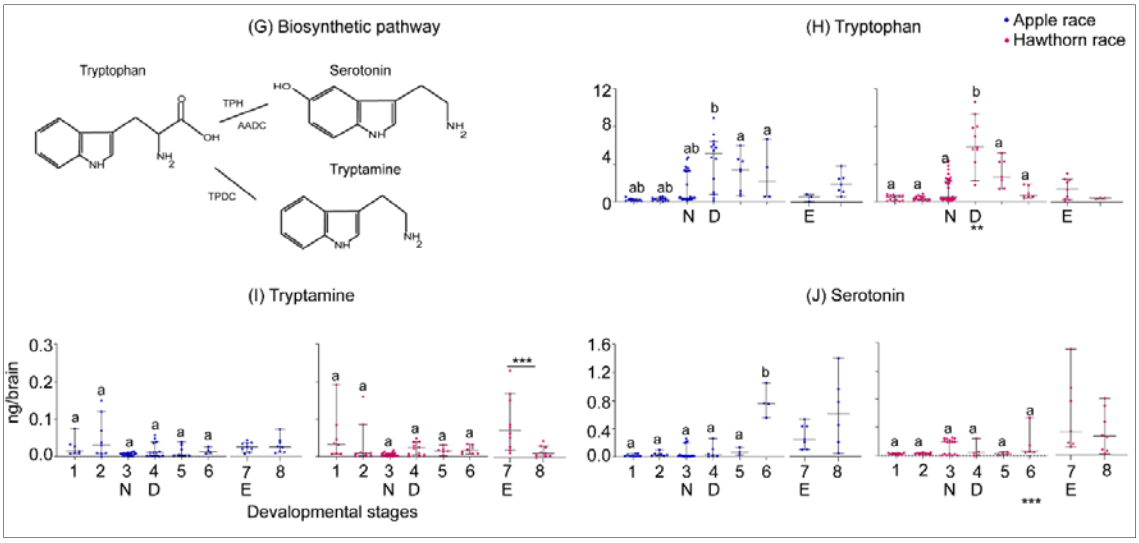

Figure 4: Quantification of biogenic amines and their precursors across the different stages of development as defined in Figure 1. (G) Biosynthetic pathway for serotonin; (H-J) Scatter plots of neurochemical titres for both host races at different developmental stages with 4-15 samples per stage containing five brains each, symbols represent median with 95% CI: (H) tryptophan; (I) tryptamine; (J) serotonin. Lettering, asterisks, and statistical comparisons as in Figure 3.

Like the other biogenic amine pathways, levels of the precursor tryptophan (Figure 4) also increased during the transition from larval to pharate adult brain development (stages 3 to 4) in both host races (apple race n=14, total of 70 brains, p = 0.0001; hawthorn race n=11, total of 55 brains, p = 0.0001). At the adult stage, levels of serotonin did not show any significant

change in either host race. However, the level of tryptamine decreased between sexually immature flies (stage 7) and sexually mature flies (stage 8) in the hawthorn race but not the apple race (apple race, n=10, total of 50 brains, p = 0.985; hawthorn race, n=10, total of 50 brains, p = 0.0005). We note, however, that tryptamine is not considered to be an endogenous neuromodulator in insects (Luetje and Chen, 2014).

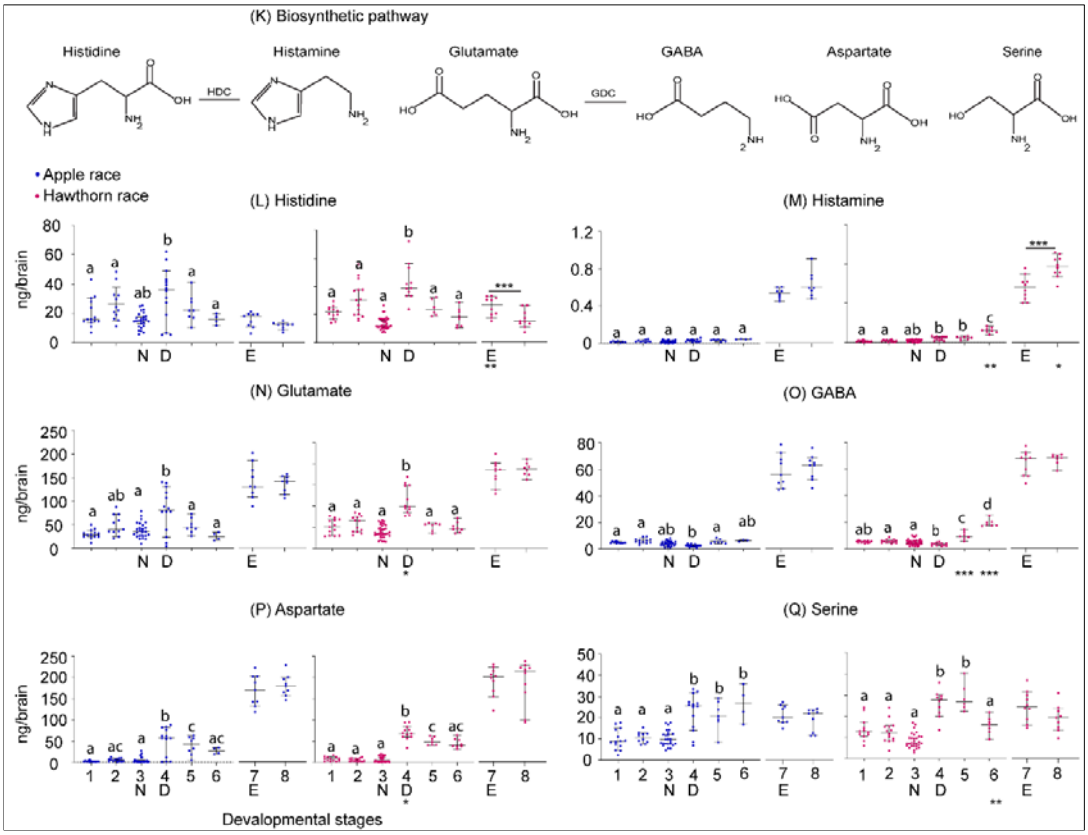

Figure 5: Quantification of biogenic amines and their precursors across the different stages of development as defined in Figure 1. (K) Biosynthetic pathway for histamine, GABA, aspartate and glutamate; (L-Q) Scatter plots of neurochemical titres for both host races at different developmental stages with 4-15 samples per stage containing five brains each, symbols represent median with 95% CI: (L) histidine; (M) histamine; (N) glutamate; (O) GABA; (P) aspartate; (Q) serine. Lettering, asterisks, and statistical comparisons as in Figure 3.

For other neurochemicals (Figure 5), levels of the precursor molecules histidine, glutamate, aspartate, and serine all increased significantly in both host races during the transition from larval/pupal to pharate-adult brain morphology (stage 4; apple race histidine, n=14, total of 70 brains, p = 0.0002; glutamate, n=14, total of 70 brains, p = 0.0006; aspartate, n=13, total of 65 brains, p = 0.0001; serine n=15, total of 75 brains, p = 0.0001; hawthorn race histidine,

n=11, total of 55 brains, p = 0.0001; glutamate, n=12, total of 60 brains, p = 0.0001; aspartate, n=12, total of 60 brains, p = 0.0001; serine n=10, total of 50 brains, p = 0.0001). However, in the hawthorn race the level of the precursor histidine significantly decreased from sexually immature flies (stage 7) to sexually mature flies (stage 8) (n=10, total of 50 brains, p = 0.0002), while the product histamine showed higher titres in sexually immature flies (stage 7) versus mature flies in both host races (stage 8) (n=10, total of 50 brain, p = 0.0001). None of these pathways showed any other changes in emerged adults (stages 7 & 8) in the apple race.

Comparison of diapause termination timing and neurochemical titres across host races

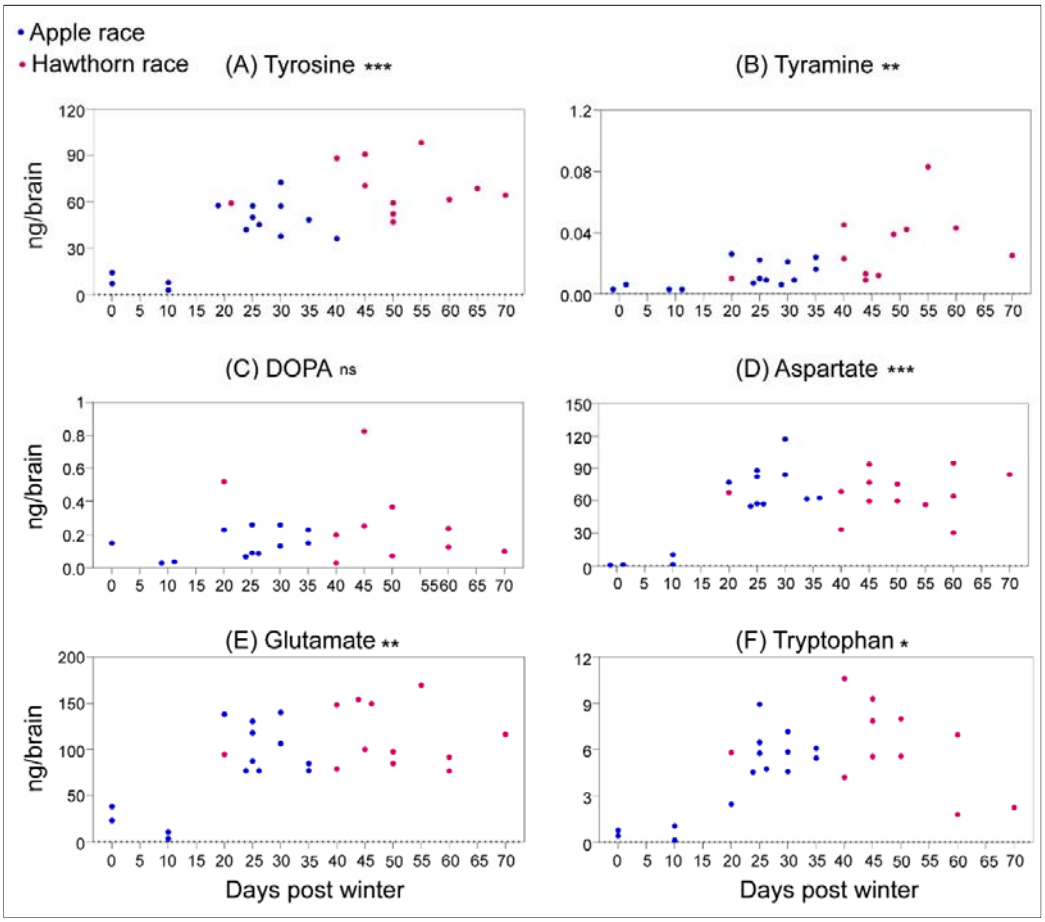

Figure 6: Quantification of precursor neurochemicals from the diapause termination stage of brain development (stage 4 only) collected between day 0 to day 70 after simulated overwintering. (A-F) Scatter plots of neurochemical titres for both host races at developmental stage 4 with five brains per sample, 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.

In general, Figures 3-5 indicate an increase in the titre of precursor molecules for several neurotransmitter pathways as *R. pomonella* development progressed, suggesting a change in titre over time after winter. Figures 3-5 also show that six precursor molecules from four different biosynthetic pathways exhibited significantly higher titres in the hawthorn race compared to the apple race at diapause termination, the first appearance of the adult brain during pupal-pharate adult metamorphic development (Stage 4, Figure 1). These chemicals were: tyrosine, tyramine, DOPA, aspartate, glutamate, and tryptophan. To compare the rate of change in these neurochemical precursor molecules over time between the hawthorn and apple races, we matched the Stage 4 brains sampled in Figures 3-5 to the day they were sampled after winter.

A two-way ANOVA was then conducted to examine the effect of host race and days after winter on the titre of these six 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, $F(12, 0.001) = 4.95$, $p = 0.008$; DOPA, $F(12, 0.254) = 0.82$, $p = 0.632$; aspartate, $F(12, 2751.0) = 10.1$, $p = 0.0001$; glutamate, $F(12, 9415.6) = 6.47$, $p = 0.002$; tryptophan, $F(12, 37.1) = 4.35$, $p = 0.013$;). These results show that while precursor molecule titres in both races increased over time after winter, several pupae in the apple race exhibited adult brain morphology and lower titres earlier after simulated winter (Figure 6). We note that in both races a portion of samples reached stage 4 in development soon after termination of the overwintering period, and their biochemistry could have been impacted by the low temperatures they were exposed to during initial neurogenesis (Stage 3) in the simulated winter period. To account for this, we also repeated this analysis removing any samples in both races that reached stage 4 up to 20 days after winter. Still, both tyrosine and tyramine (precursors for dopamine and octopamine) exhibited a significant interaction between host race and day for neurochemical titre (tyrosine, $P = 0.007$; tyramine, $p = 0.036$; DOPA, $p = 0.160$; aspartate, $p = 0.752$; glutamate, $p = 0.180$; tryptophan, $p = 0.930$; unpaired, non-parametric student t test). Therefore, neurochemical titres were significantly lower in earlier developing apple race pupae than at later time points, when hawthorn race pupae were beginning to terminate diapause. This was true for precursors of the dopamine and octopamine pathways even when accounting for pupae who might have been impacted by the temperature effects of development during winter itself (i.e. until day 20). 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 3-5)

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 sexual maturation. 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 pupal diapause ended and brain development progressed through several morphological stages to adult emergence. Respirometry data collected before artificial overwintering (figure 7) also indicated that pupae entering diapause in the pre-winter maintenance phase retain the gross morphology of the larval brain with presence of the ring gland. However, over the course of diapause the ring gland becomes disassociated and the place between the hemispheres where the dorsal vessel runs thins during the 6-month period in artificial overwintering (Figures 1 and s1). This progressive pattern of brain development appears to be similar to the thinning of the region between the two brain hemispheres observed during pupal-adult brain morphogenesis in Drosophila melanogaster (Truman and Bate, 1988), albeit massively slowed down so that processes that occur over the span of hours in D. melanogaster development occur over the course of months in the brains of diapausing *R. pomonella* pupae. The neural differentiation we observed in diapausing pupae after overwintering is also consistent with the hypothesis put forth in Dowle et al. (2020) based on transcriptomic data that slow and progressive neuronal differentiation occurs over the course of winter as diapausing pupae become capacitated to terminate diapause.

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, showing that they had already terminated

diapause in the artificial overwintering treatment (Figure 2B). Closer comparison of the proportions of apple and hawthorn race pupae showing stage 3 larval brains vs stage 4 pharate-adult brains showed that 50% of apple race pupae were terminating diapause and initiating pharate-adult brain development within 18 days of being removed from overwintering, while hawthorn pupae reached 50% adult brain morphology only after 42 days, or three weeks later (Figure 2). This finding agrees with previous work showing that the earlier seasonal adult emergence of the newly derived apple host race than the ancestral hawthorn host race is driven by the timing of the termination of pupal diapause, which occurs earlier after overwintering in the apple host race than in the hawthorn host race (Powell et al., 2020). 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 (Dowle et al., 2020).

We next examined how 14 neurotransmitter precursor and product molecule levels compared within and between these two host races at different stages of pupal development and sexual maturation. Changes at different stages of development could indicate which life history events would be most likely influenced by neuromodulation in these populations. For example, changes in neuromodulator levels at the pre-diapause phase could impact diapause timing and subsequent development, while changes during diapause maintenance could impact the onset of adult brain neurogenesis and thus the timing of diapause termination. Third, changes in neuromodulator levels at the termination of diapause could impact adult brain development and differentiation. Finally, changes in neuromodulators could directly impact adult host preference. Our results indicate that there is no evidence that neuromodulator titres change at either diapause initiation (stage 2, Figure 1) or at the onset of neurogenesis (stage 3) either within or between the host race, which indicates that levels of neuromodulators are not correlated with these events. However, we found profound changes in the titers of the 14 neurotransmitters and their precursors within both host races after diapause termination during adult brain development and differentiation (stage 4), with additional changes detectable between newly emerged and reproductively mature adults (stage 7 vs. stage 8 in Figure 1). Together, these results suggest that neuromodulators could impact, or be impacted by adult brain development and adult sexual maturity. Both of these events could impact neural wiring and processing that subsequently affect host preference behavior in the host-seeking adult populations.

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 (Monastirioti, 1999; Ramesh and Brockmann, 2019). Interestingly, precursor molecules such as tyrosine, histidine, and glutamate showed higher titres at early stages of pharate-adult development (stage 4-6 in Figure 1) compared to adult sexual maturation stages (stages 7-8), whereas product molecules like octopamine and histamine levels increased only at later stages of adult fly sexual maturation (stages 7-8). In *R. pomonella*, host preference for either apple or hawthorn fruit cues is exhibited only by sexually mature adults (Nojima et al., 2003b, 2003a; Zhang et al., 1999), thus we might expect that any differences observed between the host races in neurochemical concentrations between immature and mature emerged adults might impact host choices. In general, the levels of precursor molecules of other pathways i.e., aspartate, glutamate, and serine remained high following initiation of adult brain differentiation (stage 4 in Figure 1) but not during adult sexual maturation (stages 7 and 8). This suggests that the increase in neurochemicals observed during adult brain development and/or sexual maturation could potentially impact behavioural preferences for specific host fruits.

We therefore hypothesize that differences in neurochemical titers between the host races during adult brain development and sexual maturation could impact host-preference behavior in adult flies, a key characteristic for host-associated divergence and ecological speciation in the *R. pomonella* system. As a first step towards testing this hypothesis, we examined differences in neurochemical titres between the apple and hawthorn host races across developmental stages to identify potential candidate neurochemicals that could ultimately affect either life history timing or adult host-preference behaviour. When comparing the two host races, the titers of several neurochemicals were significantly reduced in the apple race pupae upon initiation of adult brain development (stage 4) as compared to the hawthorn race, including tyrosine, tyramine, DOPA, aspartate, glutamate, and tryptophan (Figures 3-6). In adult flies, two major pathways showed differences between the host races: tyrosine to octopamine and histidine to histamine. In both pathways, titres of neurochemicals were significantly higher in the hawthorn race than the apple race for all chemicals except tyramine. These differences in neurotransmitter titres between the races are often carried through later developmental stages. In some cases, particularly for biogenic amines, the differences persisted through the eclosion and sexual maturation in the adult fly, the stage at which host preference is exhibited (Figures 3-5).

Studies in other systems have reported that high levels of octopamine stimulate feeding and drinking behaviour in blowflies, and specific changes in the level of histaminergic system in honeybee brain suggest its role in feeding and arousal behaviour (Long and Murdock, 1982;

Ramesh and Brockmann, 2019). Further analysis showed that apple flies initiated adult brain differentiation three weeks faster than hawthorn flies (Figure 2). This earlier diapause termination and development corresponded to significantly lower levels of the aforementioned neurochemicals than later developing pupae. This difference remained for the dopamine and octopamine precursors (tyrosine and tyramine) even when accounting for the impacts of temperature on very early developing pupae that might have been biochemically impacted by development in cold temperatures. In other words, earlier developing apple race adult brains contained lower titres of several neurochemicals, particularly those in the dopamine and octopamine pathways, than later developing hawthorn race adult brains. Given the importance of these neurochemicals to both development and behaviour, this difference in neurochemical production between the host races could impact both diapause termination timing and subsequent host preference in the adult flies.

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 (Tait et al., 2021). 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 (Unbehend et al., 2021). 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 (Dowle et al., 2020). In our current study, we show that 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 within and between the host races, specifically members of the dopamine and octopamine pathways (Figures, 3 and 6). 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 (Monastirioti, 1999). 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 genes in *Rhagoletis* and ultimately lead to changes in host-fruit preference in reproductively mature adults.

Future studies that measure the expression of enzymes like tyrosine decarboxylase, tyrosine hydroxylase, tyramine hydroxylase, and DOPA decarboxylase involved in the production of dopamine and octopamine between early developmental stages in these two host races could indicate if these pathways differ between the two host races at this critical juncture in the initiation of pharate-adult development, the transition from a stage 3 pupal brain to a stage 4 early pharate-adult brain. Further, selective pharmacological treatments that act as agonists and antagonists of these biogenic amines in the early developing brain of both races is needed to test the extent to which titres of these dopamine and octopamine associated neurochemicals affect development and subsequent host-fruit selection behaviour in mature adults of these diverging host races. Such treatment with an agonist of D2-like dopamine receptors prolonged pupal diapause and delayed adult emergence in Chinese oak silkmoth *Antheraea pernyi* ,whereas the antagonist of the receptor terminated the pupal diapause and stimulated earlier adult emergence (Wang et al., 2015). Also studies have shown that feeding bee colonies with octopamine resulted in earlier onset of foraging in young honeybees (Barron and Robinson, 2005; Schulz et al., 2002). However, we note that because of the univoltine life cycle of *Rhagoletis* with facultative diapause, such pharmacological experiments would be challenging.

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. Immunohistochemical staining showed that substantial neuronal differentiation occurs between early diapause before winter and late in diapause after overwintering, supporting the transcriptomic results of (Dowle et al., 2020). We further identified profound differences in neurochemical levels, particularly biogenic amines in the dopamine and octopamine pathways, during adult brain differentiation between the hawthorn and apple races of *R. pomonella* that correspond to differences in developmental timing. Because biogenic amines have been implicated to impact both pupal diapause and adult behaviour, these changes could potentially link observed differences in life history timing with adult host-fruit 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 (Dambroski et al., 2005). Furthermore, a recent study of a similar neurological change in the European corn borer has implicated a cis-regularly element (Unbehend et al., 2021), several of which also influence biogenic amine pathways (Monastirioti, 1999). 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 (Tait et al., 2021).

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Material and methods:

Sexually immature and sexually mature adult fly collection:

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 (1). In May to August 2017, after leaving the pupae at room temperature for 15 days, they were shipped to India (with permit). 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 (Nojima et al., 2003b, 2003a; Zhang et al., 1999).

Pre diapause development: In summer 2018, after collecting infested fruits from the above field sites, fruit were transferred to a tray with a wire mesh attached an insect-rearing room at 25$\pm$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. For the pre-diapause study four different cohorts of pupae were set aside and subjected to metabolic rate measurements once they reached either 7 or 19 days after pupariation. The rest of the other cohorts of pupae were transferred to a refrigerator at 4°C in a dark refrigerator with saturated KCL solution to stimulate over-winter diapause for six months at 10 days after pupariation.

Protocol for measuring the metabolic rate at pre diapause stage and brain sample collection:

This protocol is adapted from Ragland *et* al 2009 & Powell *et* al 2021 to use metabolic rates to phenotype pupae as diapausing or non-diapausing in the pre-winter period. 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 (Dambroski and Feder, 2007). We wanted to eliminate these non-diapausing individuals from our sampling before overwintering. 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 dririte-ascarite-dririte 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.

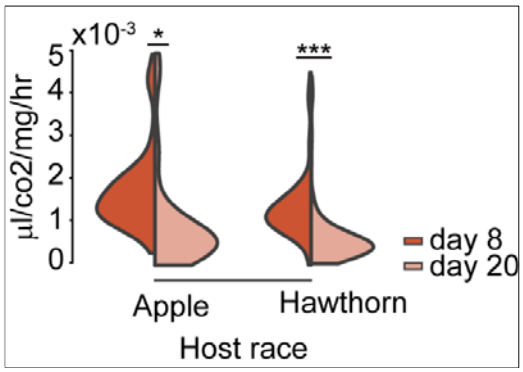

Figure 7: Violin plots showing metabolic rates of pupae at two-time points for both host races. (apple race, day 8, n=142, diapausing pupae; n=68, non-diapausing pupae; day 20, n= 145, diapausing pupae, n=62, non-diapausing pupae; hawthorn race, day 8, n=145, diapausing pupae; n=59, non-diapausing pupae; day 20, n= 152, diapausing pupae, n=17, non-diapausing pupae;). Analysis performed using a linear mixed model to test for a difference in metabolic rates between day 8 and day 20. P value is significant < 0.05*, < 0.01**, < 0.001***

Previous work had shown that metabolic rates dropped as pupae initiate diapause and become stabilized after they enter into the diapause maintenance phase, with a fraction of pupae not entering diapause and developing directly into pharate adults that are identifiable by their much higher metabolic rates (Ragland et al., 2009). We initially measured metabolic rates and then dissected individual pupae on day 6 (n=70), day 8, day 15 (n=40), and day 20

after pupariation. After this initial effort showed that on day 6 individuals were still undergoing larval-pupal metamorphosis and by day 8 they showed fully developed pupal morphology, we chose to measure individuals on day 8 after pupariation to estimate metabolic rates during diapause initiation. By day 20 after pupariation, metabolic rates were lower than on day 8, showing that pupae had transitioned from diapause initiation and entered into the metabolic depression indicative of the diapause maintenance phase (Ragland et al. 2009, Powell et al. 2020). However, high metabolic rates exhibited by some individuals on day 20 showed that some pupae were clearly following a non-diapause developmental trajectory and had already initiated pharate-adult development, as was expected from previous reports in this system (Dambroski and Feder, 2007; Ragland et al., 2009). Because we wished to conservatively remove any individuals that may not have been in diapause from the experiment, any individual with a metabolic rate higher than 0.025 ul CO_2/mg/hour was classified as not in diapause and eliminated from further sampling. When we dissected pupae in the low metabolic rate class indicative of being in the diapause maintenance phase at day 20 after pupariation, they clearly possessed the larval brain morphology seen in stage 2 (Fig. 1). However, when we dissected brains from pupae with metabolic rates higher than 0.025 ul CO_2/mg/hour 20 days after pupariation these brains were in stage 4 (Fig. 1), indicating pharate-adult development had begun.

Development after overwintering:

In early November 2018 diapausing pupae were hand-carried with ice packs to maintain a cold temperature from Gainesville, FL, USA to Bangalore, India. After keeping pupae 6-months in the refrigerator 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. From day 1 after removal from artificial overwintering and every 5th day afterwards, 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'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 and were photographed and then dissected using the above protocol.

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).

Immunohistochemistry and brain morphology

To characterize brain morphology, brain samples were dissected in 1X phosphate buffer solution, PBS (PH: 7.4) on ice with fine forceps, and then underwent immunohistochemical staining using a protocol adapted from those used in *Drosophila* (Ibba et al., 2010; Singh et al., 2013; Tait et al., 2021). Briefly, dissected brains were transferred into plastic cell culture plates containing 4% paraformaldehyde (PFA), and plates were covered with aluminium foil. Brain samples were fixed in PFA for 3 hours on a rotator at 4°C under red light and subsequently washed with 0.3% Triton X prepared in 1x PBS (PTX) solution three times at an interval of 15 minutes. Brain samples were then treated with 0.1% bovine serum albumin in PTX (PBTX) as a blocking agent for 15 minutes, followed by incubation with the primary mouse anti-Bruchpilot/mAbnc82 (1:30, DSHB, University of Iowa) antibody for 48 hours on a rotator at 4°C. After two days of incubation with antibody, samples were washed four times with PTX solution at 15-minute intervals. Secondary antibody goat anti-mouse Alexa 647 (1:400, Invitrogen, Thermo Fisher Scientific, Waltham, USA) was added and brain samples were incubated for 48 hours on a rotator at 4°C. Mounting of the brain for visualization occurred on the fifth day when samples were washed with PTX 4 times, at an interval of 15 minutes at 4°C, and then mounted in 70% Glycerol on glass slides. Optical sections (512 x 512 pixels) of each brain sample with 1 µm step size, were imaged with a confocal scanning microscope (Olympus FV3000, DSS Imagetech, Bangalore) under a 10x, 20x and 40x DIC objective. Using the 3d reconstruction of *R. pomonella* brain as a guide (Tait et al., 2021) different brain regions were visualized and identified in FIJI open-source software (Schindelin et al., 2012).

Quantification of neurotransmitters from the R. *pomonella* brain:

After classifying diapausing and non-diapausing pupae, as well as post-diapause developmental stages, on the day of extraction of neurotransmitters brain samples were taken out from -80°C storage and five brains were pulled out for each sample replicate. The 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) method (Natarajan et al., 2015; Ramesh and Brockmann, 2019; Rangiah and Palakodeti, 2013). Adult fly brains were dissected individually, pooled into a group of five, and stored at -80°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 plastic pestle. It was then immediately centrifuged (13500 rpm 4°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 point down to 50%,25%,12.5% and 6,25% of 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°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°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 the supplementary materials (table s8, s9, s10, s11, figure s2 and s3).

Statistical Analysis:

Logistic regression was used 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 after pupae were removed from artificial overwintering conditions. Respiration data were analysed using linear mixed effects models in R (V4.0.2) using the lmer function from the lme4 package. In the model, age and host race were used as a fixed effect whereas cohort was used as random effect. Further, Tukey's HSD tests with correction for multiple comparisons were performed with pairwise multiple comparisons using the lmerTest package. The level of neuromodulator per sample was calculated by a ratio of mass area of unknown sample and internal standards vs. ratio of mass area of internal standards and standards of each compound. The amount of each chemical (in ng) in the unknown sample was calculated from calibration curves. Further, outliers were identified and removed by calculating inter-quartile range (difference between Q1 and Q3 quartile) in Microsoft Excel for each age group. The lower bound was calculated by multiplying the IQR value by 1.5 then subtracting it from the Q1 data point. The upper bound was calculated by multiplying the IQR by 1.5 and adding it to the Q3 data point. In brief, the values lying 1.5 times below or above the IQR were considered outliers and removed from the further analysis. Quantities of each compound in each sample group were analysed using linear mixed model effects as mentioned above. In the model, age and host race were used as a fixed effect, whereas mass spec batch was used as a random effect. The satterthwaite approximations in the lmerTest package was used to test the significance of the effect. Further Tukey's HSD tests were performed for pairwise multiple comparisons with significance cut offs of 0.0001,0.001, and 0.05. The interaction between host race and days on amount of precursor neurochemicals at 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 whereas amount of neurochemicals was used as dependent variable. Further, Tukey's LSD test was performed for pairwise comparisons with significance cut offs of 0.0001,0.001 and 0.05.

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

Author 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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Supplementary Information:

Supplementary Data File: This file contains Figures s1-s3, and Tables s1-s11:

Figure s1: Pupal brain morphology across developmental stages.

Figure s2: Sample chromatograms obtains from LCMS neurochemical analysis.

Figure s3: Sample calibration curves for one of the LCMS runs showing all 14 neurotransmitters analysed.

Table s1: Total number of pupae sampled after winter in the apple race.

Table s2: Total number of pupae sampled after winter in the hawthorn race.

Table s3: Within and between host race metabolic rate comparisons.

Table s4: Between host race stage comparisons for each neurotransmitter.

Table s5: Apple race developmental stage comparisons for each neurotransmitter.

Table s6: Hawthorn race developmental stage comparisons for each neurotransmitter.

Table s7: Two-way ANOVA result table showing host race comparisons at stage 4 for selected neurotransmitters.

Table s8: Instrument method details for liquid chromatography (LC).

Table s9: Instrument method details for mass spectrometry (MS).

Table s10: Internal standard molecular weights (MW) and expected retention times (RT) for the 14 neurotransmitters and their internal standards used for quantification in mass spectrometry.

Table s11: Standard solution preparation concentrations for all neurotransmitters.

Movie 1: Confocal Z-projection stacks (2 µm slices, 136 µm total depth) showing nc82 staining of the pre-diapause larval brain (stage 1, Figure 1).

Movie 2: Confocal Z-projection stacks (2 µm slices, 142 µm total depth) showing nc82 staining of the early diapause larval brain (stage 2, Figure 1).

Movie 3: Confocal Z-projection stacks (2 µm slices, 114 µm total depth) showing sowing nc82 staining of the late diapause larval brain (stage 3, Figure 1).

Movie 4: Confocal Z-projection stacks (1 µm slices, 109 µm total depth) showing nc82 staining of the diapause termination stage and the early adult brain (stage 4, Figure 1).

Movie 5: Confocal Z-projection stacks (1 µm slices, 50 µm total depth) showing nc82 staining midway through pharate-adult developing brain (stage 5, Figure 1).

Movie 6: Confocal Z-projection stacks (1 µm slices, 57 µm total depth) showing nc82 staining of the late pharate-adult developing brain (stage 6, Figure 1).

Movie 7: Confocal Z-projection stacks (1 µm slices, 46 µm total depth) showing showing nc82 staining of the eclosed adult fly brain (stage 7, Figure 1).