The neural basis for host shifts in *Rhagoletis pomonella*

A THESIS TO BE SUBMITTED TO

THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY

FOR THE AWARD OF THE DEGREE OF
DOCTOR OF PHILOSOPHY

BY

Ms. HINAL B. KHARVA

UNDER THE GUIDANCE OF

DR. SHANNON OLSSON

NATIONAL CENTRE FOR BIOLOGICAL SCIENCES,
TATA INSTITUTE OF FUNDAMENTAL RESEARCH,
BANGALORE-INDIA

SUBMIITED: MAY 2023
VIVA: DECEMBER 2023

THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY

Private University Established in Karnataka by ACT 35 of 2013

BENGALURU - 560064

DECLARATION BY THE CANDIDATE

I declare that this thesis entitled "**The neural basis for host shifts in *Rhagoletis pomonella***" submitted for the award of Doctor of Philosophy to THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY, Bengaluru, is my original work, conducted under the supervision of my guide Dr. Shannon Olsson (and co-guide, Dr. Axel Brockmann). I also wish to inform that no part of the research has been submitted for a degree or examination at any university. References, help and material obtained from other sources have been duly acknowledged

I hereby confirm the originality of the work and that there is no plagiarism in any part of the dissertation.

Hinal

Place: Bengaluru **Signature of the Candidate**

Date:11/12/2023

Name of candidate: Hinal B Kharva

Reg. No.: 20916030132

(September 2016)

THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY

Private University Established in Karnataka by ACT 35 of 2013

BENGALURU - 560064

CERTIFICATE

This is to certify that the work incorporated in this thesis “**The neural basis for host shifts in *Rhagoletis pomonella***” submitted by Hinal B. Kharva was carried out under my supervision. No part of this thesis has been submitted for a degree or examination at any university. References, help and material obtained from other sources have been duly acknowledged. I hereby confirm the originality of the work and that there is no plagiarism in any part of the dissertation.

Research Supervisor

Date: 11/12/2023

Dr Shannon Olsson,
National Centre for Biological Sciences,
Tata Institute for Fundamental Research,
Bangalore

THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY
Private University Established in Karnataka by ACT 35 of 2013
BENGALURU - 560064

CERTIFICATE

This is to certify that the work incorporated in this thesis "**The neural basis for host shifts in *Rhagoletis pomonella***" submitted by Hinal B Kharva was carried out under my supervision. No part of this thesis has been submitted for a degree or examination at any university. References, help and material obtained from other sources have been duly acknowledged. I hereby confirm the originality of the work and that there is no plagiarism in any part of the dissertation.

Co-supervisor

Date:11/12/2023

Dr. Axel Brockmann,
National Centre for Biological Sciences,
Tata Institute for Fundamental Research,
Bangalore

Acknowledgement

I would like to begin by expressing my gratitude towards my advisor, Shannon B Olsson, for giving me the opportunity to explore the fascinating world of neuromodulation and speciation. She not only gave me the freedom to choose my own project but also provided me with all the resources I needed for my research. Under her guidance, I have learned a lot, from science to philosophy. She not only provided technical guidance but also taught me how to have a broader view of science and how to troubleshoot problems through collaborations. I thank her for giving me opportunity work in our collaborator's lab at the University of Florida. I am grateful to her for giving us opportunity to write blogs on NICE website. This has helped me improve my writing skills and boosted my confidence to showcase my work outside the lab. Thanks to her, I now understand the meaning of an "elevator pitch." One of my happiest times in the NICE lab was organizing an insect walk for kids, and I am grateful to her for trusting me to plan it.

I would also like to express my appreciation for Axel Brockmann, with whom I worked as a trainee and junior research fellow. Axel taught me how to build hypotheses, design experiments, and standardize new protocols in his lab. The same training later helped me standardize methods for my PhD work. I am grateful to him for being a committee member for my PhD thesis. I would also like to thank Gaiti Hasan, who has guided me on my project as a thesis committee member and helped me with manuscript outlines. Her questions and suggestions have significantly improved my research work.

I was fortunate to visit Dr. Daniel Hahn's lab at the Department of Entomology and Nematology, University of Florida. Dr. Hahn, his post-doc (Andrew), and I went to the field site in Michigan in August 2018 to observe apple and hawthorn trees. It was one of the best road trips I have ever had, and I learned a lot from both of them. Dr. Hahn is an excellent physiologist, and I cannot thank him enough for guiding me on the diapause experiment and shaping and correcting my manuscript. He has been one of the great mentors I am fortunate to work with. I would also like to thank his post-doctoral fellows, Andrew Nguyen and Chao Chen, for their help with experiments and analysis. I would also like to thank my roommate in Gainesville, Florida, Elenore Philips, who helped me with everything during my stay.

I am grateful to Jeffrey L Feder for providing flies for my research. I would like to thank Cheyenne Tait, with whom I had so much fun working. I am thankful to both Daniel Kritsch and Jürgen Rybak for sharing their work with me and Cheyenne, allowing us to publish our *Rhagoletis* manuscript.

I am grateful to all the members of the NICE lab with whom I worked for so many years. They have been my support system. I would like to thank my only intern, Khushboo, who helped me take care of flies, Geetha, Gauri, Aditi, Pragdheesh, Jagath,

Anshika, Sreeraksha, Santosh, Sebastian, Pavan, Srishti, and Shreeshubha for always being there for me.

I would also like to thank the mass spectrometry facility for helping me run all my brain samples in the machine. I am especially grateful to Dr. Padma and her assistant Tanmayee for their help in troubleshooting the instruments many times.

I would like to thank my dance group friends Sukanya, Shruti, and Dhanya for their support throughout my PhD journey.

I would also like to acknowledge the invaluable contribution of Divya Ramesh from Axels lab, who helped me with the mass spectrometry experiments. She played a critical role in standardizing the experiments, troubleshooting during the process, and even wrote the code for my analysis. I extend my sincere thanks to the Director of NCBS, Prof. L Shashidharan, for his encouragement to complete my thesis.

I am also grateful to my mother-in-law, Yogini Bhatt, and father-in-law, Nitin Bhatt, for their support and encouragement towards my career. I would like to thank my sister-in-law, Niyati Bhatt, for her consistent support and encouragement during my Ph.D. studies.

My siblings have been my rock throughout my academic journey, and I extend my heartfelt gratitude to them. I thank my brothers, Siddharth and Vivek, and my elder sister, Rameshwari, for always being there for me. I am also grateful to my kind-hearted sister-in-law, Divya, and brother-in-law, Bhavik Gajjar, for their consistent encouragement. My niece, Aadhya, has been an inspiration to me with her unquenchable curiosity and thirst for knowledge. I would also like to acknowledge the youngest member of my family, my niece, Gargi, for bringing so much joy and love into our lives.

My deepest gratitude goes to my husband, Tanay Bhatt, who has been my pillar of strength and support throughout my Ph.D. journey. His unwavering support and guidance have helped me navigate some of the most challenging situations. I have learned so much from his own Ph.D. experience, and I am grateful for his constant support and encouragement.

Lastly, I would like to express my heartfelt appreciation to my spiritual guru, Swami Smarnanandji Maharaj from Ramkrishna Mission Vivekananda Memorial Vadodara, for always inspiring me to be my best self. I am also thankful to Shree Thakur, Maa, and Swamiji for their unwavering blessings and guidance.

I dedicate my dissertation to my parents, Bhupendra Kharva and Lata ben Kharva, who have taught me the values of kindness and empathy, which are more important than any degree I will ever receive.

Table of Content

List of Figures

List of Tables

Synopsis

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 adult behavioural preference toward new hosts (Rajus *et al*., 2021). I am studying the neural mechanism for ecological speciation in *Rhagoletis sp*. (Diptera: *Tephritidae*). This speciation is being observed over the past 300 years in North America. Introduction of domesticated apples (*Malus pumila*) by European colonists around 1800-1850 caused a shift in host preference by these flies from their native host downy hawthorn (*Crataegus mollis*). However, the question of how such a rapid and specific change in host preference could occur in such a short period of evolutionary time remains unanswered.

I, along with my colleagues, have identified a neuronal switch at the first synapse in the chemosensory system in the brain associated with differential host choice behaviour (Tait *et al*., 2021). This suggests that any changes in host preference must occur via network-level changes in the brain. In addition to behavioural preference, these races exhibit differences in pupal diapause timing with respect to their hosts. The dual role of neuromodulators in regulating both insect development and host-seeking behaviour provides a potential source for linking network level changes in the brain to changes in life history timing. Thus, I also correlated life-history timing, brain development and corresponding levels of 14 neurochemicals in *Rhagoletis pomonella* (Kharva *et al*., 2022). I found that apple race pupae exhibited adult brain morphogenesis three weeks faster after an identical simulated winter than the hawthorn race, which correlated with significantly lower titres of several neurochemicals. In some cases, particularly biogenic amines, differences in titres were reflected in the mature adult stage, when host preference is exhibited. In summary, life history timing, neurochemical titre and brain development can be coupled in this spectating system, providing new hypotheses for the origins of species through host shifts.

List of Publication

Research Publication

1) **Kharva, H.,** Feder, J., Hahn, D., Olsson S.B. (2022). Rapid brain development and reduced neuromodulator titers correlate with host shifts in *Rhagoletis pomonella*. R. Soc. Open Sci.9: 220962.https://doi.org/10.1098/rsos.220962

2) Tait, C., **Kharva, H.,** Schubert, M., Kritsch, D., Sombke, A., Rybak, J., Feder, J.L., and Olsson, S.B. (2021). A reversal in sensory processing accompanies ongoing ecological divergence and speciation in *Rhagoletis pomonella*. Proc. R. Soc. B.28820210192;20210192 https://doi:10.1098/rspb.2021.0192

3) Rajus. S., Bhagavan, S.G., **Kharva, H.,** Srinivas Rao, S., and Shannon B. Olsson, S.B. (2021). Behavioral Ecology of the Coffee White Stem Borer: Toward Ecology-Based Pest Management of India's Coffee Plantations. Front. Ecol. Evol. 9:607555. https://doi: 10.3389/fevo.2021.607555

Book chapters:

4) Kashyap R., Grover S., Puri H., Kaur S., Singh J., Sandhu K.S., Kaur G., **Kharva, H.,** Kaur S., and Kaur, B. (2023). Insect and Pest Management for Sustaining Crop Production Under Changing Climatic Patterns of Drylands. In: Naorem, A., Machiwal, D. (eds) Enhancing Resilience of Dryland Agriculture Under Changing Climate. Springer. Singapore. https://doi.org/10.1007/978-981-19-9159-2_21

5) Kaur, B., Singh J., Sandhu K.S., Kaur S., Kaur G., **Kharva, H.,** Grover S., Puri H., Kaur S., and Kashyap R. (2023). Potential Effects of Future Climatic Changes in Pest Scenario. In: Naorem, A., Machiwal, D. (eds) Enhancing Resilience of Dryland Agriculture Under Changing Climate. Springer. Singapore. https://doi.org/10.1007/978-981-19-9159-2_22

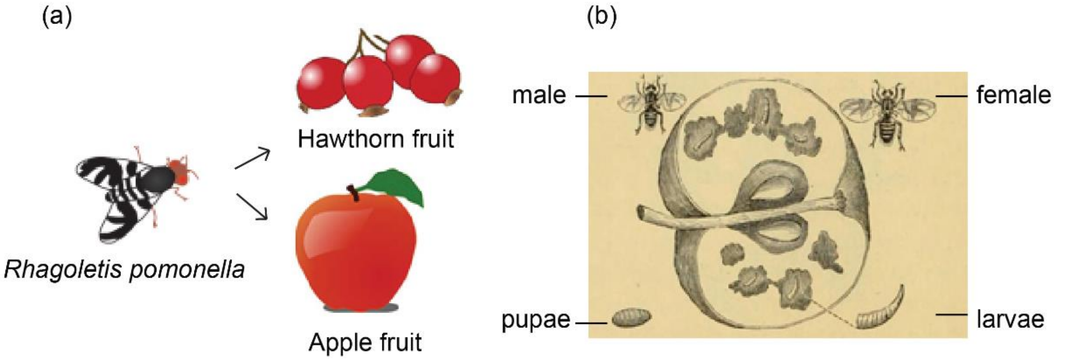
Chapter 1

1.1 Introduction

Evolution defines life on earth and understanding the evolution of different species, known as speciation, provides fundamental knowledge of the existing natural world (Barraclough, 1998). 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). Insects are one of the organisms that account for our planet's vast biodiversity. Out of 5.5 million species of insects, 25-40% of them are phytophagous insects (Mora et al., 2011; Stork, 2018). Phytophagous insects use plants for feeding and oviposition. The origin and evolution of host races in certain phytophagous insect groups is a topic of intense interest across scientific fields as these insect populations change their habitat and behavioural preference towards newly introduced host plants to eventually become separate species (Bush, 1969).

Population divergence and subsequent speciation in organisms can occur with or without geographical barriers between the two populations and is derived by reproductive isolation and genetic drift. Allopatric speciation occurs when two populations are separated by geographic barriers, which establishes reproductive isolation between them, and eventually gives rise to separate species. Speciation without any geographical isolation is called sympatric speciation. In sympatric speciation, habitat plays a very important role in the speciation process. Adaptation to different habitats (niche specialisation) may overcome the effect of gene flow and initiate population divergence. Sympatric speciation has historically been one of the most controversial issues in evolutionary biology (Coyne J. A. and Orr H. A., 2004) as there is no physical barrier to prevent gene flow, and adaptation to alternate habitats must itself establish the pre- and postzygotic reproductive barriers between populations.

The phytophagous insect *Rhagoletis pomonella* (Diptera: Tephritidae) is a model for ongoing sympatric speciation (Bush, 1969; Feder et al., 1988) and the story of this species traces back to the origins of evolutionary theory. In the 6th edition of *On the Origin of Species*, (Charles Darwin, 1872) Darwin mentions "Mr. B.D. Walsh, a distinguished entomologist of the United States" and his theory of phytophagic races. Darwin notes "differences of food apparently cause more marked and constant differences in colour or structure, or in both combined, in the larvae and in the mature insect. Forms modified to this degree are ranked by all entomologists as distinct, though allied, species of the same genus." The insects Walsh relied upon for his theory were the apple and hawthorn host races of *R. pomonella*. Walsh noted the shift of the fly from attacking its native host hawthorn (*Crataegus mollis*) to attacking introduced domesticated apple (*Malus domestica*) in the eastern USA around 1860s, exemplifying his theory for rapid ecological generation of insect diversity through adaptation to new host plants, figure 1.1 (Walsh. B, 1867). Walsh's idea intrigued Darwin. However, neither Darwin nor Walsh could deduce in the 1860s exactly what changes could happen between insects feeding on different hosts that could isolate them and establish separate species. In this dissertation, I examine the neural mechanisms, life history timing, development, and role of neurochemicals in the ongoing host shift and ecological speciation of *Rhagoletis pomonella*.



Host shift in *Rhagoletis pomonella*

Walsh's depiction of new "Apple Maggot species"
(1867, Am J Hort2, 338-343)

Figure 1.1 a) host shift in *Rhagoletis pomonella* from native hawthorn (*Crataegus mollis*) fruit to newly introduced apples (*Malus domestica*). b) Walsh's depiction of new apple maggot spices with different stages of life cycle.

1.2 Host shifts in phytophagous insects.

Host shifts in phytophagous insects are driven by the three factors:
1) change in life history timing of the insect with changes in development and physiology of the new host plant.
2) behavioural preference towards the new host.
3) maintenance of assortative mating differences between the new and former host race (Craig et al., 1993).

1.2.1 Life history timing and host shifts

In temperate climates, a difference in life history timing is an important ecological factor contributing to a host shift (Craig et al., 1993; Feder et al., 1993; Groman and Pellmyr, 2000; Itami et al., 1998; Pratt, 1994; Smith, 1988; Wood and Keese, 1990). Examples of this include the origin of the new *Solidago gigantea* population via host shift from *Solidago altissima* in the stem-galling tephritid fly. The mating isolation between these flies has arisen due to a change in their adult emergence time. The *S. gigantea*-associated flies eclose nearly 2 weeks (10-14 days) earlier than the *S. altissima*-associated population (Craig et al., 1993).

Another example of changes in life history timing is the lycaenid butterfly, which is specialised on a few species of Wild Buckwheat for mating, obtaining nectar, and oviposition. These populations have synchronized their life history timing with the flowering time of wild buckwheat. However, some sympatric subspecies can alter their flight timing, which provides the opportunity to adapt to different hosts with different blooming times, resulting in reproductive isolation and finally speciation (Pratt, 1994). Host colonisation in the moth *Prodoxus quinquepunctellus* (Lepidoptera: *Prodoxidae*) is also an excellent example of host shifts via changes in life history timing. On the east coast of the United States, a highly specialized yucca moth larvae used to feed on native *Y. filalamentosa* until *Y. aloifolia* was introduced by humans. The newly derived *Y. aloifolia*-specific population differs in the timing of emergence with differentiation in allozyme as well as ovipositor characters from the ancestral population (Groman and Pellmyr, 2000).

1.2.2 Life history timing in the *Rhagoletis* species complex

The host shift in *R. pomonella* from hawthorn to apple fruit was facilitated by a change in life history timing. Apple and hawthorn flies are univoltine (one generation emerges per year) and the adults are short lived (~1month). Thus, each host race must emerge synchronized with the fruiting time of its host plant. Several phytophagous insects like *Rhagoletis* synchronize their developmental rate with the timing of availability of host plants using a physiological process called diapause. The diapause process includes defined physiological phases such as pre-diapause, diapause maintenance, and post-diapause termination (Figure 1.2) (Andreatta et al., 2018). *Rhagoltis* adult flies mate near or on fruit and female lay eggs inside the fruit. The larvae eat the fruit and exit it once the overripe fruit falls to the ground, and burrow into the soil. The larvae then generate puparia. The pupae enter the diapause phase and remain until next summer season when they terminate diapause and pharate adult development continues (Feder et al., 1993, 1994; Smith, 1988). A few pupae do not enter diapause and direct development occurs in the same season. These non-diapausing flies emerge when there is hardly any fruit available and so they cannot reproduce. Diapause is therefore functionally obligatory in the case of *Rhagoletis* (Dambroski and Feder, 2007). Temperature acts as an important environmental factor for diapause termination and timing of adult emergence in *R. pomonella*. Photoperiod does not have any role since the pupae reside in the soil almost at depths where there is not much light (E. Boller and R.J. Prokopy, 1976).

Due to the earlier fruiting time of apples, apple flies initiate overwintering diapause earlier than hawthorn flies, and emerge as adults about one month earlier as well (Feder et al., 1988). 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 (Powell et al., 2020). Recent transcriptome work shows that apple race has a shorter post-winter diapause duration and rapid up-regulation of growth and development-related transcript genes compares to the long post-winter diapause duration of the hawthorn race (Dowle 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).

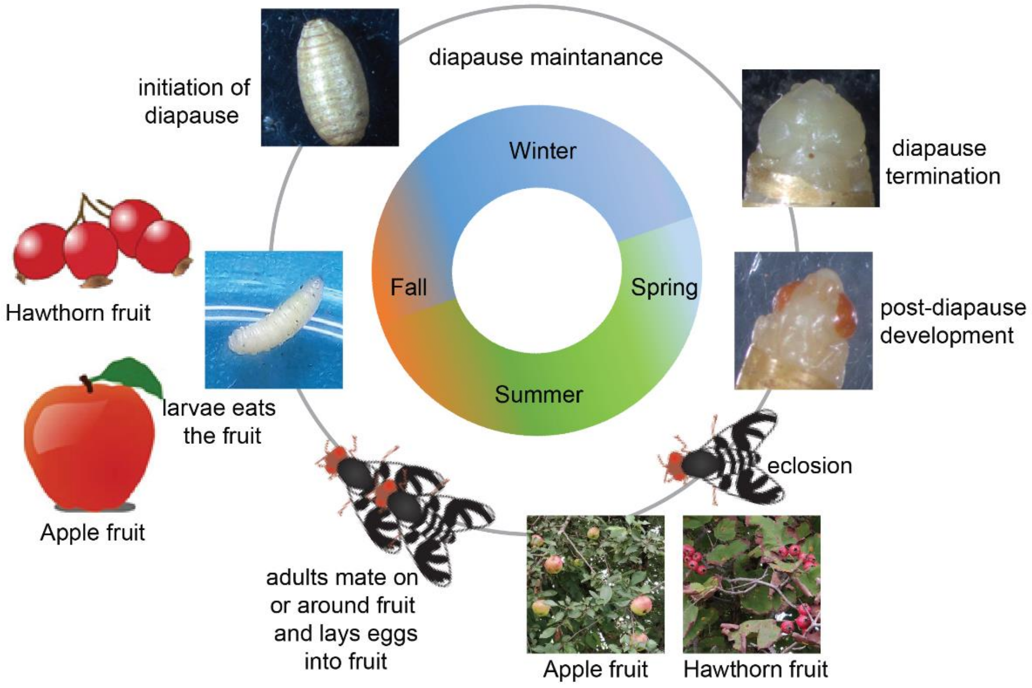


Figure 1.2: Life-history timing in *Rhagoletis pomonella* (egg, larvae, pupal diapause and adult emergence) corresponding to different seasons.

1.2.3 Olfactory preference and host shifts

Another factor apart from life history timing that impacts host shifts is a change in behavioural preference towards new hosts. Changes in behavioural preference can lead to a shift in habitat choice and affect reproductive isolation. (Date et al., 2017). Many phytophagous insects identify their host plant through host volatiles. Olfactory cues are one of the most common sensory modalities insects use to identify hosts. The overall structure of the olfactory system is highly conserved among insect groups, and indicates that the evolution of chemosensory preference and neuronal processing could be very similar across different species (Martin et al., 2011).

The insect olfactory system includes the antenna, the main organ of odour detection at the peripheral system, and central nervous system, the brain. The antenna harbours hundreds of cuticular structures called sensilla. Within sensilla, odour binding proteins (OBPs), odorant degrading enzymes (ODEs) and olfactory sensory neurons are found in the sensillar lymph (Shanbhag et al., 2001; Younus et al., 2014). Odour molecules enter through pores on the surface of the sensilla. OBPs carry odour molecules to the OSNs (Shanbhag et al., 2001). Chemosensory receptors, which are present on the dendritic side of OSNs, bind to odour molecules (Hallem and Carlson, 2006; Vosshall et al., 1999). OSNs subsequently send electrical signals via axonal projection to the brain to indicate the presence of specific volatile compounds (Keil, 1999).

OSNs expressing similar chemoreceptors project onto the same area of the central brain called the antennal lobe. There are two antennal lobes corresponding for each antenna. Each antennal lobe has grape-like structures called glomeruli (Couto et al., 2005). For each insect, the glomerular number is almost equal to the number of different types of chemoreceptors. OSNs synapse with projection neurons in glomeruli, and from here glomeruli send signals via axons to higher brain centres like the lateral horn and mushroom bodies. Glomeruli are interconnected by a network of local interneurons within and between the two lobes. This neuronal network can also be modulated via neuromodulators which can further affect behavioural output (Wilson et al., 2004). A feedback loop connection from higher olfactory centres (lateral horn and mushroom body) also exists (Hu et al., 2010).

Any change in the olfactory system at the peripheral or central nervous system (for example, change in OSNs number, type of ORs, sensitivity of neurons, size of glomerulus, reversal in glomerular arborisation) can affect odour preference and accompany a host shift. For example, a host shift in *Drosophila sechellia* is coupled with changes at both the peripheral and central olfactory systems. *Drosophila sechellia,* the specialist sibling species of *Drosophila melanogaster*, oviposits specially on toxic morinda fruit found in the Seychelles. *Drosophila sechellia* is behaviourally attracted to the host volatile methyl hexanoate found in morinda fruit. Studies have found that preference for methyl hexanoate involves overexpression of the OR22a-expressing ab3a neuron on the antenna of *D. sechellia* compared to *D. melanogaster,* which does not prefer the morinda fruit. This difference is also correlated to a 2.9x increase in the volume of DM2 glomeruli in the antennal lobe which processes the methyl hexanoate odour (Auer et al., 2020; Dekker et al., 2006). The cactophilic *Drosophila mojavensis* group is specialised on different cacti for feeding and oviposition substrates and identify their host cacti based on the volatiles emitted from the hosts (Date et al., 2017). The preference for cacti is coupled to changes in the specificity and sensitivity of olfactory sensory neurons (OSNs) as well as differential gene expression of the associated olfactory receptors (ORs) for the cactus volatiles (Crowley-Gall et al., 2016; Date et al., 2013, 2017). Finally, studies using gas chromatographic-electroantennographic detection (GC-EAD) in ephemeral gall midges show that different species of gall midges exhibit changes in specificity and sensitivity to host plant volatiles in their peripheral olfactory system (Molnár et al., 2018).

1.2.4 Olfactory preference in the *Rhagoletis* species complex

Rhagoletis flies use olfactory cues from long range to identify their host plants (Green et al., 1994). However, they also use visual and tactile cues once they are near or on the host plant (Prokopy, 1968a; Prokopy and Bush, 1973). Adults of the two host races of *Rhagoletis pomonella* also exhibit distinct behavioural 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., 2003a, 2004; Prokopy et al., 1971).

The behaviourally active host blend for apple and hawthorn fruits has been identified with the help of gas chromatography coupled with electroantennographic detection (GC-EAD) and flight tunnel and field assays. The synthetic fruit blends in the laboratory were also able to induce similar responses to whole fruit extracts (Linn et al., 2003a; Nojima et al., 2003a; Zhang et al., 1999). Field trapping studies and laboratory flight tunnel assays confirmed that both apple and hawthorn races prefer their own host volatile blend and avoid the other non-host odours in both field and lab (Forbes et al., 2005; Linn et al., 2005a). Assays employing sequential removal or addition of volatiles from the blends further provided information about which chemicals are behaviourally important (Linn et al., 2005a). These studies showed that the addition of the hawthorn volatile 3-methyl-1-butanol (3MB) into the apple blend removed attraction to the apple blend in apple flies and removing 3-methyl-1-butanol from the hawthorn blend entirely removed attraction in the hawthorn flies (Nojima et al., 2003a). Interestingly, the addition or removal of apple volatile butyl hexanoate (BH) from both the apple and hawthorn blend also showed exactly the opposite affect (Forbes et al., 2005). Thus, 3MB and BH volatiles show reciprocal agonist/antagonist behavioural response in these two host races as shown in figure 1.3.

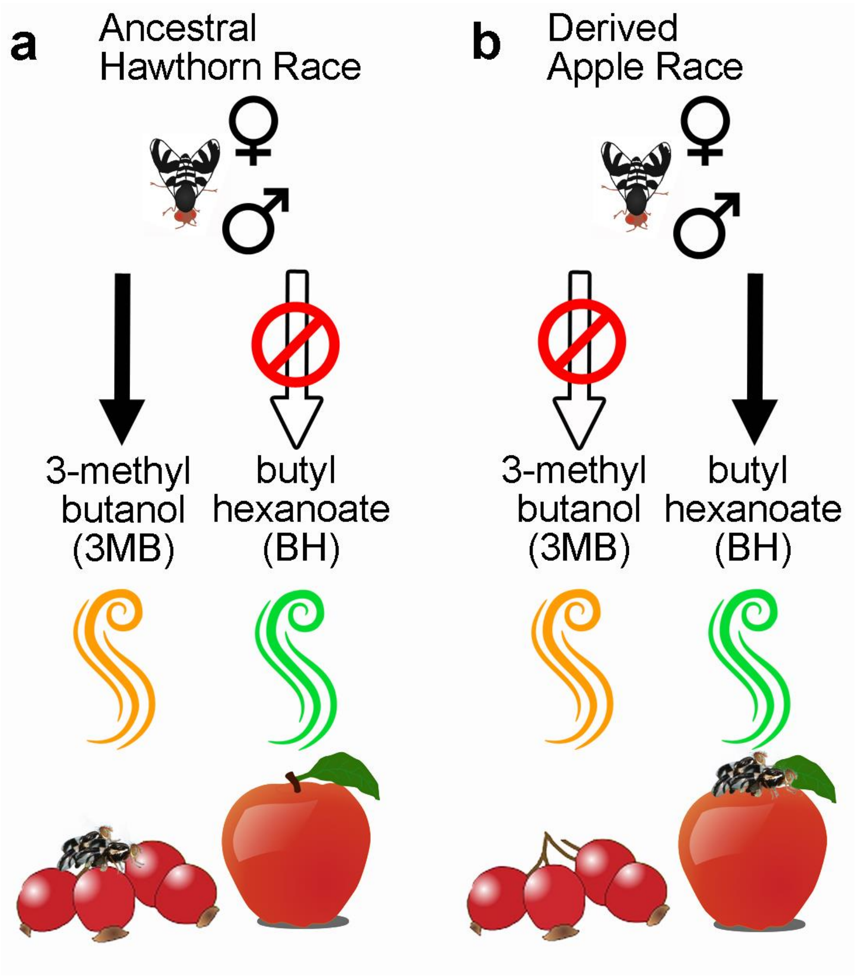


Figure 1.3: *Rhagoletis pomonella* host fidelity. (a) Both sexes of the ancestral hawthorn race are attracted to volatiles from hawthorn fruit. 3MB is essential for hawthorn fly attraction, while BH, a key apple volatile, is an antagonist. (b) The same volatiles exhibit opposite valences for the apple race, which uses BH to locate apples and is antagonized by 3MB. The mating flies' image was adapted from a photo by Joseph Berger, Bugwood.org, licensed under CC BY 3.0.

Flight tunnel assays have also indicated that the majority of flies prefer their host blend while some proportion of flies also prefer the non-host blend (Linn et al., 2005b). About 25% of both apple and hawthorn flies do not discriminate between apple and hawthorn blends and show behavioural response to both the blends. Around 20% of flies from both the races do not respond to either of the blends. Hybridisation studies report that all F1 hybrid crosses between the host races and close to 60% of F2 offspring do not respond to either blend (Dambroski et al., 2005; Linn et al., 2004). This behavioural data from hybrid flies suggests that the hybrid flies cannot find host fruit for mating/oviposition, which will contribute to postzygotic isolation in the *Rhagoletis* system (Olsson et al., 2006, 2009). These behavioural studies on host volatiles also provide information that olfaction plays a very important role in locating host plants in the case of *Rhagoletis* flies.

Previous studies on the olfactory peripheral system in *Rhagoletis* confirmed that both apple and hawthorn races have similar numbers, types, distribution, and sensitivity of olfactory sensory neurons on the antenna (Olsson et al., 2006, 2009; Tait et al., 2016). However, out of 28 olfactory sensory neurons identified, two colocalised olfactory sensory neurons in the sensilla B7 and B9 respond to the key host volatiles 3MB and BH for both the host races. A shift in the receptor expression between these paired neurons or the reversal in processing information from OSN to the brain could be responsible for changes in behavioural preference for host odours and for host shifts in these flies. 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 (Olsson et al., 2006, 2009).

1.3 The neuronal basis for host shifts

Like *Rhagoletis*, a similar shift in olfactory preference has been found in the sex pheromone system of the European Corn Borer moth, *Ostrinia nubilalis.* where males of Z and E pheromone stains show olfactory preference to two different pheromone blends. Interestingly, the two colocalized OSNs for these volatiles (in a similar configuration as the host volatile OSNs in *Rhagoletis*) show reversed response to the two isomers (Z and E) of the female sex pheromone blend at the antenna. This difference at the periphery is reflected by a reversal in the glomeruli of the antennal lobe responding to these two key volatiles. This suggests that the difference in behavioural preference is due to a change in the neural wiring from OSNs to the antennal lobe in the central brain (Hansson et al., 1987; Kárpáti et al., 2008; Koutroumpa et al., 2014; Lassance, 2010; Olsson et al., 2010).

Therefore, as a first principle, I hypothesise that the apple and hawthorn races of *R. pomonella* could also display a similar reversal in how volatile compounds emitted from the surface of ripe fruit are processed in the brain, which corresponds to the difference in their behavioural preference for host fruit. This difference in neurological phenotype between these recently diverged populations provides a unique opportunity to pinpoint changes initiating divergence in a still speciating system where one does not have to distinguish causative changes from those that occur after speciation is complete.

However, the mechanism by which these rapid changes could arise in such closely related races has remained elusive. One of the mechanisms that can alter neuronal network development and the behaviour is neuromodulation (Erber et al., 1993).

1.3.1 Neuromodulation and host shifts

In insects, metamorphosis brings profound reorganization and transformation of the nervous system. Neuromodulation refers to the changes of chemical cues in the brain, which in turn alter the functional traits of an organism. These changes can be modulated by amino acids, biogenic amines, neuropeptides, or nitric oxide. Neurochemicals play a role as neurotransmitters, neuromodulators and neurohormones. In the form of modulation, they have profound effects on neural circuits, development, metabolism, circadian rhythms, reproductive behaviour, learning and memory in many organisms. A small difference in the concentration or the level of neurochemicals can have a massive impact on insect development or behaviour. Some of the classical examples of neurochemical roles in insect development and behaviour are mentioned in below table 1.1.

Table 1.1 Case studies of role of neurochemicals in insect development and behaviour

Insect system	**Role of neurochemicals in development or behaviour in the system**	**References**
Fruit fly, *Drosophila melanogaster* (Dormancy and development)	down regulation of dopamine and serotonin pathways enhanced ovarian dormancy, whereas up regulation of octopamine signalling inhibited dormancy	(Andreatta et al., 2018)
Cabbage armyworm *Mamestra brassicae* (Diapause and development)	elevated levels of dopamine induce onset of pupal diapause in the armyworm	(Noguchi and Hayakawa, 1997)
Chinese oak silk moth, *Antheraea pernyi,* (Diapause and development)	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)

Cabbage butterfly, *Pieris brassicae* (Diapause and development)	elevated levels of biogenic amines dopamine, serotonin in the brain of cabbage butterfly *Pieris brassicae* are known to regulate diapausing and non-diapausing pupal development	(Isabel et al., 2001; Puiroux et al., 1990)
Bertha armyworm moth, *Mamestra configurata WLK* (Diapause and development)	increasing levels of octopamine in the brain followed diapause termination in this system	(Bodnaryk, 1980)
Mosquito, *Anopheles gambiae* (Behaviour)	low levels of octopamine also reduced egg laying in *Anopheles gambiae*	(Fuchs et al., 2014)
Honeybees, *Apis mellifera* (Behaviour)	elevated levels of octopamine lead to increased foraging activity in bees. The pollen foragers also showed lower phototaxis and it was corelated with higher level octopamine in their optic lobe of the brain. Specific changes in the level of histaminergic system in honeybee brain suggest its role in feeding and arousal behaviour	(Erber et al., 1993; Ramesh and Brockmann, 2019; Scheiner et al., 2014; Schulz et al., 2002)
Cotton leaf worm, *Spodoptera littoralis* (Behaviour)	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	(Felix et al., 2008; Kromann, 2012)
Blowflies, *Phormia regina* (Behaviour)	high levels of octopamine stimulate feeding and drinking behaviour in blowflies	(Long and Murdock, 1982)
Firefly *Lampyridae* (Behaviour)	One of the octopamine receptor adenosine 3',5'-ponophosphate, stimulates	(Nathanson J.A, 1979)

	light production in lantern of fireflies	
Fruit fly, *Drosophila melanogaster* (Behaviour)	manipulation of dopamine biosynthesis with chemical inhibitors showed colder temperature preference than normal one in flies	(Bang et al., 2011)

I hypothesize that changes in neuromodulation during adult brain development in holometabolous insects could link developmental synchronization with adult behavioural response to host plants, leading to host shifts and potentially the emergence of new species. Both apple and hawthorn races undergo pupal diapause (developmental arrest) during winter, and apple flies initiate and terminate diapause earlier than hawthorn flies to correspond with the earlier fruiting time of apples vs. hawthorn berries. Likewise, these two host races exhibit distinct behavioural preferences for their host plants. The coupled difference in pupal diapause timing, adult host preference, and adult brain physiology in *R. pomonella* provides a unique opportunity to examine if there are corresponding differences in neuromodulation during brain development between the races that could simultaneously impact both developmental rate and adult olfactory host choice.

While other studies, including a large body of work in the European Corn Borer moth, have implied that changes in gene expression during pupal development could impact adult behaviour, the phenotype of these changes has not been suggested (Unbehend et al., 2021). Therefore, our unique study on neuromodulation in this speciating system has broad implications for understanding the origins of new biodiversity. Across taxa and sensory modalities, shifts in the development of the nervous system can have important consequences for habitat and mate choice across the Tree of Life.

1.4 Objectives of this study

In this study, I used a variety of chemoanalytical, morphological, and immunohistochemical techniques to examine adult brain development, life history timing, and corresponding neurotransmitter levels in *Rhagoletis.* Specifically, I:

1) Identify the key host odour processing centres in the *Rhagoletis* brain.
2) Understand the life history timing and brain development in both host races.

3) Quantify the levels of neurochemicals from larval to adult developmental stages.
4) Compare levels of neurochemicals in diapausing apple and hawthorn race flies to non-diapausing lab-reared colony apple and wild apple flies.

Chapter 2 focuses on the identification of the key host odour processing centres in the *R. pomonella* brain.

Recent studies in our lab show that two pairs of co-localized olfactory sensory neurons (OSNs) respond specifically to key apple and hawthorn odours that mediate host preference (Tait et al., 2016). This suggests that any changes in host preference must occur via network-level changes in the olfactory circuit of the brain. To answer this, I have completed anterograde backfilling of dye to identify the glomeruli innervated by these OSNs. Here, I identify a neuronal switch at the first synapse in the chemosensory system in the brain associated with differential host choice behaviour (Tait et al., 2021).

Chapter 3 focuses on life history timing and brain development in both host races.

The results of Chapter 2 suggest that any changes in host preference must occur via network-level changes in the olfactory circuits of the brain. One of the neural mechanisms that can alter a neural network is neuromodulation (Erber et al., 1993) . As mentioned above (table 1.1) neuromodulators are known to regulate development and behaviour, I further quantified level of 14 neurochemicals in in developing apple and hawthorn race pupae. I found that the apple race adult brain develops nearly three weeks faster than the hawthorn race adult brain, and simultaneously exhibits significantly lower levels of several neuromodulators, particularly biogenic amines.

Chapter 4 then explores a study of neuromodulator levels in the brains of non-diapausing lab-reared colony wild adult apple flies.

Based on the results of Chapter 3 indicating a link between developmental timing and neuromodulator titre, I hypothesize that both early developing lab-reared colony flies and non-diapausing flies will have a lower level of neurochemicals in comparison to the diapausing hawthorn race. To test my hypothesis, I collected sexually mature lab-reared and non-diapausing apple race flies and quantified their levels of neurochemicals using mass spectrometry. My results confirm that faster-developing individuals display significantly reduced neurochemical titres, particularly biogenic amines.

Chapter 5 synthesizes the above data and presents a new hypothesis for host shifts in *R.pomonella* with a discussion of future directions for research on this topic.

In this dissertation I have identify the primary odour processing centre for key host volatiles in the brain of *Rhagoletis pomonella*, a species undergoing ecological speciation through an ongoing host shift from hawthorn to apple fruit. I also correlate life-history timing, brain development and corresponding levels of 14 neurochemicals in the brain of this species complex. I show that apple race pupae exhibit adult brain morphogenesis three weeks faster after an identical simulated winter than the hawthorn race, which correlates with significantly lower titres of several neurochemicals. In some cases, particularly biogenic amines, differences in titres are reflected in the mature adult stage, when host preference is exhibited. Similarly, the non-diapausing apple race and lab-reared colony apple flies also showed lower level of neurochemicals in the brain. In summary faster brain development and changes in neurochemicals can be coupled in this spectating system, providing new hypotheses for the origins of new species through host shifts.

Chapter 2

2.1 Introduction

Behaviour plays an important role in an animal's life, from finding food, to mating and habitat choice (Mendleson TC and Shaw KL, 2005; Smadja and Butlin, 2009; Terai et al., 2006; Wilczynski et al., 1993). Changes in behaviour also affect reproductive isolation, divergence and speciation in many organisms (Date et al., 2017). Sensory cues including vision, smell, and tactile cues play important roles in different behaviours. In many phytophagous insects, olfactory signals are particularly important in host choice behaviour, and changes in preference to olfactory cues often accompany host shifts. However, how olfactory processing is altered to accommodate these host shifts is still a subject of intense study.

In this chapter, I examine the neural mechanism for changes in host preference in *Rhagoletis pomonella*, a model for ongoing ecological speciation. *Rhagoletis* flies mate near or on host plants (Berlocher and Feder, 2002; Bush, 1969). This host-specific mating translates into reproductive isolation. Females from both host races identify their host plants using olfactory cues. This host choice behaviour affects mating, oviposition and larval feeding. (Bush, 1969; Prokopy et al., 1971). Both host races show attraction to the volatiles of their natal fruit and avoid non-natal fruit odours (Dambroski et al., 2005; Linn et al., 2003a, 2005a). Behavioural studies in the field and in the lab with synthetic fruit blends reproduced similar attraction as real fruit (Linn et al., 2003a; Nojima et al., 2003a; Zhang et al., 1999). Addition or removal of individual volatiles from these blends confirmed which volatiles are behaviourally important (Linn et al., 2005a). Apple flies are attracted to the key volatile butyl hexanoate (BH) in the apple blend (Linn et al., 2003a; Zhang et al., 1999). Similarly, hawthorn flies are attracted to the key volatile 3methyl butanol (3MB) in the hawthorn blend (Linn et al., 2003a; Nojima et al., 2003a). Interestingly, apple flies avoided the apple blend when 3MB was added to the apple blend (Forbes et al., 2005) and hawthorn flies avoided the hawthorn blend when BH was added to the hawthorn blend (Nojima et al., 2003a). Thus, 3MB and BH volatiles show reciprocal agonist/antagonist behavioural responses in these two host races as shown in Figure 1.3 (chapter 1). A similar behavioural response has been studied in the moth European Corn Borer, *Ostrinia nubilalis* a model for evolutionary dynamics of pheromone signals, where two Z and E strains of moths are attracted and repelled by the specific ratio of two components in sex pheromone blend (Roelofs et al., 1987). Hence, a small change in the detection or processing of these key volatile 3MB and BH in the olfactory system at the peripheral or central nervous system could be involved in behavioural attraction and avoidance in the apple and hawthorn races.

The olfactory system of *R. pomonella* includes the antenna (the peripheral olfactory organ) and the central nervous system (brain; figure 2.1). In insects, odours are detected by olfactory sensory neurons (OSN) on the antenna. OSNs are present within cuticular structures called sensilla. There are three types of sensilla found in *R.pomonella*: basiconic, trichoid, and coeloconic. Both basiconic and trichoid sensilla are known to harbor olfactory receptor neurons. Recordings from these sensilla identified 28 different types of OSNs in *Rhagoletis*, out of which there are two colocalized olfactory sensory neurons in sensilla B7 and B9, which respond to the key volatiles BH and 3MB in both apple and hawthorn races (Tait et al., 2016). These OSNs are similar in type, number, response profile, and dose response curve in both the apple and hawthorn race. Therefore, OSN sensitivity and specificity do not play a role in the change of olfactory preference between these races.

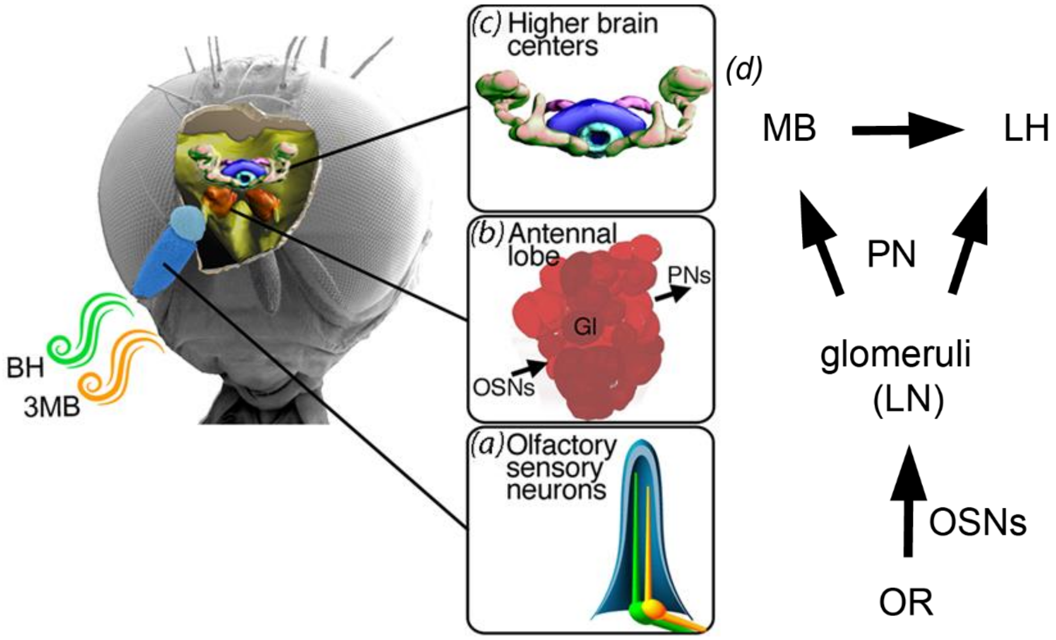


Figure 2.1: Overview of *R. pomonella* olfaction. (a) Olfactory sensory neurons (OSNs) are in stereotyped groupings within sensilla on antennae, where odorants including BH and 3MB interact with protein olfactory receptors (ORs). (b) The antennal lobe (AL) is the first olfactory synapse where OSNs from the periphery provide input to specific glomeruli (Gl). There are local interneurons among the glomeruli

(LN) which mediate complex interactions within and between the two antennal lobes. (c) Projection neurons (PNs), a specific type of antennal lobe neuron, bring olfactory information from the AL to higher brain centers such as the mushroom bodies (MB) and the lateral horn (LH). (d) Schematic of olfactory signaling in flies.

From the antenna, OSNs relay information via axonal projections to the antennal lobe in the brain (figure 2.1). In the antennal lobe, OSNs connect to antennal lobe neurons through neuropil structures called glomeruli. Similar types of OSNs arborize into the same glomerulus. In the European Corn Borer system, two colocalised OSNs show reversed responses to the two isomers of pheromone blend (Z and E) on the antenna (Koutroumpa et al., 2014). This change at the periphery corresponds to a reversal in the processing of these odours in the antennal lobe between the two pheromone races (Kárpáti et al., 2008; Koutroumpa et al., 2014; Lee et al., 2006; Wu et al., 2015). Therefore, I hypothesise that similar changes within the antennal lobes of apple versus hawthorn flies in the processing of the behaviourally relevant volatiles BH and 3MB could be involved in the change host preference between these two host races. My objective is to identify the processing centre for the two key host volatiles, 3MB and BH in the antennal lobe. To achieve this goal, I use neurophysiological techniques to trace responses to BH and 3MB from OSNs to the antennal lobe.

2.2 Methods:

Insects

Apple flies were obtained as pupae from a colony maintained at the NY Agricultural Experiment Station in Geneva, NY, USA, which relocated to the USDA-ARS Appalachian Fruit Research Station, Kearneysville, WV, USA. Additional apple flies and all hawthorn flies were collected as larvae from fruit in Michigan, USA, and reared to adulthood following established *Rhagoletis* husbandry methods (Feder JL, Chilcote CA, 1989).

Odours and their delivery

3-methyl-1-butanol(3MB) the key volatile of hawthorn blend and butyl hexanoate (BH) the key component in the apple blend were purchased from Sigma Aldrich (Fluka, 98.5% purity).

Both the chemicals were serially diluted to 10−3 w/v in mineral oil for antennal backfilling. They were administered as stimuli on filter paper circles in glass Pasteur pipettes as previously described (Tait et al., 2016). Appropriate solvent blanks were also used as controls.

Brain reconstruction

For brain reconstruction, apple flies were prepared for histology as described previously (Sombke et al., 2012). Briefly, heads were prefixed 24 h in 80% ethanol, 37% formaldehyde, 100% acetic acid (10: 4:1), washed in phosphate buffer solution (PBS, pH 7.4) and postfixed 1 h in 2% OsO_4 solution. After dehydration in acetone, heads were embedded in Araldite (Araldite epoxy resin kit, Agar Scientific). Serial sections (1.5 µm) were created with a Microm HM 355 S rotary microtome and stained using 1% toluidine blue and Pyronin G in 1% sodium tetraborate. Sections were imaged with a Zeiss AxioImager Z1 using 10× objective (Plan-NEOFLUAR, 10x/0.3; Carl Zeiss, Jena, Germany).

For antennal lobe reconstruction and immunohistochemistry, flies were prefixed in 4% paraformaldehyde (PFA) for 30 min, dissected and fixed over night at 4°C. After washing in PBS, brains were blocked with 1% BSA for 2 h. Specimens were labelled with monoclonal mouse anti-synapsin 'Synorf1' antibody (1:30, provided by E. Buchner, University of Würzburg) and polyclonal rabbit anti-FMRFamide (Sombke et al., 2011) (1 : 2000; ImmunoStar, Hudson, WI, USA) at 4°C for 4 days. After washing in PBS, brains were incubated with Alexa Fluor-488, goat anti-rabbit (1: 250; Invitrogen Life Technologies, Darmstadt, Germany) and Cy3, anti-mouse (1: 250; Jackson Laboratories, West Grove, PA, USA) at 4°C for 4 days. Specimens were then washed in PBS and mounted in VectaShield (Vector Laboratories Inc., Burlingame, CA, USA). Z-stacks were scanned in 1 µm intervals at 1024 × 1024 pixel.resolution with a Zeiss LSM 710 NLO confocal microscope (Carl Zeiss, Jena, Germany) using a 20× water immersion objective (W Plan-Apochromat 40x/1.0 DIC M27; Carl Zeiss). For three-dimensional (3D) reconstruction and volume estimation, images from histological methods were aligned and brain structures reconstructed using the segmentation software. AMIRA (Grabe et al., 2015; Rybak et al., 2010) (FEI Visualization Sciences Group, Burlington, MA, USA; Advanced 3D Visualization and Volume Modelling, RRID: nif-0000-00262). After surface models were digitized, they were also segmented using AMIRA. Regions of the brain were identified, and glomeruli named according to standard insect brain nomenclature (Ito et al., 2014). Note: Brain reconstruction and antennal lobe reconstruction was done by Daniel Kritsch and Jürgen Rybak.

Antennal olfactory sensory neuron backfilling

To identify target neurons for backfilling, I performed single sensillum recordings from neurons on the antennae, stimulating with BH and 3MB, as described previously (Tait et al., 2016). Target OSNs were passively filled for 2 h with 4% neurobiotin (Vector Laboratories, Inc.) (Ghaninia et al., 2007), then flies were stored at 4°C for 2 h for further tracer diffusion, decapitated and dissected in PBS. In a protocol adapted from *Drosophila melanogaster* (Ibba et al., 2010; Singh et al., 2013), dissected brains were first fixed in 4% PFA for 3 h on a rotator at 4°C. They were washed with 0.3% Triton X in PBS (PTX) and blocked with 0.1% bovine serum albumin in PTX for 15 min. Brains were incubated with DyLight 488 Streptavidin (1: 125, Vector Laboratories Inc., USA) and mouse anti Bruchpilot/ mAbnc82 (1:30, DSHB, University of Iowa) to highlight specific neuronal tissues for 48 h on a rotator at 4°C. After washing in PTX, I added goat anti-mouse Alexa 647 (1: 400, Invitrogen) and incubated for another 48 h on a rotator at 4°C. Samples were washed in PTX at 4°C then mounted in 70% glycerol. Optical sections (512 × 512 pixels) were imaged with a confocal scanning microscope (Olympus FV1000, DSS Imagetech, Bangalore) under a 40×, 1.4 oil-immersion DIC objective, with 1 µm step size. Using the AL reconstruction for guidance, glomerular identification and production of maximum projections was performed in FIJI.

2.3 Result

Input to the antennal lobe

The 3D reconstruction of *Rhagoletis* brain and antennal lobe is mentioned in (Tait et al., 2021). The *Rhagoletis* brain structures are very similar to *Drosophila melanogaster*. Using standard Drosophila nomenclature, all brain regions and glomeruli in the antennal lobe were identified (Grabe et al., 2015; Ito et al., 2014). There are 47±2 glomeruli in *R. pomonella* (n = 6 reconstructions; figure 2.2 a–c; (Tait et al., 2021), similar to *D. melanogaster* (n = 54) (Grabe et al., 2015) and *Ceratitis capitata* (n = 53) (Solari et al., 2016).

Using this reconstruction of the *Rhagoletis* brain as a reference, I performed single-sensillum electrophysiology combined with anterograde backfilling of the OSNs responsive to BH and 3MB to determine whether there was a difference in input to the AL between the host races (Tait et al., 2016). I found that BH and 3MB responsive neurons innervated the same set of three glomeruli in apple (n = 9) and hawthorn (n = 10) flies, identified as the dorsal medial 1 (DM1), and ventral posterior 1 and 2 (VP1 and VP2) glomeruli (figure 2.2d–f; figure 2.3). Note that while all three glomeruli were not labelled in every trial (table 2.1), only these glomeruli were identified in targeted backfills. Thus, there appears to be no difference in the overall input to the antennal lobe by OSNs responding to BH or 3MB between the apple and hawthorn races. However, backfilling resulted in the indiscriminate labelling of both BH and 3MB responsive neurons as they are colocalized together within sensilla. Consequently, I was unable, with this method, to discern which volatile activated which glomerulus.

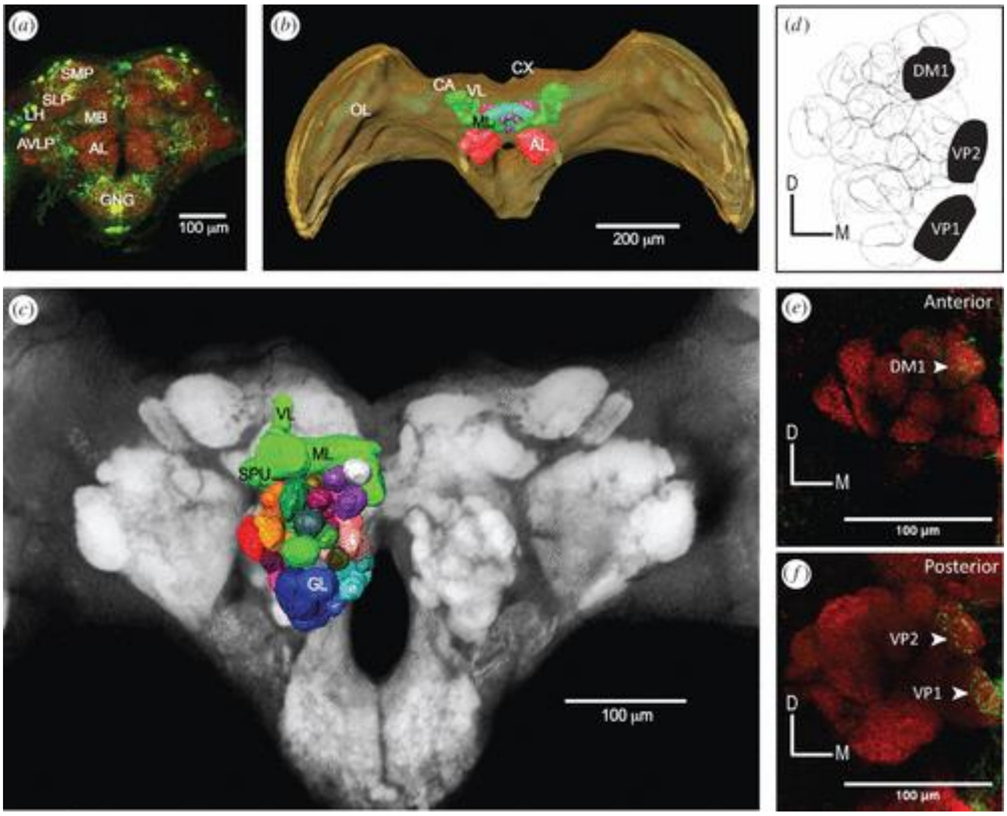
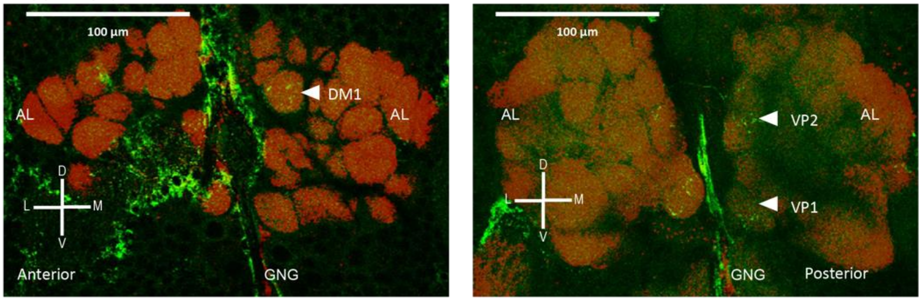


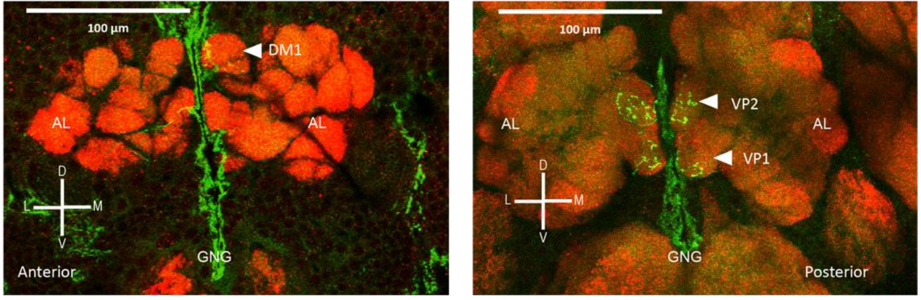
Figure 2.2: *Rhagoletis pomonella* brain anatomy and identification of glomeruli innervated by host volatile responsive OSNs. (a) Confocal Z-projection micrograph (75 μm depth) showing synapsin (red) and FMRFamide-like neuron immunoreactivity (green). Anatomical regions are identified: superior medial protocerebrum (SMP), superior lateral protocerebrum (SLP), lateral horn (LH), anterior venterolateral protocerebrum (AVLP), mushroom body (MB), antennal lobe (AL) and gnathal ganglia (GNG). (b) Three-dimensional reconstruction based on histological sections showing the optic lobe (OL), mushroom body calyx (CA), mushroom body ventral lobe (VL), mushroom body medial lobe (ML), central complex (CX) and AL. (c) Three-dimensional reconstruction of the AL and the MB indicating the spur (SPU) overlaid onto a Z-projection micrograph (300 μm depth). The glomeruli (GL) are labelled with different colours. (d) Three-dimensional reconstruction with glomeruli (DM1, VP1 and VP2) targeted by OSNs responsive to the volatiles BH and 3MB in black. (e,f) Confocal Z-projection of the AL following neurobiotin backfilling of OSNs (green), targeting glomerulus DM1 (e, arrowhead) and

targeting glomeruli VP1 and VP2 (f, arrowheads). The synaptic marker nc82 (red) labels background neuropil.

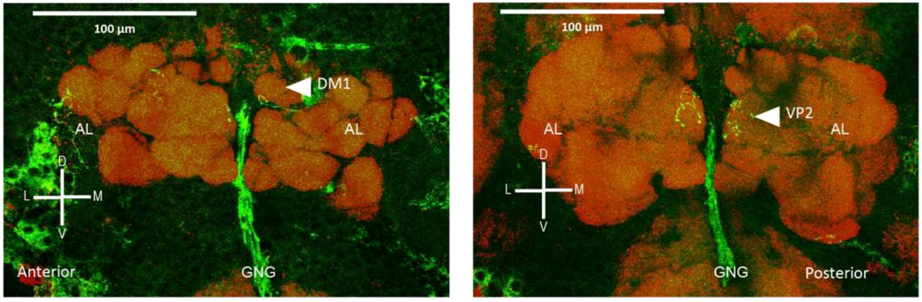
a Apple fly6, sensillum backfill



b Apple fly 7, sensillum backfill



c Hawthorn fly11, sensillum backfill



d Hawthorn fly14, sensillum backfill

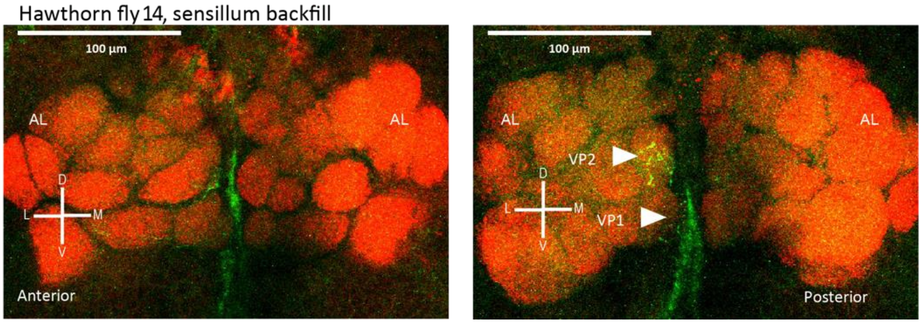


Figure 2.3: Additional replicates of backfilling of OSNs responding to BH and 3MB. Images are showing neurobiotin filling (green). Antennal lobe glomeruli were labelled against the synaptic marker nc82 (red). (a), (b) apple race OSN backfills corresponding to individuals in table 2.1 as noted; (c), (d) hawthorn race OSNs. Innervated glomeruli are indicated with arrowheads. Note that labelling always included some subset of the glomeruli DM1, VP1, and/or VP2. Note that in most cases, OSNs also projected contralaterally to the same glomerulus in the other antennal lobe, as occurs in *D. melanogaster*.

Table 2.1: Summary of olfactory sensory neuron backfilling. Total replicates of backfilling of OSNs responding to BH and 3MB in apple and hawthorn race.

ID	**Host Race**	**Collection Site**	**Glomerulus/i Innervated**
1	Apple	Grant	VP2
2	Apple	Grant	VP1, VP2
3	Apple	Grant	VP2
4	Apple	Grant	DM1, VP1, VP2
5	Apple	Grant	DM1, VP1, VP2
6	Apple	Grant	DM1, VP1, VP2
7	Apple	Grant	DM1, VP1, VP2
8	Apple	Grant	DM1
9	Apple	Grant	DM1
10	Hawthorn	Lansing	VP1, VP2
11	Hawthorn	Lansing	DM1, VP2
12	Hawthorn	Lansing	VP1, VP2
13	Hawthorn	Lansing	VP1
14	Hawthorn	Lansing	VP1, VP2
15	Hawthorn	Lansing	DM1
16	Hawthorn	Lansing	DM1
17	Hawthorn	Lansing	VP1
18	Hawthorn	Lansing	DM1
19	Hawthorn	Lansing	DM1

2.4 Discussion

The goal of this chapter was to identify whether there is a difference in the input from OSNs to the antennal lobe between the host races. I found both colocalized OSNs innervate the same three glomeruli DM1, VP1, and VP2 and thus there is no difference in in the location of processing for key host volatiles 3MB and BH in the antennal lobe.

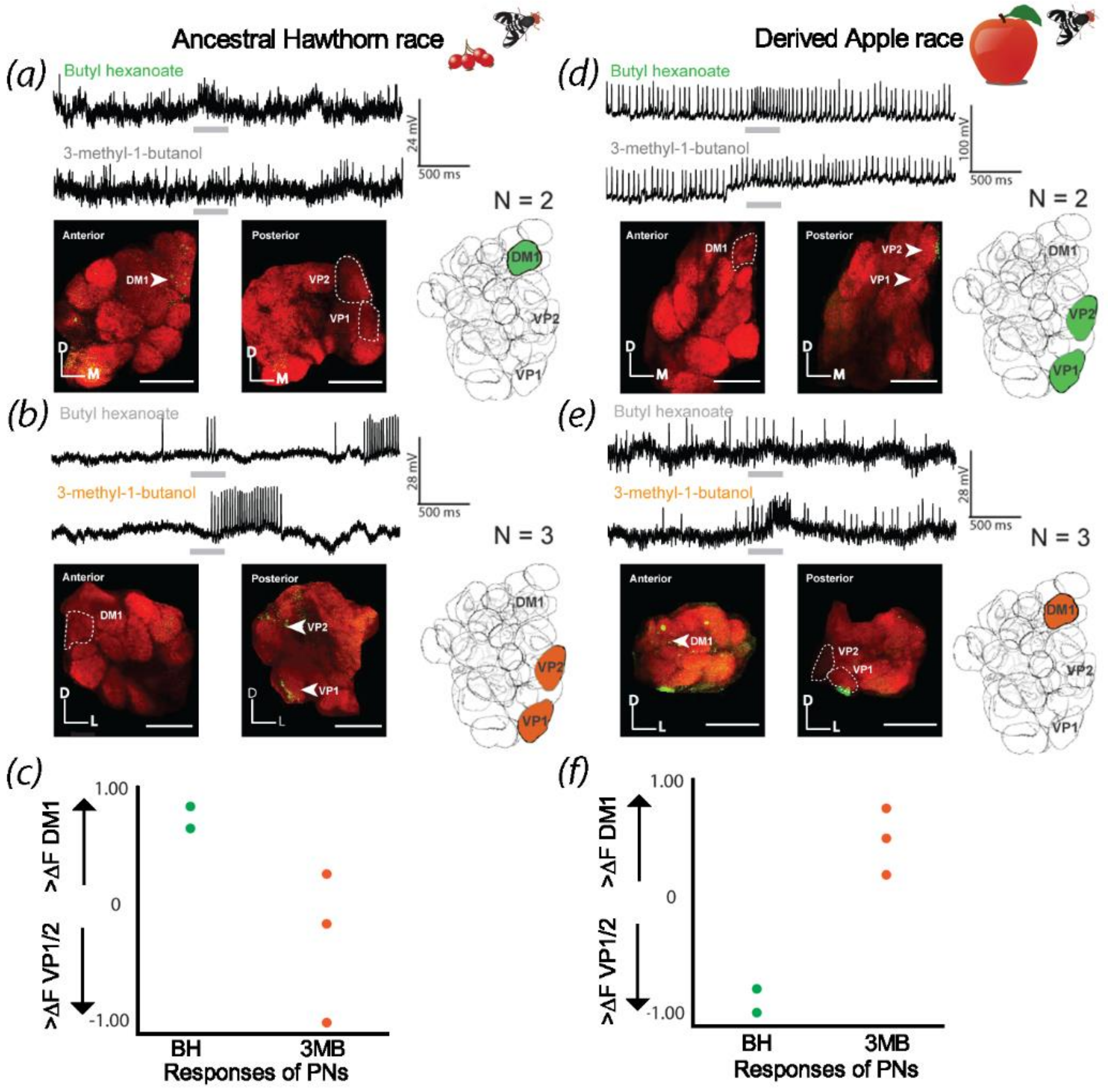


Figure 2.4. Targeting of ALNs responding to key volatiles. (a, b, d, e) (top) Paired 5 s intracellular recording traces for ALNs responding to BH (top) or 3MB (bottom). A volatile prompted a response if the volatile's name is in colour. Grey lines below indicate the 0.5 s stimulus. (bottom) Maximum confocal Z-projections (10–20 μm anterior, left, and 10–20 μm posterior, right) of labelled ALNs corresponding to the spike recordings, using neurobiotin (green), counter-labelled with synaptic marker nc82 (red). Scale bars, 50 μm. ALNs targeting DM1, VP1 or VP2 glomeruli indicated with white arrowheads; glomeruli lacking arbourization are outlined by dotted lines. Insets for each panel summarize glomeruli targeted by ALNs responding to BH (green; a, d) or 3MB (orange; b, e). (c, f) Scatter plots showing the normalized difference index of average fluorescence intensity within the three target glomeruli (ΔF DM1 – ΔF VP1and2)/ (ΔF DM1 + ΔF VP1and2) for confocal Z-projections of the ALNs responding to either BH or 3MB in each race. This figure has been reproduced from Tait *et* al 2021.

Further studies in *R pomonella* by my colleagues using calcium imaging and intracellular recording (Figure 2.4; calcium imaging not shown) indicated that antennal lobe neurons (ALNs) responding to 3MB in hawthorn flies arborized in VP1 and VP2 glomeruli while ALNs responding to BH only arborized in DM1 glomeruli in the hawthorn race. Conversely, in ALNs responding to BH in apple flies only arborized in VP1 and VP2 glomeruli, and ALNs responding to only 3MB arborized in DM1 glomeruli in the apple race. No identified neurons arborized all three glomeruli, suggesting that VP1 and VP2 specifically process one host odour, and DM1 the other (Tait et al., 2021). Therefore, our combined results indicate that a reversal in the processing of two critical host volatiles (BH and 3MB) affecting host fruit choice in apple and hawthorn race flies occurs at the first olfactory synapse in the brain, the antennal lobe. Similar features in olfactory processing, including colocalization of key OSNs on the antennae and reversals in odour-evoked activity at the level of the antennal lobe, are associated with changes in sex pheromone systems in moths (Kárpáti et al., 2008; Koutroumpa et al., 2014; Lee et al., 2006; Wu et al., 2015). These similarities suggest evolutionary convergence in the neural mechanisms associated with behavioural divergence in mate and habitat choice, generating prezygotic ecological reproductive isolation. In *R. pomonella*, host fruit volatiles are the critical first signal and are sufficient for finding mates on host plants without the need for long distance sex pheromones.

The *Rhagoletis* genus contains many specialist taxa attracted to the volatiles of specific hosts, providing the opportunity to expand analysis to the immediate sister species to apple and hawthorn flies, the flowering dogwood fly. These flies specialise on dogwood, *Cornus florida*, fruit. Behavioural studies suggest that dogwood flies are more attracted to 1-octen-3-ol. Interestingly they are also attracted to 3MB and avoid BH like hawthorn race (Nojima et al., 2003b). How 1-octen-3-ol is processed in the brain of dogwood flies and hawthorn flies could provide information about changes in neurophysiology between dogwood and hawthorn flies as well. This will help us better understand the mechanism for the evolution of the olfactory system and divergence in host shifts across this species group.

The known history of the recent shift of *R. pomonella* to apple and the partial but not complete isolation of the apple from the hawthorn race are important, because this indicates the reversal in BH and 3MB processing is contributing to active divergence rather than evolving after speciation is complete. Thus, changes in the antennal lobe can be directly tied to initiating rapid population divergence. Future work is still needed to identify the genetic cause and developmental basis for the reversal in processing of BH and 3MB. Leading candidates include differential expression of receptor proteins on the OSNs (figure 2.1a), as appears to be the case in the sex pheromone systems of heliothine moths (Lee et al., 2006; Wu et al., 2015) and/or developmental changes in neuronal targeting of OSNs from the antennae glomeruli in the antennal lobe glomeruli (input to figure 2.1b), as in European corn borer moth (Kárpáti et al., 2008; Koutroumpa et al., 2014). It remains possible that more complex changes in processing within the antennal lobe itself, prior to the ALN level output (figure 2.1 b, c), contribute to the observed differences via interneurons, neuromodulators or centrifugal input from higher processing centres. One mechanism that regulates both behaviour and development in insects is neuromodulators. Neurotransmitters like dopamine, serotonin, and octopamine, which are known to modulate olfactory cues in insects, could also be involved in this reversal at the antennal lobe (Kromann, 2012). For example, in the American cockroach, *Periplaneta americana* octopamine released from neurons in the brain regulates both the behaviour and response of olfactory sensory neurons in the antenna (Jung et al., 2013; Zhukovskaya, 2008, 2012).

In conclusion, we have identified a reversal in processing at the first olfactory synapse, the antennal lobe of the brain, associated with odours key for differential host choice behaviour and thus involved in the rapid ecological divergence of *R. pomonella*. Coupling of habitat and mate choice is common for host specialist phytophagous insects and their parasites (Bush, 1969), and often involves olfactory cues. The reversal in olfactory processing in *Rhagoletis* may be a common theme contributing to the reproductive isolation of many phytophagous insects. Host-seeking behaviour connecting adult reproduction directly with larval feeding and survival, here shown to involve relatively simple changes in sensory processing, could be part of the explanation for the great diversity of insect specialists noted by Walsh and Darwin. Indeed, such changes in sensory processing may be a broad mechanism for generating biodiversity across sensory modalities and for many groups in addition to phytophagous insects, constituting a major factor contributing to the origin of species.

Chapter 3

3.1 Introduction:

Life history timing is one of the most important ecological barriers separating host plants and their specific insects (Craig et al., 1993; Feder et al., 1993; Groman and Pellmyr, 2000; Itami et al., 1998; Pratt, 1994; Smith, 1988; Wood and Keese, 1990). For phytophagous insects shifting to new host plants, populations may 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 (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).

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 (Kostal, 2006; Kostal et al., 2017). It is still unclear which aspect of insect diapause responds to environmental cues to synchronize life history timing with the insect's host plant. It could be the pre-diapause phase that directly effects timing of adult emergence, or it could be the diapause termination or post-diapause development, which is controlled by environmental factors like photoperiod/temperature (Dambroski and Feder, 2007). Another factor could be the duration of diapause, which plays an important role in the resumption of active growth and reproduction (Dowle et al., 2020). However, it is also possible that life-history timing requires little genetic change, and it is a phenotypic response to varied environmental conditions. (e.g., egg hatch in *Enchenopa binotata* treehoppers is cued to spring sap flow in host trees) (Wood et al., 1990).

Rhagoletis pomonella provides a unique opportunity to identify associations between pupal development, life history timing, and adult host choice. Due to the earlier fruiting time of apples, apple flies initiate their overwintering dormancy, diapause, earlier than hawthorn flies, and emerge as adults about one month earlier as well (Feder et al., 1988). 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 (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., 2003b, 2004; Prokopy et al., 1971) In chapter 2 my colleagues and I found a reversal in processing of the host key volatile of apple and hawthorn in the antennal lobe of the brain associated with host choice behaviour. Apple and hawthorn flies also differ in their life history timing. To identify the causative changes between *R.pomonella* host races I hypothesise that there can be a change in brain development, level of neurochemicals or both.

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). 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 (Andreatta et al., 2018; Noguchi and Hayakawa, 1997; Wang et al., 2015; Zhang et al., 2013). Increasing levels of octopamine in the brain followed diapause termination in the Bertha armyworm moth *Mamestra configurata WLK* (Bodnaryk, 1980).

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 (Felix et al., 2008; Kromann, 2012; Schulz et al., 2002; Schwaerzel et al., 2003). These studies suggest that neuromodulators can have effects on development and behaviour.

In this study, I 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. There are a number of potential neurochemical candidates and life history stages that could play a role in this host shift, and also a general lack of knowledge regarding how neuromodulators could impact this process. Therefore, my goal is to track brain development from larval to adult stages in the apple and hawthorn host races of *R. pomonella*, and identify which stages, if any, exhibit differences in neural development or neuromodulation between the host races, and how these differences are reflected in the mature adult fly, when host preference is exhibited.

3.2 Methods:

(a) 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 (Walsh. B, 1867). 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 (Nojima et al., 2003b, 2003a; Zhang et al., 1999).

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 (Dambroski and Feder, 2007). To eliminate these non-diapausing individuals from my sampling before overwintering, I used a protocol adapted from (Powell et al., 2020; Ragland et al., 2009) to phenotype pupae as diapausing or non-diapausing by measuring g metabolic rates in the pre-winter period.

First, I 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. I 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. The total number of brains dissected are denoted as $\mathbf{n}_{\mathbf{dissections}}$, and total number of brains used for staining denoted as $\mathbf{n}_{\text{staining}}$ for each sample.

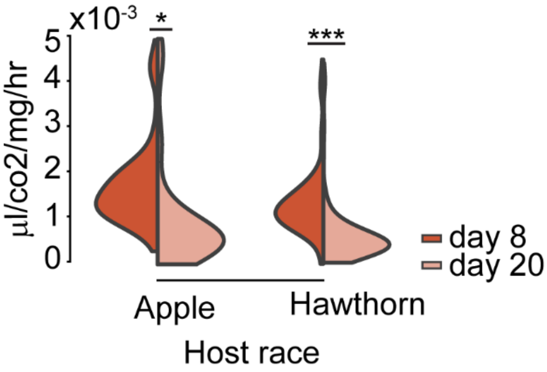


Figure 3.1: Violin plots showing metabolic rates of pupae at two-time points for both host races. Apple race, day 8, n=142, diapausing pupae; n=68, non-diapausing pupae; day 20, n= 145, diapausing pupae, n=62, non-diapausing pupae; hawthorn race, day 8, n=145, diapausing pupae; n=59, non-diapausing pupae; 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***

Table 3.1: Within and between host race metabolic rate comparisons.

comparisons	estimate	SE	df	t.ratio	p.value	
Apple d 8 - Apple d 20	-0.0013	0.0004	237.8	-3.112	0.0111	*
Apple d 20 - Hawthorn d 20	0.0016	0.0005	136.0	3.522	0.0032	**
Apple day 8 - Hawthorn d 8	0.0002	0.0004	75.16	0.549	0.9465	
Hawthorn d 8 - Hawthorn d 20	-0.0027	0.0004	784.8	-6.286	0.0001	***

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). I initially measured metabolic rates and dissected individual pupae on day 6 (n=70), day 8, day 15 (n=40), and day 20 after pupariation. This initial effort showed that day 6 individuals were still undergoing larval-pupal metamorphosis and by day 8 they showed fully developed pupal morphology. I therefore measured 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 (Figure 3.1) (Powell et al., 2020; Ragland et al., 2009). Because I 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 eliminated from further sampling. When I 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. 3.3). However, when I 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. 3.3), indicating pharate-adult development had begun.

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. 3.3, 3.4). 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* in Chapter 2 (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 morphologically determining developmental stage, brain samples were removed from -80°C storage and five brains of the same stage were pooled for each sample replicate (denoted as $\mathbf{n_{samples}}$). In one sample, only a single brain at stage 4 was observed in the hawthorn race prior to day 35 after the artificial winter. To assess the neurochemistry of this brain, I was required to pool it with other hawthorn stage 4 brains from days 35 and 40 to obtain enough material for analysis. This unique data point is shown at day 30 in Figure 3.7. The sample processing was performed as in Ramesh *et* al, 2019 (Natarajan et al., 2015; Ramesh and Brockmann, 2019; Rangiah and Palakodeti, 2013). 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. (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^{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 3.5). 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 to the standard solution tubes. 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. 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 3.2,3.33.4,3.5 Fig 3.2 and A.1.

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.

Table 3.2: Instrument method details for liquid chromatography (LC).

Instrument	Agilent 1290 Infinity UHPLC
Column	Waters Acquity HSS C18, 1.8u, 100mmx2.1mm
Mobile Phase A	10mM Ammonium Acetate in Water (0.1%FA)
Mobile Phase B	Acetonitrile (0.1%FA)
Flow Rate	0.2ml/min
Column Oven	40°C
Auto-sampler Temp.	10°C
Injection Volume	10ul
Run Time	35mins
Gradient	0-3mins:2%B, 3-20mins:2-20%B, 20-25mins:35%B, 25.1mins:80%B, 27mins:80%B, 27.1-35:2%B

Table 3.3: Instrument method details for mass spectrometry (MS).

Instrument	Thermo Fisher- TSQ Vantage
Spray Voltage (+ve)	3700V
Vaporizer temp	80°C
Sheath gas flow rate	20Arb
Aux gas flow rate	10Arb
Peak width settings	0.70FWHM
Scan time	0.05s
Injector settings	0-3mins: waste, 3-28.5mins: load, 28.5-35mins: waste

Table 3.4: Internal standard molecular weights (MW) and expected retention times (RT) for the 14 neurotransmitters and their internal standards used for quantification in mass spectrometry.

compound	MW	RT
Histidine-D3	329	7.53
Histidine	326.11	7.56
serine-D3	279.2	8.53
Serine	276.1	8.59
Histamine-D4	286.37	9.33
Histamine	282.37	9.36
Aspartate-D3	307.1	9.62
Aspartate	304.1	9.68
Glutamate-D5	323.26	10.48
Glutamate	318.1	10.54
GABA-D6	280.32	12.96
GABA	274.28	13.06
Dopa-D3	371.37	15.29
Dopa	368.35	15.33
Octopamine-D3	327.17	15.44
Octopamine	324.17	15.5
Tyrosine-D7	359.4	17.25
Tyrosine	352.2	17.35
Dopamine-D4	328.37	17.85
Dopamine	324.3	17.94
Tyramine-D4	312.36	20.18
Tyramine	308.36	20.37
Serotonin-D4	351.4	22.03
Serotonin	347.38	22.11
Tryptophan-D5	380.43	23.83
Tryptophan	375.19	23.91
Tryptamine-D4	335.4	26.19
Tryptamine	331.38	26.25

Table 3.5: Standard solution preparation concentrations for all neurotransmitters. *

	Standards			Internal standards
Compound	Pre diapause	Post diapause	Adult fly	ISTD
name	on column (ng)	on column (ng)	on column (ng)	on column (ng)
Histidine	20	50	50	1
Serine	8	80	80	1
Histamine	3.2	3.2	3.2	1
Aspartate	25	250	250	1
Glutamate	25	250	250	1
GABA	10	100	100	1
Dopa	0.16	0.16	0.1	1
Octopamine	1	1	1	1
Tyrosine	4	40	40	1
Dopamine	1	1	1	1
Tyramine	0.32	0.32	0.64	1
Serotonin	1	1	1.6	1
Tryptophan	1	8	8	1
Tryptamine	1	1	0.16	1

*The 14 neurotransmitters were weighed individually and standard stock solutions of 1mg/ml in 0.1N HCL were prepared. The final concentration for each chemical for each experiment was calculated by running a preliminary experiment with calibration curves used for honey bee whole brain as in Ramesh et al 2019 and three replicates of apple/ hawthorn brain samples. The internal standard (ISTD) stock solution of 1mg/ml in 0.1NHCL was also prepared.

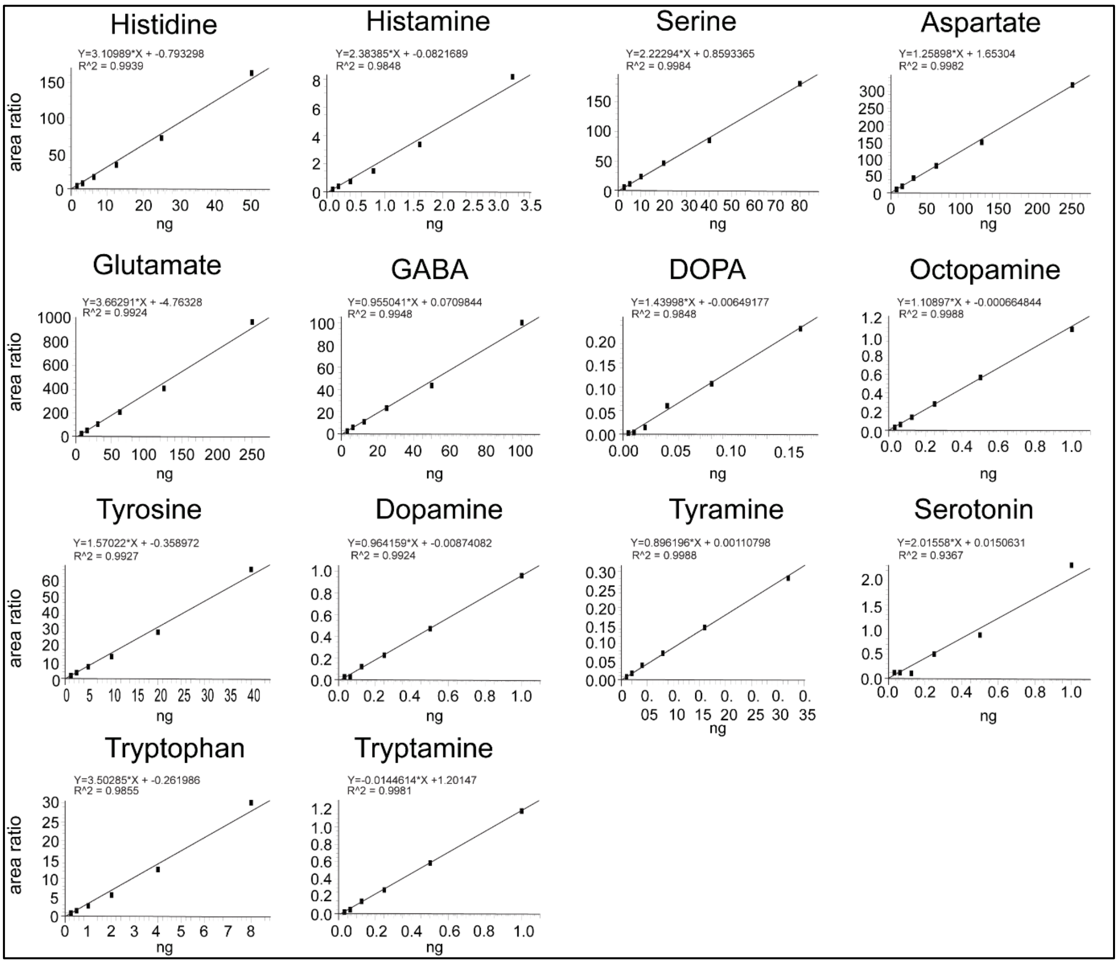


Figure 3.2: Sample calibration curves for one of the LC-MS runs showing all 14 neurotransmitters analysed.

Statistical Analysis:

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, I 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 estimates. 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 effect 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. 3) The interaction between host race and days on amount of precursor neurochemicals at brain development stage 4 was done using, generalised linear model, Univariate analysis of variance 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 0.05.

3.3 Results:

Rhagoletis pomonella host races exhibit distinct brain development stages during pupation

I 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 3.3 and 3.4). 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 3.3, 3.1 and 3.4). The pre-winter diapause initiation stage 1, (8 days after puparium formation, Methods & Figure 3.1, 3.4 apple $\mathbf{n}_{\textbf{dissections}}$ = 75, $\mathbf{n}_{\text{staining}}$ = 7, hawthorn $\mathbf{n}_{\textbf{dissections}}$ = 65, $\mathbf{n}_{\text{staining}}$ = 8), and the pre-winter diapause maintenance stage 2, (20 days after puparium formation, Figure 3.1, 3.3, 3.4 apple $\mathbf{n}_{\textbf{dissections}}$ = 65, $\mathbf{n}_{\text{staining}}$ = 7, hawthorn $\mathbf{n}_{\textbf{dissections}}$ = 75, $\mathbf{n}_{\text{staining}}$ = 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 3.1, (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 3.4, A). After a six-month artificial overwintering period at 4-5^{o}C, we observed pandiculation with substantial neural differentiation and remodelling had occurred compared to pre-winter diapausing brains (stage 3, Figure 3.4, B, apple $\mathbf{n}_{\textbf{dissections}}$ = 134, $\mathbf{n}_{\text{staining}}$ = 2, hawthorn $\mathbf{n}_{\textbf{dissections}}$ = 171, $\mathbf{n}_{\text{staining}}$ = 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 3.4, A, B).

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, Figure 3.4, C, apple $\mathbf{n}_{\mathbf{dissections}}$ = 70, $\mathbf{n}_{\text{staining}}$ = 4, hawthorn $\mathbf{n}_{\mathbf{dissections}}$ = 60, $\mathbf{n}_{\text{staining}}$ = 1). At this stage 4, brain region boundaries were apparent, but the regions themselves remained undefined (Figure 3.4, C). 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 3.4, apple $\mathbf{n}_{\mathbf{dissections}}$ = 43, $\mathbf{n}_{\text{staining}}$ =1, hawthorn $\mathbf{n}_{\mathbf{dissections}}$ = 35, $\mathbf{n}_{\text{staining}}$ = 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, apple $\mathbf{n}_{\mathbf{dissections}}$ = 20, $\mathbf{n}_{\text{staining}}$ = 1, hawthorn $\mathbf{n}_{\mathbf{dissections}}$ = 41, $\mathbf{n}_{\text{staining}}$ = 1; stage 7, apple race $\mathbf{n}_{\mathbf{dissections}}$ = 45, hawthorn race $\mathbf{n}_{\mathbf{dissections}}$ = 50 Movie 7, and stage 8 apple race $\mathbf{n}_{\mathbf{dissections}}$ = 45, hawthorn race $\mathbf{n}_{\mathbf{dissections}}$ = 45, hawthorn $\mathbf{n}_{\text{staining}}$ = 1 Figure 3.4, D).

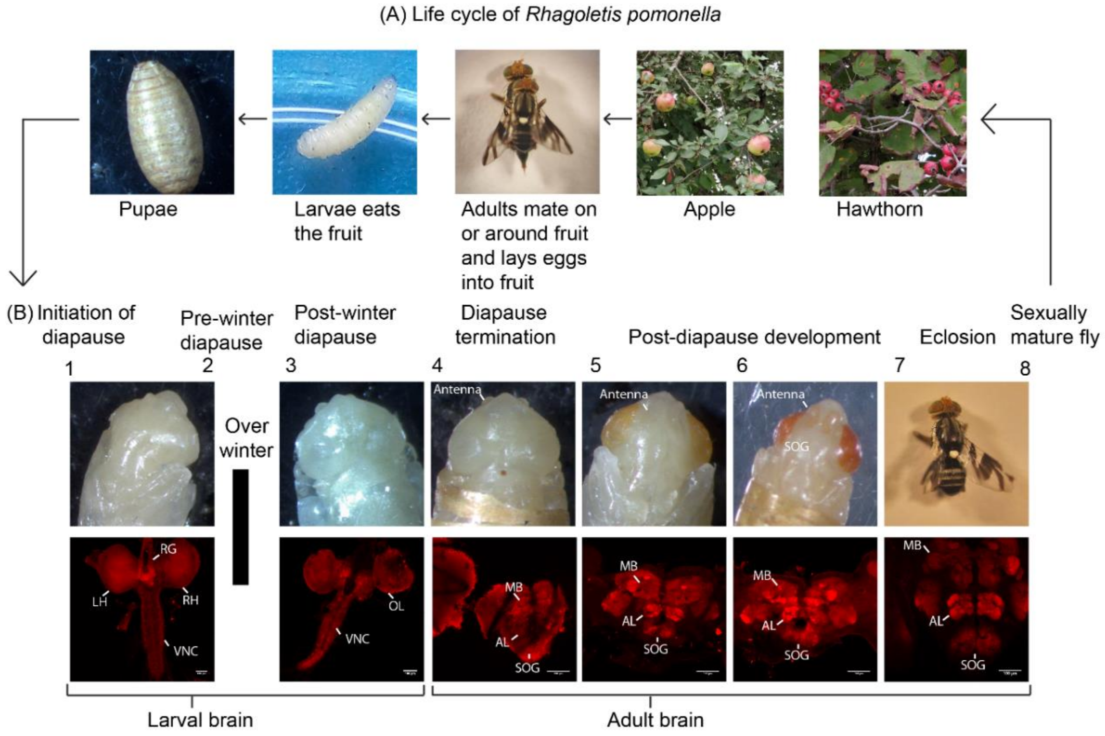


Figure 3.3: The life cycle of *Rhagoletis pomonella.* (A) Life stages of the fly from mating to oviposition from right to left. (B) Micrographic images of pupal, pharate-adult, and adult developmental stages from left to right. The upper stereomicrographs in the bottom panels show the head morphology 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. My 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 3.1, 3.4, apple race, stage 1); **2**, pre-winter diapause induction stage when pupae have entered metabolic depression (Figure 3.1, 3.3,3.4, hawthorn race, stage 2); **3**, post-winter diapause maintenance phase (Figure 3.3, 3.4, apple race, stage 3); **4**, end of diapause (Figure 3.4, hawthorn race, stage 4); **5**, midway through pharate-adult development (Figure 3.4, apple race, stage 5); **6**, late pharate-adult development (Figure 3.4, apple race, stage 6); **7**, sexually immature adult fly, less than 7 days old (Figure 3.4, hawthorn race, stage 7); **8**, sexually mature fly, more than 12 days old; The anatomical brain regions are identified as left and right brain hemispheres (LH and RH), ring gland (RG), optic lobe (OL), ventral nerve code (VNC), the antennal lobes (AL), mushroom bodies (MB), and suboesophageal ganglion (SOG).

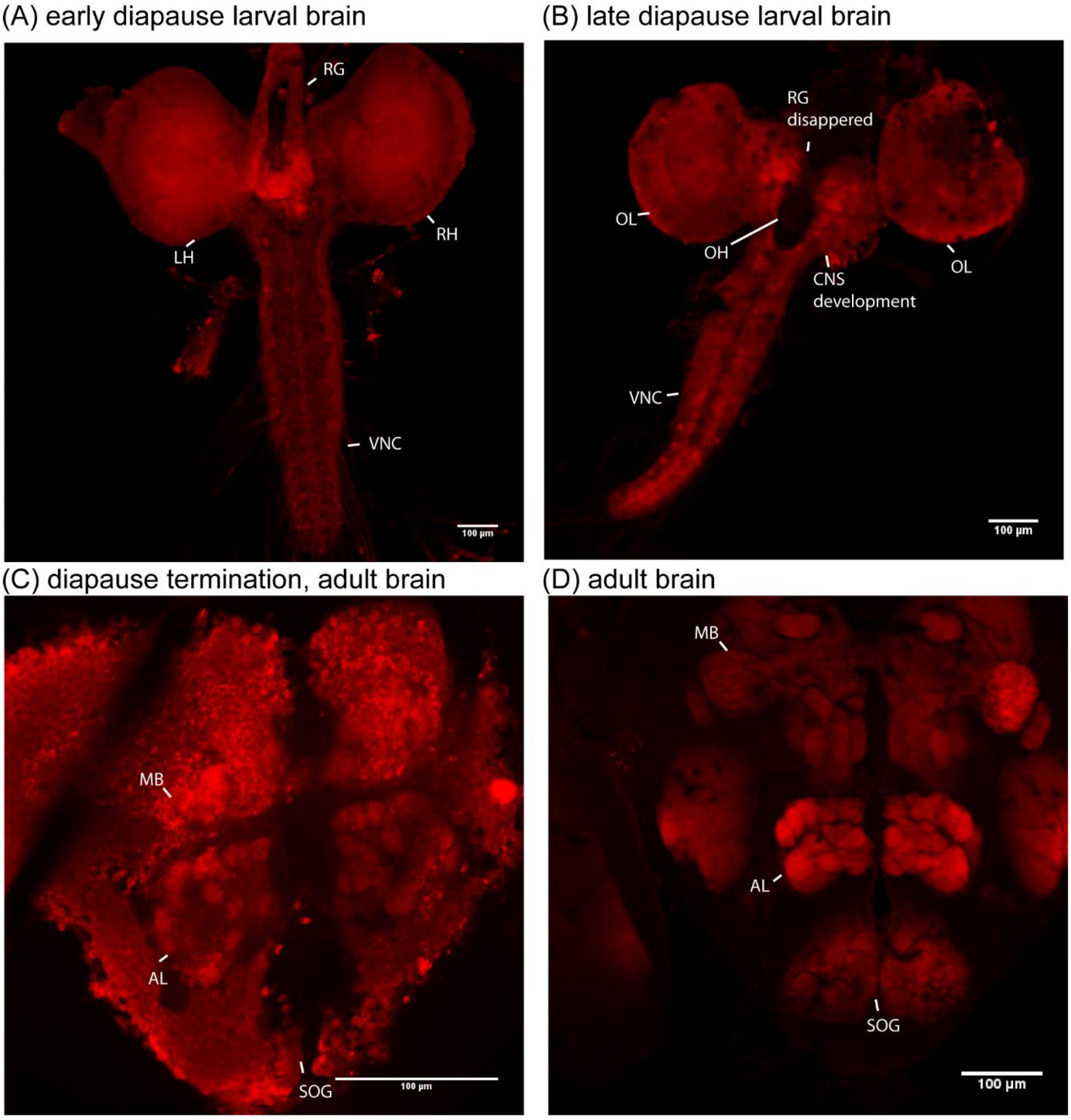


Figure 3.4: Pupal brain morphology across developmental stages. Confocal micrographs (1 µm depth) showing nc82 (red) staining of pupal brains at different stages. These are the same confocal images represented in Figure 3.3. The four images provide (A) an overview of the larval brain at diapause initiation (stage1, Figure 3.3) with two, left and right brain hemispheres (LH and RH), the ring gland (RG) and ventral nerve code (VNC), (B) the intermediate developing pupal brain at late diapause (stage 3) with clear formation of the optic lobe (OL), disappearance of the ring gland, oesophageal hole (OH) with CNS development, and VNC, (C) early adult brain (stage 4) following diapause termination with the first appearance of the boundaries of brain region like the antennal lobes (AL), mushroom bodies (MB), and suboesophageal ganglion (SOG), and (D) the adult brain (stage 7) with completely developed CNS after adult eclosion.

Apple race pupae exhibit more rapid onset of adult brain morphogenesis 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°C) after 6 months of simulated overwintering (4-5°C) synchronized to their respective diapause initiation timing (Figure 3.5 A, B and Tables 3.6 and 3.7). By sampling a subset of pupae every 5 days from day 0 to day 70 after removal from artificial overwintering conditions, I observed that both host races exhibited onset of adult neurogenesis during the post-winter phases, as shown in Figure 3.3. In the apple race, a substantial number of pupae exhibited adult brain morphology (Stage 4-6 in Figure 3.3) 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 3.5 A, B and Tables 3.6 and 3.7). Logistic regression analysis (Figure 3.5 C) showed that apple race individuals began initiating adult brain development significantly more rapid 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 faster than diapausing hawthorn race pupae, with 18.34 ($\underline{+}$0.56-0.42, 95%CI) days for 50% pupae exhibiting adult brains in apple vs. 42.10 ($\underline{+}$0.61-0.42, 95%CI) days for hawthorn (Figure 3.5). Therefore, even though the artificial overwintering period was of the same duration for both the races, the transition from pupal to adult brain development was significantly more rapid 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 post winter to the initiation of adult brain morphogenesis has become more rapid.

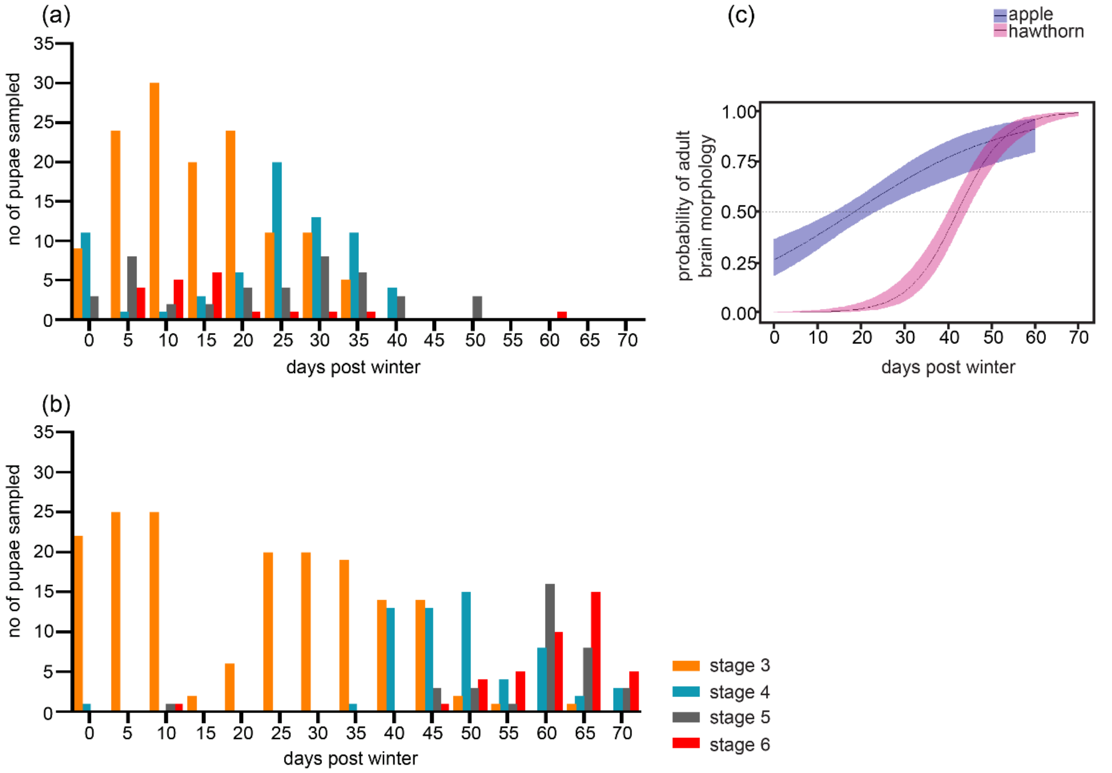


Figure 3.5: Post-winter brain development over time between the two host races. Brains were dissected from pupae and assessed every 5 days after simulated overwintering. A) Apple race, stages 3, $\mathbf{n}_{\text{dissections}}$ = 134; stage 4, $\mathbf{n}_{\text{dissections}}$ = 70; stage 5, $\mathbf{n}_{\text{dissections}}$ = 43; stage 6, $\mathbf{n}_{\text{dissections}}$ = 20; B) Hawthorn race, stages 3, $\mathbf{n}_{\text{dissections}}$ = 171; stage 4, $\mathbf{n}_{\text{dissections}}$ = 60; stage 5, $\mathbf{n}_{\text{dissections}}$ = 35; stage 6, $\mathbf{n}_{\text{dissections}}$ = 41; 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.

Table 3.6: Total number of pupae sampled after winter in the apple race

day	total pupae	stage 3	stage 4	stage 5	stage 6	dead pupae	dead fly	wasp larvae	adult wasp
D 0	27	9	11	3	0	3	0	0	1
D 5	46	24	1	8	4	5	0	2	2
D 10	46	30	1	2	5	8	0	0	0
D 15	70	20	3	2	6	38	0	1	0
D 20	65	24	6	4	1	29	0	1	0
D 25	60	11	20	4	1	21	0	3	0
D 30	37	11	13	8	1	0	3	0	1
D 35	37	5	11	6	1	10	1	2	1
D 40	10	0	4	3	0	3	0	0	0
D 45	0	0	0	0	0	0	0	0	0
D 50	6	0	0	3	0	0	3	0	0
D 55	0	0	0	0	0	0	0	0	0
D 60	3	0	0	0	1	0	1	1	0
D 65	0	0	0	0	0	0	0	0	0
D 70	0	0	0	0	0	0	0	0	0
total	407	134	70	43	20	117	8	10	5

Table 3.7: Total number of pupae sampled after winter in the hawthorn race

day	total pupae	stage 3	stage 4	stage 5	stage 6	dead pupae	dead fly	wasp larvae	adult wasp
D 0	26	22	1	0	0	0	0	2	1
D 5	28	25	0	0	0	1	0	2	0
D 10	56	25	0	1	1	0	3	15	11
D 15	10	2	0	0	0	0	0	1	7
D 20	9	6	0	0	0	0	0	0	3
D 25	50	20	0	0	0	2	6	9	13
D 30	41	20	0	0	0	3	2	7	9
D 35	30	19	1	0	0	2	1	3	4
D 40	55	14	13	0	0	14	3	4	7
D 45	78	14	13	3	1	18	9	14	6
D 50	74	2	15	3	4	16	4	16	14
D 55	31	1	4	1	5	4	1	7	8
D 60	51	0	8	16	10	1	5	6	5
D 65	47	1	2	8	15	8	3	10	0
D 70	19	0	3	3	5	1	4	3	0
total	605	171	60	35	41	70	41	99	88

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

I next examined a total of 14 neurochemicals across 6 biochemical pathways in the developing brains of both hawthorn and apple race of *R. pomonella.* These neurochemicals included both precursor molecules and their products (Figures, 3.6, A.2, A.3, A.4). I 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, only four chemicals showed higher titres in the apple race, (dopamine, stage 5, p= 0.0001; serotonin, stage 6, p= 0.0001; serine, stage 6, p= 0.009, table 3.8). For all other molecules, the hawthorn race exhibited higher titres than the apple race. One exception is tyramine which showed higher titres in the hawthorn race at the pupal stages and higher tires at the sexually mature adult stage in the apple race (tyramine, stage 4, p= 0.0001; stage 6, p= 0.039; stage 8, p= 0.0114, table 3.8). 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. 3.3), 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 $\mathbf{n_{samples}}$ =10, $\mathbf{n_{dissections}}$ = 50; hawthorn race $\mathbf{n_{samples}}$ =10, $\mathbf{n_{dissections}}$ = 50; histidine, p = 0.005; stage 8, apple race $\mathbf{n_{samples}}$ =10, $\mathbf{n_{dissections}}$ = 50; hawthorn race $\mathbf{n_{samples}}$ =10, $\mathbf{n_{dissections}}$ = 50; tyramine, p= 0.011; octopamine, p= 0.011; histamine, p= 0.020, table 3.8).

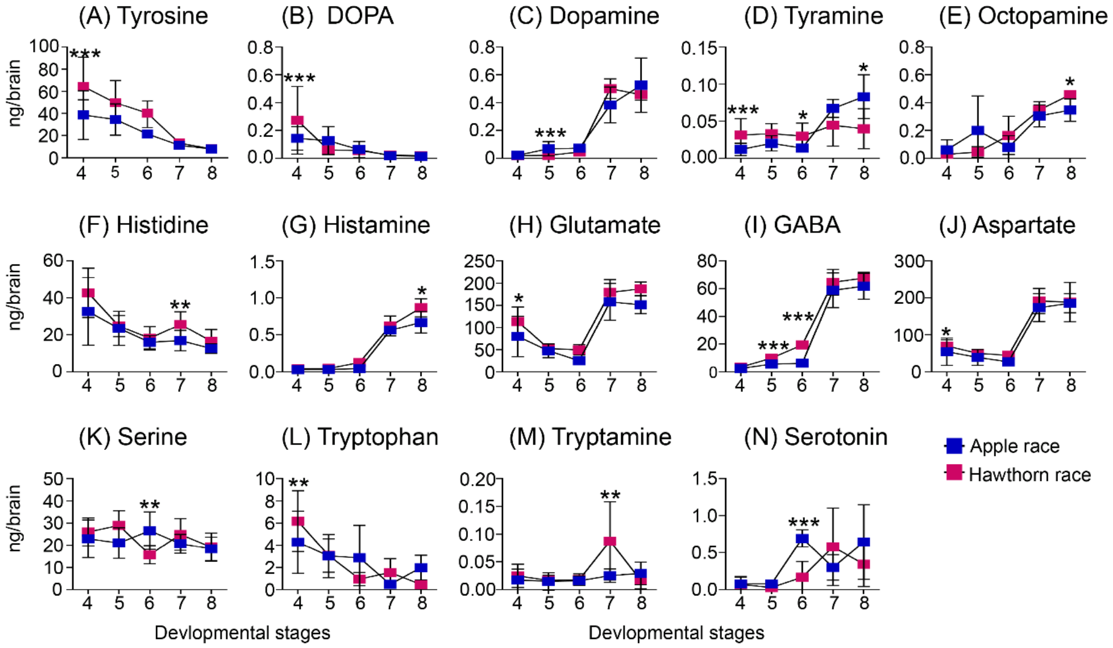


Figure 3.6: Quantification of biogenic amines and their precursors from the onset of adult brain development (stage 4) all the way up to sexually mature fly (stage 8) as defined in Figure 3.3 (A-N). Line graph of neurochemical titres for both host races at different developmental stages with 4-15 $\mathbf{n}_{\mathbf{samples}}$ per stage, containing a pool of five brains in each sample, symbols represent mean with SE: 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.

Table 3.8: Between host race developmental stage comparisons for each neurotransmitter. Generalised linear mixed models, Tukey's HSD tests was performed for pairwise multiple comparisons with a significance cut off of 0.0001***, 0.001**, or 0.05* as indicated.

Histidine	estimate	SE	df	t. ratio	p.value	
Apple stage 1 - Hawthorn stage 1	-1.324	3.40	151.0	-0.38	1.0000	
Apple stage 2 - Hawthorn stage 2	-4.229	3.34	150.2	-1.26	0.9823	
Apple stage 3 - Hawthorn stage 3	2.757	2.29	152.9	1.20	0.9883	
Apple stage 4 - Hawthorn stage 4	-9.151	3.50	154.1	-2.61	0.2835	
Apple stage 5 - Hawthorn stage 5	-1.358	4.70	153.6	-0.28	1.0000	
Apple stage 6 - Hawthorn stage 6	-1.494	5.41	157.3	-0.27	1.0000	
Apple stage 7 - Hawthorn stage 7	-7.165	2.00	34.5	-3.57	0.0055	**
Apple stage 8 - Hawthorn stage 8	-1.529	2.06	35.34	-0.74	0.8794	

Serine	estimate	SE	df	t. ratio	p.value	
Apple stage 1 - Hawthorn stage 1	-4.348	1.854	138.3	-2.34	0.4502	
Apple stage 2 - Hawthorn stage 2	-1.866	1.964	140.9	-0.95	0.9984	
Apple stage 3 - Hawthorn stage 3	2.041	1.281	139.9	1.59	0.9087	
Apple stage 4 - Hawthorn stage 4	-2.241	1.962	141.3	-1.14	0.9921	
Apple stage 5 - Hawthorn stage 5	-7.339	2.636	141.0	-2.78	0.1984	
Apple stage 6 - Hawthorn stage 6	11.687	3.035	146.8	3.85	0.0093	**
Apple stage 7 - Hawthorn stage 7	-2.288	2.129	33.54	-1.07	0.7069	
Apple stage 8 - Hawthorn stage 8	1.446	2.227	34.27	0.64	0.9151	

Histamine	estimate	SE	df	t. ratio	p.value	
Apple stage 1 - Hawthorn stage 1	0.002	0.005	123.6	0.45	1.0000	
Apple stage 2 - Hawthorn stage 2	0.007	0.005	122.3	1.56	0.9186	
Apple stage 3 - Hawthorn stage 3	0.001	0.003	125.6	0.27	1.0000	
Apple stage 4 - Hawthorn stage 4	-0.011	0.004	123.2	-2.49	0.3553	
Apple stage 5 - Hawthorn stage 5	-0.009	0.006	124.1	-1.48	0.9409	
Apple stage 6 - Hawthorn stage 6	-0.077	0.007	124.0	-11.5	0.0001	***
Apple stage 7 - Hawthorn stage 7	-0.023	0.048	33.15	-0.48	0.9617	
Apple stage 8 - Hawthorn stage 8	-0.154	0.050	35.55	-3.07	0.0201	*

Aspartate	estimate	SE	df	t. ratio	p.value	
Apple stage 1 - Hawthorn stage 1	-3.695	3.992	150.1	-0.92	0.9988	
Apple stage 2 - Hawthorn stage 2	-0.847	3.765	148.7	-0.22	1.0000	
Apple stage 3 - Hawthorn stage 3	2.891	2.643	149.8	1.09	0.9945	
Apple stage 4 - Hawthorn stage 4	-14.201	3.969	150.4	-3.57	0.0228	*
Apple stage 5 - Hawthorn stage 5	-4.448	5.270	149.2	-0.84	0.9995	
Apple stage 6 - Hawthorn stage 6	-11.889	6.189	150.9	-1.92	0.7444	

Apple stage 7 - Hawthorn stage 7	-8.640	12.07	34.01	-0.71	0.8902	
Apple stage 8 - Hawthorn stage 8	9.157	12.47	34.48	0.73	0.8826	

Glutamate	estimate	SE	df	t. ratio	p.value	
Apple stage 1 - Hawthorn stage 1	-15.347	7.376	150.9	-2.08	0.6371	
Apple stage 2 - Hawthorn stage 2	-11.188	7.504	150.0	-1.49	0.9410	
Apple stage 3 - Hawthorn stage 3	2.563	4.863	150.9	0.52	1.0000	
Apple stage 4 - Hawthorn stage 4	-30.956	7.270	152.3	-4.25	0.0021	**
Apple stage 5 - Hawthorn stage 5	-5.329	9.593	151.8	-0.55	1.0000	
Apple stage 6 - Hawthorn stage 6	-24.041	11.50	153.2	-2.09	0.6303	
Apple stage 7 - Hawthorn stage 7	-15.494	10.32	33.08	-1.50	0.4481	
Apple stage 8 - Hawthorn stage 8	-21.453	11.40	34.66	-1.88	0.2550	

GABA	estimate	SE	df	t. ratio	p.value	
Apple stage 1 - Hawthorn stage 1	-0.323	0.663	149.8	-0.48	1.0000	
Apple stage 2 - Hawthorn stage 2	0.510	0.659	146.8	0.77	0.9998	
Apple stage 3 - Hawthorn stage 3	-0.828	0.431	147.5	-1.92	0.7444	
Apple stage 4 - Hawthorn stage 4	-0.651	0.666	153.4	-0.97	0.9980	
Apple stage 5 - Hawthorn stage 5	-4.357	0.887	150.7	-4.91	0.0001	***
Apple stage 6 - Hawthorn stage 6	-13.474	1.148	151.4	-11.7	0.0000	***
Apple stage 7 - Hawthorn stage 7	-3.480	3.118	32.82	-1.11	0.6822	
Apple stage 8 - Hawthorn stage 8	-0.453	3.466	34.21	-0.13	0.9992	

Dopa	estimate	SE	df	t. ratio	p.value	
Apple stage 1 - Hawthorn stage 1	-0.031	0.026	149.9	-1.18	0.9896	
Apple stage 2 - Hawthorn stage 2	0.007	0.027	151.0	0.24	1.0000	
Apple stage 3 - Hawthorn stage 3	0.036	0.018	150.3	2.00	0.6888	
Apple stage 4 - Hawthorn stage 4	-0.138	0.029	151.3	-4.68	0.0004	***
Apple stage 5 - Hawthorn stage 5	0.059	0.037	151.8	1.60	0.9048	
Apple stage 6 - Hawthorn stage 6	0.008	0.048	153.2	0.15	1.0000	
Apple stage 7 - Hawthorn stage 7	-0.001	0.002	32.46	-0.56	0.9429	
Apple stage 8 - Hawthorn stage 8	0.001	0.002	33.36	0.77	0.8672	

Octopamine	estimate	SE	df	t. ratio	p.value	
Apple stage 1 - Hawthorn stage 1	-0.001	0.048	76.51	-0.01	1.0000	
Apple stage 2 - Hawthorn stage 2	0.000	0.045	78.24	0.00	1.0000	
Apple stage 3 - Hawthorn stage 3	0.026	0.035	83.56	0.73	0.9999	
Apple stage 4 - Hawthorn stage 4	0.056	0.033	80.04	1.68	0.8688	
Apple stage 5 - Hawthorn stage 5	0.166	0.045	83.08	3.66	0.0206	*
Apple stage 6 - Hawthorn stage 6	-0.046	0.051	81.59	-0.89	0.9990	
Apple stage 7 - Hawthorn stage 7	-0.033	0.027	32.55	-1.21	0.6219	
Apple stage 8 - Hawthorn stage 8	-0.101	0.030	34.53	-3.30	0.0113	*

Tyrosine	estimate	SE	df	t.ratio	p.value	
Apple stage 1 - Hawthorn stage 1	-0.827	3.144	143.1	-0.26	1.0000	
Apple stage 2 - Hawthorn stage 2	-0.452	3.558	144.4	-0.12	1.0000	
Apple stage 3 - Hawthorn stage 3	2.007	2.284	150.3	0.87	0.9992	
Apple stage 4 - Hawthorn stage 4	-29.120	3.396	151.2	-8.57	0.0000	***
Apple stage 5 - Hawthorn stage 5	-13.221	4.473	145.9	-2.95	0.1329	
Apple stage 6 - Hawthorn stage 6	-18.429	5.225	152.3	-3.52	0.0267	
Apple stage 7 - Hawthorn stage 7	-1.309	1.004	33.56	-1.30	0.5674	
Apple stage 8 - Hawthorn stage 8	0.694	1.049	34.44	0.66	0.9109	

Dopamine	estimate	SE	df	t.ratio	p.value	
Apple stage 1 - Hawthorn stage 1	0.010	0.006	128.4	1.52	0.9304	
Apple stage 2 - Hawthorn stage 2	-0.011	0.007	131.8	-1.67	0.8778	
Apple stage 3 - Hawthorn stage 3	0.003	0.005	138.6	0.57	1.0000	
Apple stage 4 - Hawthorn stage 4	0.002	0.007	131.5	0.31	1.0000	
Apple stage 5 - Hawthorn stage 5	0.044	0.009	134.1	4.92	0.0001	***
Apple stage 6 - Hawthorn stage 6	0.021	0.011	135.6	1.96	0.7168	
Apple stage 7 - Hawthorn stage 7	-0.083	0.053	31.29	-1.56	0.4102	
Apple stage 8 - Hawthorn stage 8	0.093	0.051	31.19	1.82	0.2823	

Tyramine	estimate	SE	df	t.ratio	p.value	
Apple stage 1 - Hawthorn stage 1	0.002	0.003	137.0	0.56	1.0000	
Apple stage 2 - Hawthorn stage 2	0.005	0.003	134.6	1.53	0.9277	
Apple stage 3 - Hawthorn stage 3	0.001	0.002	135.1	0.48	1.0000	
Apple stage 4 - Hawthorn stage 4	-0.017	0.003	136.4	-6.10	0.0001	***
Apple stage 5 - Hawthorn stage 5	-0.007	0.004	136.8	-1.85	0.7850	
Apple stage 6 - Hawthorn stage 6	-0.015	0.005	137.4	-3.40	0.0393	*
Apple stage 7 - Hawthorn stage 7	0.013	0.011	31.29	1.15	0.6577	
Apple stage 8 - Hawthorn stage 8	0.036	0.011	32.09	3.32	0.0114	*

Serotonine	estimate	SE	df	t.ratio	p.value	
Apple stage 1 - Hawthorn stage 1	0.001	0.028	89.59	0.03	1.0000	
Apple stage 2 - Hawthorn stage 2	0.008	0.029	85.70	0.29	1.0000	
Apple stage 3 - Hawthorn stage 3	-0.013	0.022	87.66	-0.61	1.0000	
Apple stage 4 - Hawthorn stage 4	0.047	0.042	94.96	1.12	0.9930	
Apple stage 5 - Hawthorn stage 5	0.066	0.046	88.54	1.44	0.9519	
Apple stage 6 - Hawthorn stage 6	0.453	0.046	91.09	9.79	0.0001	***
Apple stage 7 - Hawthorn stage 7	-0.103	0.171	24.54	-0.60	0.9299	
Apple stage 8 - Hawthorn stage 8	0.330	0.170	26.85	1.94	0.2352	

Tryptophan	estimate	SE	df	t.ratio	p.value	
Apple stage 1 - Hawthorn stage 1	-0.168	0.369	153.5	-0.45	1.0000	
Apple stage 2 - Hawthorn stage 2	-0.100	0.366	153.1	-0.27	1.0000	

Apple stage 3 - Hawthorn stage 3	0.424	0.241	153.2	1.75	0.8378	
Apple stage 4 - Hawthorn stage 4	-1.585	0.380	153.9	-4.17	0.0028	**
Apple stage 5 - Hawthorn stage 5	0.096	0.491	153.9	0.19	1.0000	
Apple stage 6 - Hawthorn stage 6	1.123	0.601	154.2	1.86	0.7770	
Apple stage 7 - Hawthorn stage 7	-1.065	0.640	22.00	-1.66	0.3651	
Apple stage 8 - Hawthorn stage 8	1.524	0.662	22.00	2.30	0.1283	
Tryptamine	estimate	SE	df	t. ratio	p.value	
Apple stage 1 - Hawthorn stage 1	-0.016	0.013	117.2	-1.21	0.9872	
Apple stage 2 - Hawthorn stage 2	0.007	0.012	116.3	0.64	1.0000	
Apple stage 3 - Hawthorn stage 3	0.000	0.008	115.6	0.04	1.0000	
Apple stage 4 - Hawthorn stage 4	-0.005	0.010	117.6	-0.47	1.0000	
Apple stage 5 - Hawthorn stage 5	0.004	0.014	115.5	0.29	1.0000	
Apple stage 6 - Hawthorn stage 6	0.007	0.015	118.8	0.48	1.0000	
Apple stage 7 - Hawthorn stage 7	-0.057	0.017	33.83	-3.38	0.0094	**
Apple stage 8 - Hawthorn stage 8	0.023	0.018	36.00	1.28	0.5786	

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

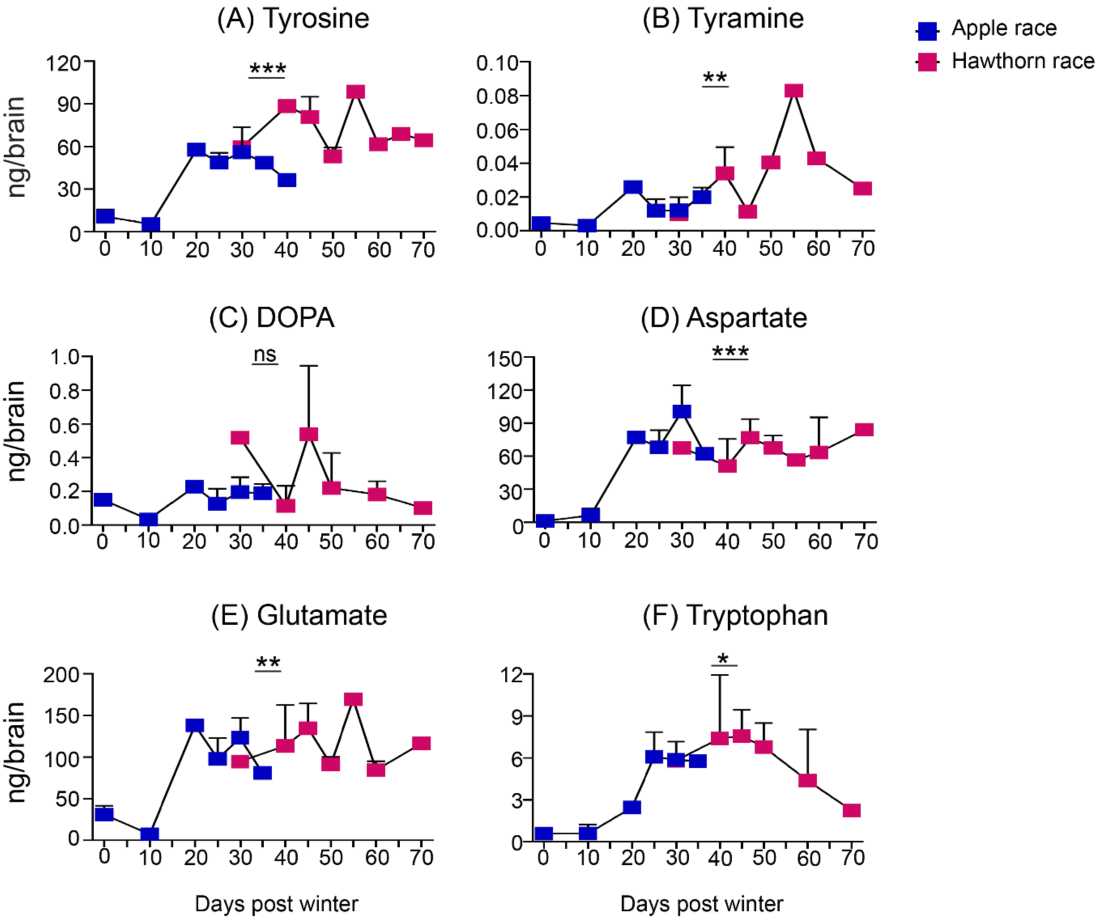


Figure 3.7: Quantification of precursor neurochemicals during onset of adult brain morphogenesis. (A-F) Line graph of neurochemical titres for both host races at different developmental stage 4 with 1-4 $n_{samples}$ per time point, containing a pool of five brains in each sample, symbols represent mean with SE: 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 the apple race, the rate of adult brain development was significantly more rapid than the hawthorn race (figure 3.5), and also exhibited lower titres of several neuromodulators. I therefore assessed whether the rate of development corresponded to lower levels of neuromodulators, particularly at the first stage of adult brain development (stage 4). My results show that six precursor molecules including tyrosine, tyramine, DOPA, aspartate, glutamate and tryptophan from four different biosynthetic pathways showed significant differences in titre between the host races at the first appearance of the adult brain during pupal-pharate adult metamorphic development, stage 4 in figure 3.3 (Figure 3.6, A.2, A.3, A.4; stage 4, apple race $\mathbf{n_{samples}}$ = 14, $\mathbf{n_{dissections}}$ = 70; hawthorn race $\mathbf{n_{samples}}$ = 11, $\mathbf{n_{dissections}}$ = 55; 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 with the apple race.

To compare how these precursor neurochemicals titres changed over time between the developing hawthorn and apple race pupae, I compared neurochemical titres of Stage 4 brains against the day they were sampled after winter. A univariate analysis of variance, UNIANOVA was conducted to examine the effects of host race and days after winter on the titres of these six precursor neurochemicals. Except for the neurochemical DOPA, there was a significant interaction between host race and day for each neurochemical titre (Figure 3.7, table 3.9, tyrosine, F12,10 = 10.9, p = 0.0001; tyramine, F12,10 = 4.95, p = 0.008; DOPA, F12,7= 0.82, p = 0.632; aspartate, F12,11 = 10.1, p = 0.0001; glutamate, F12,11 = 6.47, p = 0.002; tryptophan, F12,10 =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 3.10, tyrosine, F8,9 =38.14, p = 0.0001; tyramine, F7,10= 4.46, p =0.017; DOPA, F7,8 = 0.84, p = 0.581; aspartate, F8,10=14.25, p = 0.0001; glutamate, F8,9 =25.36, p = 0.0001; tryptophan, F7,10= 7.19, p = 0.003). As a result, more rapidly developing brains in apple race pupae exhibited significantly lower titres of neuromodulators (especially biogenic amine precursor molecules) than later developing hawthorn race brains, and these differences were also reflected in product molecules at the adult fly stage at which host preference is exhibited (compare stages 4 and 8 in figure 3.6, A.2, A.3, A.4) (Nojima et al., 2003a; Zhang et al., 1999).

Table 3.9: Two-way ANOVA result table showing host race comparisons at stage 4 for selected neurotransmitters.

Tyrosine	df	F	P	error
Hostrace	1	13.231	0.005	
Days	12	4.936	0.008	
Hostrace*Days	12	10.978	0.0001	10
Tyramine	df	F	P	error
Hostrace	1	9.705	0.011	
Days	12	3.153	0.039	
Hostrace*Days	12	4.957	0.008	10
DOPA	df	F	P	error
Hostrace	1	0.649	0.447	
Days	12	0.639	0.764	
Hostrace*Days	12	0.828	0.632	7
Aspartate	df	F	P	error
Hostrace	1	2.082	0.177	
Days	12	4.118	0.013	
Hostrace*Days	12	10.129	0.0001	11
Glutamate	df	F	P	error
Hostrace	1	2.476	0.144	
Days	12	2.918	0.043	
Hostrace*Days	12	6.479	0.002	11
Tryptophan	df	F	P	error
Hostrace	1	1.517	0.246	
Days	12	2.285	0.1	
Hostrace*Days	12	4.359	0.013	10

Table 3.10: Two-way ANOVA result table showing host race comparisons at stage 4 for selected neurotransmitters after removing early time point till day20.

Tyrosine	df	F	P	error
Hostrace	1	67.113	0.0001	
Days	8	5.786	0.008	
Hostrace*Days	8	38.149	0.0001	9
Tyramine	df	F	P	error
Hostrace	1	9.261	0.012	
Days	8	2.052	0.142	
Hostrace*Days	7	4.463	0.017	10
DOPA	df	F	P	error
Hostrace	1	0.4	0.545	
Days	8	0.198	0.983	
Hostrace*Days	7	0.846	0.581	8
Aspartate	df	F	P	error
Hostrace	1	5.087	0.048	
Days	8	0.863	0.574	
Hostrace*Days	8	14.25	0.0001	10
Glutamate	df	F	P	error
Hostrace	1	26.94	0.001	
Days	8	4.382	0.02	
Hostrace*Days	8	25.367	0.0001	9
Tryptophan	df	F	P	error
Hostrace	1	1.272	0.286	
Days	8	1.716	0.209	
Hostrace*Days	7	7.193	0.003	10

3.4 Discussion:

In this study, I 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 in the adult fly. These two closely related populations differ in both life history timing and adult host preference, 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, I 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. In particular, pre-diapause individuals were identical in both host race and neurochemical titres (stages 1 and 2). This indicates that any difference in neuromodulation between the host races occurs only after diapause, which is 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). When I 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 3.5B). In contrast, some apple race pupae were already progressing to stage 4 pharate adult development as soon as they were removed from overwintering (Figure 3.5B). This finding indicates not only has the apple race shifted its entire life cycle to coincide with fruit phenology, but adult brain morphogenesis itself occurs roughly three weeks faster in the apple race as compared to the hawthorn race even when the overwintering period is synchronised between the host races. This change in adult brain morphogenesis is not necessarily predicted from its overall host shift and suggests a unique phenotype in the derived apple host race.

To better understand how neurochemical signaling could be associated with the different stages of development in *R. pomonella*, I examined six major biosynthetic pathways known to impact brain development as well as behaviour in insects

(Monastirioti, 1999; Ramesh and Brockmann, 2019). My results indicate that the titres of 11 out of 14 neurochemicals were significantly reduced in the apple race pupae across multiple developmental stages as compared to the hawthorn race (Figure 3.6, e.g., tyrosine, tyramine, octopamine, DOPA, histidine, histamine, aspartate, glutamate, GABA, tryptophan and tryptamine). Further analysis showed that these lower titres also corresponded with earlier development of the adult brain in the apple race post diapause (Figure 3.7, e.g., tyrosine, tyramine, aspartate, glutamate, tryptophan).

Our main goal of this study was to link life history timing, brain development and neurochemistry in the *Rhagoletis* system by identifying particular developmental stages and neurochemicals that differ between the host races. Here, I have identified specific differences in several neuromodulators, particularly the biogenic amine pathways for octopamine and dopamine, at the first appearance of the adult brain (stage 4) and again at sexual maturity in the adult fly, when host preference is exhibited (stage 8). These differences are further coupled with a difference in developmental timing of adult brain morphogenesis between the two races. This data therefore provides specific timepoints and chemical pathways that could be assessed to determine if and how they impact life history and physiology in these populations.

For example, I show that 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. In Chapter 2, I and my colleagues compared olfactory neurophysiology between the apple and hawthorn races of *R. pomonella* and 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). Interestingly, in *Drosophila* cis-regulatory variation in two genes involved in these pathways, tyrosine hydroxylase and dopa decarboxylase (Figure A.2), have been shown to impact neurogenesis (Monastirioti, 1999).

To conclude, I 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. I 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 morphogenesis in this new host race. These differences in neurochemical titre between the races in the developing pupal stages are also apparent in the adult brains at stages when flies are reproductively mature. Because biogenic amines have been implicated to impact both pupal diapause and adult behaviour, this study offers a new hypothesis that could correlate life-history timing and adult host preference through developmental differences in neuromodulation. During onset of diapause in the cabbage armyworm *Mamestra brassicae*, the level of dopamine was also higher in the brain of diapausing pupae compared to non-diapausing pupae (Noguchi and Hayakawa, 1997). Interestingly, non-diapausing apple flies and lab-reared colony apple flies emerge even earlier than diapausing flies. Further studies on these flies can confirm if neurochemical titre corresponds to earlier brain development. 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). In summary, life history timing, neurochemical titre and brain development can be coupled in this speciating system, providing new hypotheses for the origins of new species through differences in neuromodulation during brain development.

Chapter 4

4.1 Introduction

In phytophagous insects, photoperiod and temperature play important roles in diapause regulation (Dambroski and Feder, 2007; Matsumoto and Takeda, 2002). In temperate regions, phytophagous insects like *Rhagoletis* use diapause to avoid the harsh winter season and to match the timing of the availability of their host plant. Biogenic amines and their precursor molecules are known to regulate diapause in many insects in response to changes in abiotic factors like temperature and photoperiod. In the *Rhagoletis* system, temperature is important abiotic factor for diapause termination in diapausing flies as well as for direct development in non-diapausing flies. Interestingly the biogenic amine dopamine plays a role at the onset of pupal diapause in the cabbage armyworm *Mamestra brassicae*, where the level of dopamine regulates whether the pupae will enter diapause or not in respect to changes in day length. (Noguchi and Hayakawa, 1997). The biogenic amines dopamine and serotonin in the cabbage butterfly *Pieris brassicae* are known to regulate diapausing and non-diapausing pupal development in response to longer or shorter photoperiod. During direct development in non-diapausing cabbage butterfly dopamine levels were significantly higher, and in diapausing insects dopamine and serotonin levels were very low (Puiroux et al., 1990). Similarly, in the Chinese oak silk moth, *Antheraea pernyi*, levels of serotonin increased during diapause maintenance phase and manipulation experiments with the D2-like dopamine receptor showed that an agonist of the receptor delayed moth emergence and treatment with an antagonist terminated pupal diapause faster. (Matsumoto and Takeda, 2002; Wang et al., 2015). Finally, in *Drosophila melanogaster*, increased dopamine and serotonin receptor signalling enhanced adult ovarian dormancy, whereas increased octopamine receptors signalling inhibited dormancy (Andreatta et al., 2018).

As mentioned in chapters 1 and 3, both apple and hawthorn races undergo pupal diapause (developmental arrest) during winter, and apple flies initiate and terminate diapause earlier than hawthorn flies to correspond with the earlier fruiting time of apples vs. hawthorn berries. In chapter 3, I investigated the role of neurochemicals in brain development of both apple and hawthorn races. I identified significantly lower neurochemical levels, particularly biogenic amines in the dopamine and octopamine pathways, in the apple race of *R. pomonella*. These lower levels of neuromodulators corresponded to more rapid adult brain morphogenesis in this new host race. The differences in neurochemical titre between the races in the developing pupal stages were also apparent in the adult brains at stages when flies are reproductively mature.

At the pre-winter stage, some *Rhagoletis* pupae are known to skip diapause and develop directly into the adults during the same season females oviposit on the fruit (Feder et al., 1997; Prokopy, 1968b). This second generation of flies is called “non-diapausing” flies. In nature, such flies are considered to be at a severe fitness disadvantage because once they complete their development there are hardly any host fruit available (Dambroski and Feder, 2007). Because biogenic amines have been implicated to impact both pupal diapause and adult behaviour, I wanted to further study their role in the non-diapausing populations. It should be noted that non-diapausing populations like the lab-reared colony of apple flies show similar behavioural responses as diapausing flies (Dambroski et al., 2016; Linn et al., 2003b; Zhang et al., 1999).

Here, I compared early emerging sexually mature non-diapausing wild apple flies and lab-reared colony apple flies to diapausing adult flies from both host races of *Rhagoletis pomonella*. Both non-diapausing apple flies and lab-reared colony apple flies show direct development in warm temperature. My objective was to identify whether non-diapausing wild flies and lab-reared colony apple flies have similar levels of neuromodulators as diapausing apple and hawthorn races. As seen in chapter 3, the early developing apple race had lower levels of neurochemicals in the brain at multiple developmental stages. I therefore hypothesize that non-diapausing flies will also have lower levels of neurochemicals as compared to later developing flies.

4.2 Methods:

Insects:

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 2018, and flies were reared from larvae to adulthood following previously established *Rhagoletis* husbandry methods (Walsh. B, 1867). The eclosed non diapausing flies 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). Note: Only apple race had non-diapausing flies in 2018, from last few years there were no hawthorn non-diapausing flies emerging. Another group of apple flies were obtained as pupae from a colony maintained at the NY Agricultural Experiment Station in Geneva, NY, USA, which relocated to the USDA-ARS Appalachian Fruit Research Station, Kearneysville, WV, USA. Post eclosion flies were classified into two groups as mentioned above.

Quantification of neurochemicals from non-diapausing fly brain:

After collecting 12–14-day old flies, 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. To identify and quantify the level neuromodulator from the samples, they were further processed as similar method mentioned in chapter 3, method section.

4.3 Results

Levels of neurochemicals from non-diapausing apple, lab-reared colony apple and diapausing apple and hawthorn flies

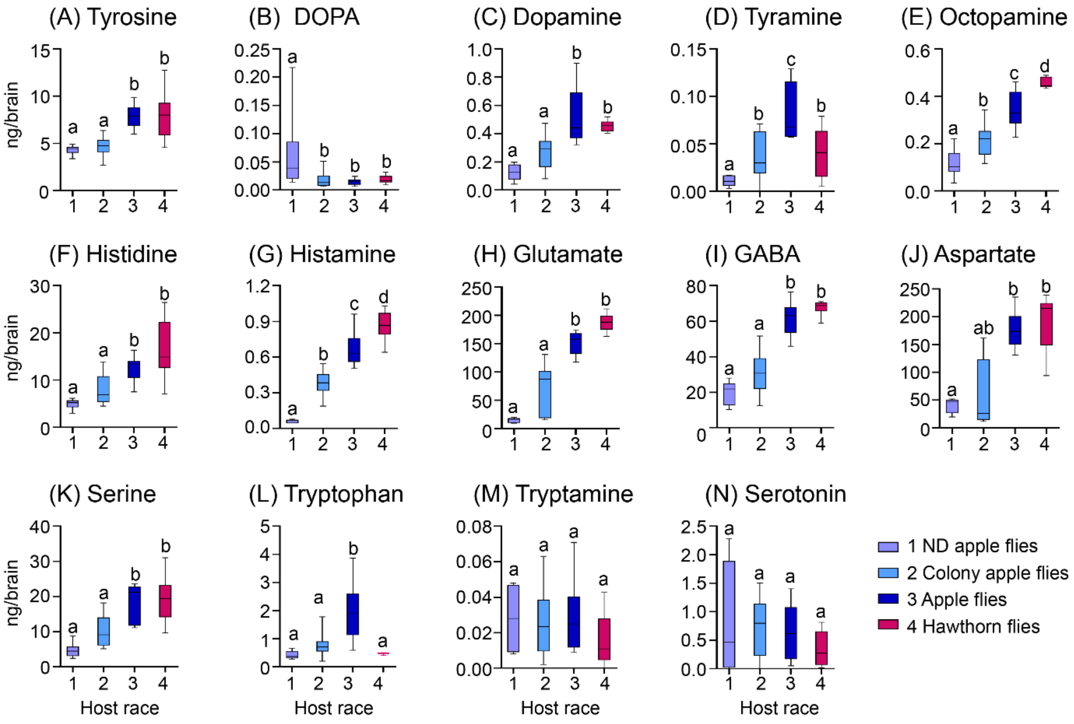


Figure 4.1: Quantification of biogenic amines and their precursors from sexually mature adult flies (stage 8) as defined in chapter 3, Figure 3.1 (A-N). Box plots of neurochemical titres for sexually mature non-diapausing wild apple flies, colony apple flies, and diapaused wild apple and wild hawthorn race flies with 9 $\mathbf{n}_{\mathbf{samples}}$ per race, containing a pool of five brains in each sample, symbols represent mean with 95% CI: 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; Letters above each population indicate similar titres of mentioned neurochemicals 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.

Table 4.1: Generalised linear mixed models, Tukey's HSD tests was performed for pairwise multiple comparisons with a significance cut off of 0.0001***, 0.001**, or 0.05* as indicated.

(Abbreviation used in this table, NDapple= non-diapausing apple flies, Coapple= colony apple flies, Apple= diapausing apple race and Hawthorn= diapausing hawthorn race)

Histidine	estimate	SE	df	t.ratio	p.value	
NDApple -Apple	8.287	2.471	10.493	3.354	0.0296	*
NDApple-CoApple	2.851	2.364	8.890	1.206	0.6385	
NDApple-Hawthorn	10.846	2.471	10.214	4.389	0.0060	**
CoApple-Apple	5.436	1.408	39.409	3.861	0.0022	**
CoApple - Hawthorn	-7.995	1.487	41.878	-5.377	0.0000	***
Apple - Hawthorn	-2.559	1.499	37.082	-1.707	0.3345	

Serine	estimate	SE	df	t.ratio	p.value	
NDApple -Apple	14.322	2.971	10.325	4.821	0.0030	**
NDApple-CoApple	5.801	2.857	8.755	2.030	0.2481	
NDApple-Hawthorn	13.287	2.999	10.226	4.431	0.0056	**
CoApple-Apple	8.521	1.838	38.834	4.636	0.0002	***
CoApple - Hawthorn	-7.486	2.013	39.423	-3.719	0.0033	**
Apple - Hawthorn	1.035	1.947	35.621	0.531	0.9508	

Histamine	estimate	SE	df	t.ratio	p.value	
NDApple -Apple	0.587	0.071	10.186	8.294	0.0001	***
NDApple-CoApple	0.329	0.067	8.223	4.947	0.0046	**
NDApple-Hawthorn	0.764	0.069	9.287	11.030	0.0001	***
CoApple-Apple	0.258	0.041	34.849	6.223	0.0001	***
CoApple - Hawthorn	-0.435	0.043	38.999	-10.199	0.0001	***
Apple - Hawthorn	-0.177	0.045	36.369	-3.897	0.0022	**

Aspartate	estimate	SE	df	t.ratio	p.value	
NDApple -Apple	110.427	38.397	8.245	2.876	0.0774	
NDApple-CoApple	42.014	38.094	7.987	1.103	0.6979	
NDApple-Hawthorn	107.224	38.446	8.276	2.789	0.0875	
CoApple-Apple	68.413	9.765	36.149	7.006	0.0001	***
CoApple - Hawthorn	-65.210	10.683	37.121	-6.104	0.0001	***
Apple - Hawthorn	3.203	10.165	35.402	0.315	0.9890	

Glutamate	estimate	SE	df	t.ratio	p.value	
NDApple -Apple	111.338	29.495	7.893	3.775	0.0231	*
NDApple-CoApple	61.562	29.416	7.806	2.093	0.2356	
NDApple-Hawthorn	136.065	29.639	8.044	4.591	0.0076	**
CoApple-Apple	49.776	5.113	33.684	9.736	0.0001	***

CoApple - Hawthorn	-74.503	5.726	33.487	-13.012	0.0001	***
Apple - Hawthorn	-24.726	5.734	33.285	-4.312	0.0007	***
GABA	estimate	SE	df	t.ratio	p.value	
NDApple -Apple	37.668	7.817	7.667	4.819	0.0064	**
NDApple-CoApple	14.933	7.742	7.345	1.929	0.2948	
NDApple-Hawthorn	38.242	7.934	8.087	4.820	0.0056	**
CoApple-Apple	22.735	2.432	34.974	9.349	0.0001	***
CoApple - Hawthorn	-23.309	2.730	34.693	-8.537	0.0001	***
Apple - Hawthorn	-0.574	2.745	33.868	-0.209	0.9967	
DOPA	estimate	SE	df	t.ratio	p.value	
NDApple -Apple	-0.047	0.015	39.000	-3.109	0.0176	*
NDApple-CoApple	-0.044	0.013	39.000	-3.244	0.0124	*
NDApple-Hawthorn	-0.043	0.015	39.000	-2.909	0.0292	*
CoApple-Apple	-0.004	0.013	39.000	-0.264	0.9935	
CoApple - Hawthorn	-0.001	0.013	39.000	-0.057	0.9999	
Apple - Hawthorn	-0.004	0.015	39.000	-0.291	0.9913	
Octopamine	estimate	SE	df	t.ratio	p.value	
NDApple -Apple	0.226	0.028	38.000	8.110	0.0001	***
NDApple-CoApple	0.101	0.025	38.000	3.963	0.0017	**
NDApple-Hawthorn	0.342	0.030	38.000	11.526	0.0001	***
CoApple-Apple	0.125	0.024	38.000	5.112	0.0001	***
CoApple - Hawthorn	-0.241	0.027	38.000	-9.092	0.0001	***
Apple - Hawthorn	-0.116	0.029	38.000	-4.018	0.0015	**
Tyrosine	estimate	SE	df	t.ratio	p.value	
NDApple -Apple	3.625	1.088	9.368	3.333	0.0348	*
NDApple-CoApple	0.303	1.069	8.685	0.284	0.9915	
NDApple-Hawthorn	3.209	1.096	9.515	2.927	0.0641	
CoApple-Apple	3.322	0.492	35.394	6.755	0.0001	***
CoApple - Hawthorn	-2.906	0.557	37.967	-5.222	0.0001	***
Apple - Hawthorn	0.415	0.510	32.895	0.815	0.8471	
Dopamine	estimate	SE	df	t.ratio	p.value	
NDApple -Apple	0.350	0.078	7.746	4.522	0.0090	**
NDApple-CoApple	0.152	0.074	6.512	2.051	0.2619	
NDApple-Hawthorn	0.264	0.079	8.266	3.324	0.0406	*
CoApple-Apple	0.198	0.043	37.390	4.561	0.0003	***
CoApple - Hawthorn	-0.111	0.049	40.223	-2.282	0.1194	
Apple - Hawthorn	0.087	0.049	34.621	1.759	0.3100	
Tyramine	estimate	SE	df	t.ratio	p.value	
NDApple -Apple	0.072	0.011	36.000	6.786	0.0001	***
NDApple-CoApple	0.026	0.010	36.000	2.520	0.0737	

NDApple-Hawthorn	0.029	0.011	36.000	2.661	0.0538	*
CoApple-Apple	0.047	0.010	36.000	4.732	0.0002	***
CoApple - Hawthorn	-0.004	0.010	36.000	-0.344	0.9858	
Apple - Hawthorn	0.043	0.011	36.000	4.048	0.0014	**
Serotonin	estimate	SE	df	t.ratio	p.value	
NDApple -Apple	-0.021	0.412	10.047	-0.050	1.0000	
NDApple-CoApple	-0.101	0.389	8.073	-0.260	0.9933	
NDApple-Hawthorn	-0.499	0.399	8.996	-1.250	0.6133	
CoApple-Apple	0.081	0.253	29.199	0.318	0.9887	
CoApple - Hawthorn	0.398	0.250	31.761	1.594	0.3963	
Apple - Hawthorn	0.479	0.274	29.404	1.747	0.3184	
Tryptophan	estimate	SE	df	t.ratio	p.value	
NDApple -Apple	1.607	0.311	9.305	5.167	0.0024	**
NDApple-CoApple	0.375	0.281	7.599	1.335	0.5696	
NDApple-Hawthorn	0.082	0.396	17.896	0.207	0.9967	
CoApple-Apple	1.232	0.278	27.501	4.436	0.0007	***
CoApple - Hawthorn	0.293	0.371	28.979	0.790	0.8582	
Apple - Hawthorn	1.525	0.386	24.148	3.954	0.0031	**
Tryptamine	estimate	SE	df	t.ratio	p.value	
NDApple -Apple	-0.003	0.010	8.148	-0.296	0.9903	
NDApple-CoApple	-0.004	0.009	7.323	-0.454	0.9668	
NDApple-Hawthorn	-0.012	0.010	7.460	-1.296	0.5915	
CoApple-Apple	0.001	0.008	31.252	0.178	0.9980	
CoApple - Hawthorn	0.008	0.008	33.422	1.067	0.7119	
Apple - Hawthorn	0.010	0.008	33.921	1.175	0.6462	

Table 4.2: A summary of significantly different neurochemicals between tested populations. Blue chemicals indicate commonalities across comparisons.

	ND apple	**Co apple**	**Apple**	**Hawthorn**
ND apple		DOPA, **Octopamine,** **histamine**	histidine, serine, **histamine**, glutamate, GABA, DOPA, **octopamine,** dopamine, tyrosine, **tyramine,** tryptophan	histidine, serine, **histamine**, glutamate, GABA, DOPA, **octopamine,** dopamine, **tyramine**, tryptophan
Co apple			histidine, serine, **histamine,** aspartate, glutamate, GABA, **octopamine,** tyrosine dopamine, **tyramine,** tryptophan	histidine, serine, **histamine**, aspartate, glutamate, GABA, **octopamine**, tyrosine
Apple				**histamine,** **tyramine,** **octopamine**
Hawthorn				

Figure 4.1, table 4.1 and 4.2 show that 12 out of 14 neurochemicals showed significant differences in titre between these four groups of flies. Non-diapausing wild apple and lab-reared colony apple flies were similar with only three neurochemicals (DOPA, octopamine and histamine) exhibiting significant differences between them, (table 4.1, tyrosine, $p = 0.9915$; DOPA, $p = 0.01$; dopamine, $p = 0.2619$; tyramine, $p = 0.07$; octopamine, $p = 0.001$; histidine, $p = 0.6385$; histamine, $p = 0.004$; glutamate, $p = 0.2356$; GABA, $p = 0.2948$; aspartate, $p = 0.6979$; serine, $p = 0.2481$; tryptophan, $p = 0.5696$; tryptamine, $p= 0.9668$; serotonin, $p = 0.9933$;).

When we compared wild non-diapausing apple vs diapausing apple and hawthorn flies, a total of 10 neurochemicals showed significant differences. Except for DOPA, all other chemicals showed significantly lower titres in non-diapausing apple populations compared to diapausing flies (table 4.1, tyrosine, Apple, p = 0.03, hawthorn, p = 0.06; DOPA, Apple, p = 0.01, hawthorn, p = 0.02; dopamine, Apple, p = 0.009, hawthorn, p = 0.04; tyramine, Apple, p = 0.0001, hawthorn, p = 0.053; octopamine, Apple, p = 0.0001, hawthorn, p = 0.0001; histidine, Apple, p = 0.02, hawthorn, p = 0.006; histamine, Apple, p = 0.0001, hawthorn, p = 0.0001; glutamate, Apple, p = 0.02, hawthorn, p = 0.007; GABA, Apple, p = 0.006, hawthorn, p = 0.005; aspartate, Apple, p = 0.077, hawthorn, p = 0.087; serine, Apple, p = 0.003, hawthorn, p = 0.005; tryptophan, Apple, p = 0.002, hawthorn, p = 0.99; tryptamine, Apple, p = 0.9903, hawthorn, p = 0.5915; serotonin, Apple, p = 1.000, hawthorn, p = 0.6133;).

Similarly, the lab-reared colony flies also showed several differences in neurochemical titre as compared to wild diapausing apple and hawthorn flies. 11 out 14 chemicals were significantly lower in colony apple flies compared to wild diapausing apple and 8 out 14 were significantly lower in colony apple flies compared to wild diapausing hawthorn flies.

Histamine and octopamine were consistently different in titre across all four groups. Both chemicals are known to regulate both development and behaviour in insects (Bodnaryk, 1980; Erber et al., 1993; Hong et al., 2006; Ramesh and Brockmann, 2019; Scheiner et al., 2006).

4.4 Discussion

I have shown that non-diapausing wild apple and lab-reared colony flies exhibited lower levels of neurochemicals compared to diapaused wild apple and hawthorn flies (Figure 4.1). Our results also suggest that all three different populations of the apple race (non-diapausing wild apple, lab-reared colony apple and diapausing wild apple flies) had lower neurochemicals compared to the hawthorn race.

A consistently lower level of neurochemicals in apple flies may play a role in their choice of apple as a host compared to hawthorn. As shown in chapter 2, I and my colleagues identified a switch between the odour processing of key volatiles (3MB and BH) in apple and hawthorn race flies at the level of the antennal lobe. In line with this result, I also showed in chapter 3 that changes in neurochemicals correspond to the morphogenesis and emergence of the antenna and antennal lobe in the developing adult fly. This suggests that there could be connection between the level of neurochemicals and the development of neural wiring in this system. Biogenic amines have been shown to play a role in the developmental of neural circuits involved in olfactory behavioural preference (Jung et al., 2013; Kloppenburg and Hildebrand, 1995). For example, a study on *Drosophila* showed that the release of serotonin and development of serotonergic neurones and their arborisation in the antennal lobe triggers the routing of sensory inputs according to the odour preference behaviour (Singh et al., 2013).

My results show differences in the titres of several neurochemicals between diapausing and non-diapausing flies, out of which two specific neurochemicals showed consistent differences - octopamine, and histamine (table 4.1 and 4.2). The titres of both of these neurochemicals were higher in diapausing flies compared to non-diapausing flies. As shown in chapter 3, these pathways are also shown to be consistently different at both stage 4 (stage where adult brain differentiation) and stage 8 (sexually mature flies). So, these are likely candidates for potential effects on *Rhagoletis* neural development and host choice.

Future studies that measure the expression of enzymes involved in the production of octopamine and histamine 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.

The experiments could include the injection/feeding of different concentrations of octopamine agonists and antagonists in larvae and/or up to stage 3 apple and hawthorn pupae (just before development of the adult antennal and antennal lobe) and assessing the olfactory response of sexually mature adult flies to BH and 3MB odours in behavioural assays (figure 4.2). My results suggest apple flies have lower levels of octopamine in comparison to hawthorn flies. Injecting octopamine would increase the level in the developing brain of apple flies. If apple flies will have a similar level to hawthorn flies, will they develop the reversed olfactory processing to show attraction to hawthorn odour (3MB)? Similarly, if we can decrease octopamine in developing hawthorn flies by injecting octopamine antagonists, will hawthorn flies develop the olfactory processing to show attraction to apple odour (BH) in flight tunnel assay? Such role of octopamine and its antagonists are extensively studied in honeybees (Behrends and Scheiner, 2012; Roeder, 2005; Roeder et al., 1998; Scheiner et al., 2002, 2006; Schulz et al., 2002).

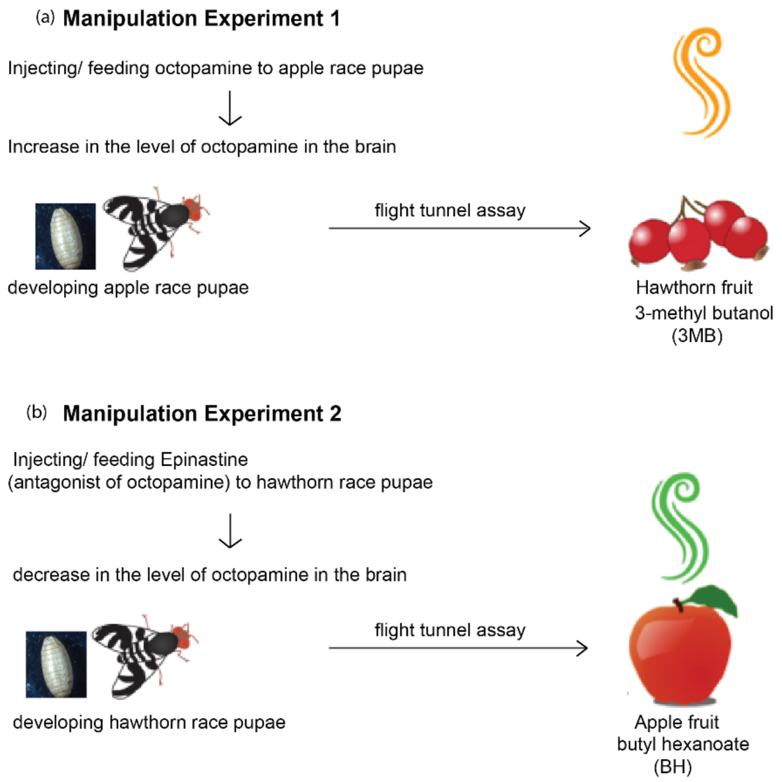


Figure 4.2: Future experiment a) injecting/feeding octopamine agonist to developing apple race pupae b) injecting/feeding epinastine/mianserin antagonist of octopamine to the developing hawthorn race pupae.

In summary my result on non-diapausing wild apple, lab-reared non-diapausing colony apple, and wild diapausing apple flies suggests that neurochemical levels are different in the derived apple race as compared to the hawthorn race.

Chapter 5

5.1 Dissertation Summary

Both apple and hawthorn races exhibit differences in diapause timing as well as behavioural preference towards their host plant. Thus, the goal of my dissertation was to identify the neural mechanisms connecting life history timing, development, and neurochemicals in ongoing ecological speciation in *Rhagoletis pomonella*. In Chapter 2, the objective was to determine whether there was a difference in input to the antennal lobe between the host races using single-sensillum electrophysiology combined with anterograde backfilling of the OSNs responsive to BH and 3MB. I found that both BH and 3MB responsive neurons innervated the same set of three glomeruli identified as the dorsal medial 1 (DM1), and ventral posterior 1 and 2 (VP1 and VP2) glomeruli (figure 2.2, 2.3). Only these glomeruli were identified in targeted backfills. Hence, there appears to be no difference in overall input to the antennal lobe by OSNs responding to BH or 3MB between the apple and hawthorn races. However, subsequent intracellular recording and calcium imaging data confirmed that there is a difference in neural processing of the major apple (BH) and hawthorn (3MB) volatiles at the level of the antennal lobe, in the form of a reversal of response between the three glomeruli (Tait et al., 2021). In other words, the response to the BH apple volatile was found in the DM1 glomerulus of the ancestral hawthorn race, and the VP1 and VP2 glomeruli in the derived apple race, and vice versa for the 3MB hawthorn volatile, indicating a switch in neural processing at the level of the antennal lobe.

In the next chapter, I showed that onset of adult brain differentiation (Stage 4) occurs parallel to the development of the adult antenna. I also found that adult neurogenesis occurs three weeks faster in the apple race compared to the hawthorn race even when the overwintering period is synchronized between the host races. Further, I identified specific differences in several neuromodulators, particularly biogenic amine pathways for octopamine and dopamine, at the first appearance of the adult brain and antenna (Chapter 3, stage 4, Figure, 3.7) and again at sexual maturity in the adult fly when host preference is exhibited (stage 8, Figure 3.6). My data indicates that the faster developing apple race exhibited lower levels of neurochemicals than the hawthorn race. This suggests that there could be connection between the level of neurochemicals and the development of neural wiring in this system.

To further investigate the connection between neurochemicals, development, and diapause regulation, I next quantified neurochemicals in non-diapausing flies that undergo direct development without diapause, and compared it with diapausing apple and hawthorn race flies. I found that both non-diapausing wild apple flies and lab-reared colony flies had lower levels of neurochemicals compared to diapausing apple and hawthorn flies (Figure 4.1). This finding supports the previous evidence from Chapter 3 that flies which develop faster and come out earlier contain a lower level of neurochemicals in their brain compared to later developing diapausing flies. My result also indicates that all three different populations of the apple race (non-diapausing wild apple, lab-reared non-diapausing colony apple, and wild diapausing apple flies) had lower neurochemicals compared to the hawthorn race.

My research, therefore, has identified specific differences in biogenic amine pathways at the first appearance of the adult brain (stage 4) and again at sexual maturity in the adult fly that differ between the two host races. Specifically, the derived apple race exhibits both faster development and lower titres of neurochemicals during these two key developmental stages. Further, this difference in neural development corresponds to a switch in host odor processing identified between the races for key host volatiles, providing a potential developmental mechanism for the host switch to be generated in this system.

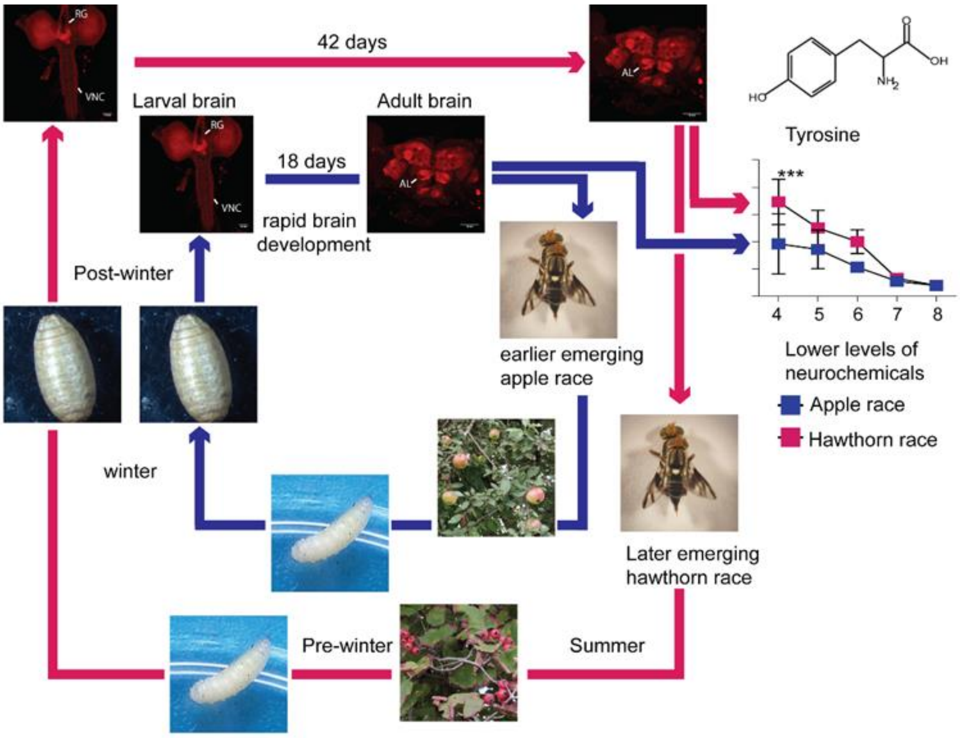


Figure 5.1: summary of this research

Biogenic amines have been implicated to impact both pupal diapause and adult behaviour. This study offers a new hypothesis that could correlate 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 (Tait et al., 2021).

5.2: A new hypothesis for host shift in *Rhagoletis pomonella*

Hypothesis "A faster developing, and early emerging hawthorn fly is an apple fly."

A percentage of non-diapausing phenotypes in the hawthorn population is known to occur in nature (Dambroski and Feder, 2007). Although, by the time they complete development, hardly any hawthorn fruit will be available to mate or reproduce. These non-diapausing flies are proof that a small percentage of *Rhagoletis* flies can develop earlier than their host availability. The lab-reared colony apple flies are also another example of *Rhagoletis pomonella* that can develop and emerge earlier than the fruiting time of apples. Likewise, it is also known that polymorphisms exist in the hawthorn race such that they can respond to both hawthorn and apple odours, only to hawthorn odours, or none of the odours (Dambroski et al., 2005; Linn et al., 2003b, 2005a, 2005b; Powell et al., 2012).

Based on the results of my research, I propose that specialization to apple fruit could have occurred because flies that developed faster and had a lower level of neurochemicals developed altered neural wiring that allow them to select apples as a host once apples were introduced to North America 300 years ago. I hypothesize that the earlier brain development (and early emergence) combined with lower levels of neurochemicals could affect adult brain development through a switch in the processing of odours in the antennal lobe and host preference to allow flies to shift from their usual host hawthorn (table 5.1). In such an event, the availability of other hosts (such as apples in North America) would facilitate the natural selection of such flies and contribute to the speciation. Thus, I predict that the faster developing and early emerging non-diapausing hawthorn fly with a lower level of neurochemicals found the apple fruit when there was no hawthorn fruit available at that earlier time of the year.

This two-step hypothesis (table 5.1), could allow four different phenotypes based on the rate of development with changes in neural wiring and levels of neurochemicals. Phenotype 1, flies developing faster and lower-level of neurochemicals would prefer Apple, phenotype 2, flies developing later with higher levels of neurochemicals would prefer hawthorn. In phenotypes 3 and 4, both of the processes will allow flies to prefer hawthorn.

	Faster brain development	Slower brain development
Lower level of neurochemicals	Phenotype1 Apple	Phenotype 3 Hawthorn
Higher level of neurochemicals	Phenotype 4 Hawthorn	Phenotype 2 Hawthorn

Table 5.1: Two step hypothesis for how the host shift could have occurred in *Rhagoletis pomonella*. The table shows how change in the developmental time would impact production of neurochemicals and the only hawthorn, only apple phenotypes in these flies.

Phenotypes 1 and 2 are already suggested in Chapter 3. Phenotype 3 flies, developing later with lower levels of neurochemicals, would also prefer hawthorn. An example of this phenotype is the earliest cohort of diapausing hawthorn flies shown in Chapter 3. These flies exhibited lower level of neurochemicals (some at the same level as the apple race, see Figure 3.7) but they still developed later than the apple race, giving the regulatory pathways and associated biochemistry longer to impact the developing adult brain. This is the perfect example of two-step hypothesis. Here the development time drives the host choice rather than the level of neurochemicals alone.

There are two possibilities that could give rise to phenotype 4, where flies have higher level neurochemicals independent of development. One of them could be a maternal effect. A hybridization study on non-diapausing apple and hawthorn flies showed that there is a maternal effect involved in host preference (Dambroski et al., 2005). This maternal effect could be due to higher levels of biogenic amines transferred from the mother to the eggs. For example, in the flash fly, both GABA and octopamine are involved in the transfer of information about diapause from mother to progeny. Injection of octopamine and picrotoxin (antagonist of GABA) have shown to contradict the maternal effects and permit the expression of diapause in flash flies (Webb and Denlinger, 1998).

The other possibility is the nutritional value and the amount of amnio acids found in the fruit. For example, *D. sechellia* specialised on toxic *Morinda citrifolia* fruit is endemic to Seychelles. Morinda provides *D. sechellia* with the dopamine precursor l-DOPA necessary for the progression of oogenesis. Similarly, it is possible that apples and hawthorns might have a different level of amino acids. An amino acid profiling study on Apple, (*Malus dometicus*) showed a lower amount of tyrosine and histidine less than 1mg/100 gram of the fruit pulp (di Maro et al., 2011). The apples found in Saudi Arabia also showed a similar result with a lower level of tyrosine (6.84 mg/l). Interestingly there was no histidine found in the pulp (Alnuwaiser and Nora, 2017). Similarly, hawthorn (*Crataegus pinnatifida*) fruit contained tyrosine 4.2 mg/100gram and histidine 2.4 mg/100 gram (Li et al., 2015). Hence, it is possible that apple and hawthorn fruit found in North America might have different nutritional values with different levels of tyrosine and histidine, which are important for production of the biogenic amines octopamine and histamine.

To test this two-step hypothesis, it will be interesting to see the brain development of non-diapausing hawthorn flies. Do they take a longer time than the non-diapausing apple race? It is also important to identify the adult brain differentiation stage (stage 4) in correlation with metabolic rate measurement for both non-diapausing lab-reared colony and non-diapausing wild apple and hawthorn flies. Secondly, if there is a maternal effect, we can look at the level of neurochemicals in the eggs of both host races. We can also examine levels of biogenic amine precursors in the larvae of both races after feeding from fruit. Parallel to these experiments, manipulation experiments with neurochemicals as mentioned in chapter 4 are needed to examine the behavioural preference of non-diapausing hawthorn and apple flies to both host blends.

Further studies could also investigate diapause adaptation and host shifts in other *Rhagoletis* populations like *R. mendax* (the blueberry maggot) or *R. pomonella dogwood*. The fruiting time of their host fruit slightly overlaps between these flies. The *R.mendax* species, which infests blueberry and deer berry (Vaccinium spp.), fruits 2-3 weeks earlier than apples (Feder et al., 1998; Payne and Berlocher, 1995). and the *Cornus florida* fruit infested by the flowering dogwood fly fruits 2-3 weeks later than hawthorn fruit (Berlocher, 1999; Smith, 1988). It will be interesting to study the rate of brain development and level of neurochemicals in these sibling species to see whether they show a similar pattern for brain development and whether the same biosynthetic pathways show differences in the levels shown in two different host races of R *pomonella*.

5.3 Broader significance of my dissertation

My studies are the first to connect life history timing, brain development, the role of neurochemicals in host shifts and speciation. In temperate regions, winter is shrinking, and overnight temperatures are rising with impacts on the duration and timing of growing seasons for plants and animals. A fundamental understanding of how organisms time their seasonal lifecycles is needed to predict and mitigate the effects of climate change. In the case of *Rhagoletis*, temperature plays an important role in diapause termination and post-diapause development (Dambroski and Feder, 2007). Our study suggests that phytophagous insects like *Rhagoletis* can develop their brain faster and emerge earlier than their parent population depending on diapause timing. As such, understanding the mechanisms regulating life history timing can have profound global impacts on predicting and mitigating the impacts of changes in climate, maintenance of biodiversity, health, and agriculture.

5.4 References

Alnuwaiser, M.A., and Nora, P. (2017). Study and comparison of Amino acids in Red and Green apples. IJPSR, *8,* 4097–4105.

Andreatta, G., Kyriacou, C.P., Flatt, T., and Costa, R. (2018). Aminergic Signaling Controls Ovarian Dormancy in Drosophila. Sci. Rep. *8*, 1–14.

Auer, T.O., Khallaf, M.A., Silbering, A.F., Zappia, G., Ellis, K., Álvarez-Ocaña, R., Arguello, J.R., Hansson, B.S., Jefferis, G.S.X.E., Caron, S.J.C., et al. (2020). Olfactory receptor and circuit evolution promote host specialization. Nature *579*, 402–408.

Bang, S., Hyun, S., Hong, S.T., Kang, J., Jeong, K., Park, J.J., Choe, J., and Chung, J. (2011). Dopamine signalling in mushroom bodies regulates temperature-preference behaviour in Drosophila. PLoS Genet. *7*.

Barraclough, T.G. (1998). Revealing the factors that promote speciation. Philos. Trans. R. Soc. B Biol. Sci. *353*, 241–249.

Behrends, A., and Scheiner, R. (2012). Octopamine improves learning in newly emerged bees but not in old foragers. J. Exp. Biol. *215*, 1076–1083.

Berlocher, S.H. (1999). Host race or species? Allozyme characterization of the "flowering dogwood fly", a member of the Rhagoletis pomonella complex. Heredity (Edinb). *83*, 652–662.

Berlocher, S.H., and Feder, J.L. (2002). Sympatric Speciation in Phytophagous Insects : Moving Beyond Controversy ? Annu. Rev. Entomol. *47*, 773–815.

Bodnaryk, R.P. (1980). Changes in brain octopamine levels during metamorphosis of the moth, Mamestra configurata Wlk. Insect Biochem. *10*, 169–173.

Bush, G.L. (1969). Sympatric Host Race Formation and Speciation in Frugivorous Flies of the Genus Rhagoletis (Diptera, Tephritidae). Evolution (N. Y). *23*, 237.

Charles Darwin (1872). On the origin of species, 6th edn. Econ. (United Kingdom).

Couto, A., Alenius, M., and Dickson, B.J. (2005). Molecular, anatomical, and functional organization of the Drosophila olfactory system. Curr. Biol. *15*, 1535–1547.

Craig, T.P., Itami, J.K., Abrahamson, W.G., and Horner, J.D. (1993). Behavioral evidence for host-race formation in Eurosta solidaginis. Evolution (N. Y). *47*, 1696–1710.

Crowley-Gall, A., Date, P., Han, C., Rhodes, N., Andolfatto, P., Layne, J.E., and Rollmann, S.M. (2016). Population differences in olfaction accompany host shift in Drosophila mojavensis. Proc. R. Soc. B Biol. Sci. *283*.

Dambroski, H.R., and Feder, J.L. (2007). Host plant and latitude-related diapause variation in Rhagoletis pomonella: A test for multifaceted life history adaptation on different stages of diapause development. J. Evol. Biol. *20*, 2101–2112.

Dambroski, H.R., Linn, C., Berlocher, S.H., Forbes, A.A., Roelofs, W., and Feder, J.L. (2005). The genetic basis for fruit odor discrimination in Rhagoletis flies and its significance for sympatric host shifts. Evolution (N. Y). *59*, 1953–1964.

Dambroski, H.R., Linn, C., Berlocher, S.H., Andrew, A., Dambroski, H.R., Linn, C., Berlocher, S.H., Roelofs, W., and Feder, J.L. (2016). The Genetic Basis for Fruit Odor Discrimination in Rhagoletis Flies and Its Significance for Sympatric Host Shifts Forbes , Wendell Roelofs and Jeffrey L . Feder Published by : Society for the Study of Evolution Stable URL : http://www.jstor.org/stable/34. *59*, 1953–1964.

Date, P., Dweck, H.K.M., Stensmyr, M.C., Shann, J., Hansson, B.S., and Rollmann, S.M. (2013). Divergence in Olfactory Host Plant Preference in D. mojavensis in Response to Cactus Host Use. PLoS One *8*, 1–10.

Date, P., Crowley-Gall, A., Diefendorf, A.F., and Rollmann, S.M. (2017). Population differences in host plant preference and the importance of yeast and plant substrate to volatile composition. Ecol. Evol. *7*, 3815–3825.

Dekker, T., Ibba, I., Siju, K.P., Stensmyr, M.C., and Hansson, B.S. (2006). Olfactory shifts parallel superspecialism for toxic fruit in Drosophila melanogaster sibling, D. sechellia. Curr. Biol. *16*, 101–109.

Denlinger, D.L. (2002). Regulation of Diapause. Annu. Rev. Entomol. *47*, 93–122.

Denlinger, D.L., Hahn, D.A., Merlin, C., Holzapfel, C.M., and Bradshaw, W.E. (2017). Keeping time without a spine: What can the insect clock teach us about seasonal adaptation? Philos. Trans. R. Soc. B Biol. Sci. *372*.

Dowle, E.J., Powell, T.H.Q., Doellman, M.M., Meyers, P.J., Calvert, M.B., Walden, K.K.O., Robertson, H.M., Berlocher, S.H., Feder, J.L., Hahn, D.A., et al. (2020). Genome-wide variation and transcriptional changes in diverse developmental processes underlie the rapid evolution of seasonal adaptation. Proc. Natl. Acad. Sci. U. S. A. *117*, 23960–23969.

E. Boller and R.J. Prokopy (1976). Bionomics and Management of Rhagoletis. Annu. Rev. Entomol. 223–246.

Erber, J., Kloppenburg, P., and Scheidler, A. (1993). Neuromodulation by serotonin and octopamine in the honeybee: behaviour, neuroanatomy and electrophysiology. Experientia *49*, 1073–1083.

Feder, J.L., and Filchak, K.E. (1999). It’s about time: The evidence for host plant-mediated selection in the apple maggot fly, Rhagoletis pomonella, and its implications for fitness trade-offs in phytophagous insects. Entomol. Exp. Appl. *91*, 211–225.

Feder, J.L., Chilcote, C.A., and Bush, G.L. (1988). Genetic differentiation between sympatric host races of the apple maggot fly Rhagoletis pomonella. Nature *336*, 61–64.

Feder, J.L., Hunt, T.A., and Bush, L. (1993). The effects of climate, host plant phenology and host fidelity on the genetics of apple and hawthorn infesting races of Rhagoletis pomonella. Entomol. Exp. Appl. *69*, 117–135.

Feder, J.L., Opp, S.B., Wlazlo, B., Reynolds, K., Go, W., and Spisak, S. (1994). Host fidelity is an effective premating barrier between sympatric races of the apple maggot fly. Proc. Natl. Acad. Sci. U. S. A. *91*, 7990–7994.

Feder, J.L., Roethele, J.B., Wlazlo, B., and Berlocher, S.H. (1997). Selective maintenance of allozyme differences among sympatric host races of the apple maggot fly. Proc. Natl. Acad. Sci. U. S. A. *94*, 11417–11421.

Feder, J.L., Berlocher, S.H., and Opp, S.B. (1998). Sympatric Host-Race Formation and Speciation in Rhagoletis (Diptera: Tephritidae): A Tale of Two Species for Charles D. Genet. Struct. Local Adapt. Nat. Insect Popul. 408–441.

Feder JL, Chilcote CA, B.G. (1989). J. L Feder, C. A. Chilcote, and G. L. Bush. J. Hered *80*, 277–283.

Felix, A., Kromann, S.H., Gramsbergen, J., Hansson, B.S., and Ignell, R. (2008). Dopamine regulates the mating switch in olfactory coding and preference in a female moth. 1–9.

Forbes, A.A., Fisher, J., and Feder, J.L. (2005). Habitat avoidance: Overlooking an important aspect of host-specific mating and sympatric speciation? Evolution (N. Y). *59*, 1552–1559.

Fuchs, S., Rende, E., Crisanti, A., and Nolan, T. (2014). Disruption of aminergic signalling reveals novel compounds with distinct inhibitory effects on mosquito reproduction, locomotor function and survival. Sci. Rep. *4*, 1–11.

Ghaninia, M., Ignell, R., and Hansson, B.S. (2007). Functional classification and central nervous projections of olfactory receptor neurons housed in antennal trichoid sensilla of female yellow fever mosquitoes, Aedes aegypti. Eur. J. Neurosci. *26*, 1611–1623.

Grabe, V., Strutz, A., Baschwitz, A., Hansson, B.S., and Sachse, S. (2015). Digital in vivo 3D atlas of the antennal lobe of Drosophila melanogaster. J. Comp. Neurol. *523*, 530–544.

Green, T.A., Prokopy, R.J., and Hosmer, D.W. (1994). Distance of response to host tree models by female apple maggot flies, Rhagoletis pomonella (Walsh) (Diptera: Tephritidae): Interaction of visual and olfactory stimuli. J. Chem. Ecol. *20*, 2393–2413.

Groman, J.D., and Pellmyr, O. (2000). Rapid evolution and specialization following host colonization in a yucca moth. J. Evol. Biol. *13*, 223–236.

Hallem, E.A., and Carlson, J.R. (2006). Coding of Odors by a Receptor Repertoire. Cell *125*, 143–160.

Hansson, B.S., Löfstedt, C., and Roelofs, W.L. (1987). Inheritance of olfactory response to sex pheromone components in Ostrinia nubilalis. Naturwissenschaften *74*, 497–499.

Hong, S.T., Bang, S., Paik, D., Kang, J., Hwang, S., Jeon, K., Chun, B., Hyun, S., Lee, Y., and Kim, J. (2006). Histamine and its receptors modulate temperature-preference behaviors in Drosophila. J. Neurosci. *26*, 7245–7256.

Hu, A., Zhang, W., and Wang, Z. (2010). Functional feedback from mushroom bodies to antennal lobes in the Drosophila olfactory pathway. Proc. Natl. Acad. Sci. U. S. A. *107*, 10262–10267.

Ibba, I., Angioy, A.M., Hansson, B.S., and Dekker, T. (2010). Macroglomeruli for fruit odors change blend preference in Drosophila. Naturwissenschaften *97*, 1059–1066.

Isabel, G., Gourdoux, L., and Moreau, R. (2001). Changes of biogenic amine levels in haemolymph during diapausing and non-diapausing status in Pieris brassicae L. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. *128*, 117–127.

Itami, J.K., Craig, T.P., and Horner, J.D. (1998). Factors Affecting Gene Flow between the Host Races of Eurosta solidaginis. In Genetic Structure and Local Adaptation in Natural Insect Populations, (Boston, MA: Springer US), pp. 375–407.

Ito, K., Shinomiya, K., Ito, M., Armstrong, J.D., Boyan, G., Hartenstein, V., Harzsch, S., Heisenberg, M., Homberg, U., Jenett, A., et al. (2014). A systematic nomenclature for the insect brain. Neuron *81*, 755–765.

Jerry A. Coyne, and H. Allen Orr (2004). Speciation (Sinauer, Sunderland.).

Jung, J.W., Kim, J.H., Pfeiffer, R., Ahn, Y.J., Page, T.L., and Kwon, H.W. (2013). Neuromodulation of olfactory sensitivity in the peripheral olfactory organs of the American cockroach, Periplaneta americana. PLoS One *8*, 1–11.

Kárpáti, Z., Dekker, T., and Hansson, B.S. (2008). Reversed functional topology in the antennal lobe of the male European corn borer. J. Exp. Biol. *211*, 2841–2848.

Keil, T.A. (1999). Morphology and Development of the Peripheral Olfactory Organs. Insect Olfaction 5–47.

Kloppenburg, P., and Hildebrand, J.G. (1995). Neuromodulation by 5-hydroxytryptamine in the antennal lobe of the sphinx moth Manduca sexta. J. Exp. Biol. *198*, 603–611.

Kostal, V. (2006). Eco-physiological phases of insect diapause. J. Insect Physiol. *52*, 113–127.

Kostal, V., Stetina, T., Poupardin, R., Korbelova, J., and Bruce, A.W. (2017).

Conceptual framework of the eco-physiological phases of insect diapause development justified by transcriptomic profiling. Proc. Natl. Acad. Sci. U. S. A. *114*, 8532–8537.

Koutroumpa, F.A., Kárpáti, Z., Monsempes, C., Hill, S.R., Hansson, B.S., Jacquin-Joly, E., Krieger, J., and Dekker, T. (2014). Shifts in sensory neuron identity parallel differences in pheromone preference in the European corn borer. Front. Ecol. Evol. *2*, 1–12.

Kromann, A. (2012). Modulation of olfactory information in the antennal lobe of Spodoptera littoralis.

Lassance, J.M. (2010). Journey in the Ostrinia World: From Pest to Model in Chemical Ecology. J. Chem. Ecol. *36*, 1155–1169.

Lee, S.G., Carlsson, M.A., Hansson, B.S., Todd, J.L., and Baker, T.C. (2006). Antennal lobe projection destinations of Helicoverpa zea male olfactory receptor neurons responsive to heliothine sex pheromone components. J. Comp. Physiol. A Neuroethol. Sensory, Neural, Behav. Physiol. *192*, 351–363.

Li, W.Q., Hu, Q.P., and Xu, J.G. (2015). Changes in physicochemical characteristics and free amino acids of hawthorn (Crataegus pinnatifida) fruits during maturation. Food Chem. *175*, 50–56.

Linn, C., Feder, J.L., Nojima, S., Dambroski, H.R., Berlocher, S.H., and Roelofs, W. (2003a). Fruit odor discrimination and sympatric host race formation in Rhagoletis. Proc. Natl. Acad. Sci. U. S. A. *100*, 11490–11493.

Linn, C., Feder, J.L., Nojima, S., Dambroski, H.R., Berlocher, S.H., and Roelofs, W. (2003b). Fruit odor discrimination and sympatric host race formation in Rhagoletis. Proc. Natl. Acad. Sci. U. S. A. *100*, 11490–11493.

Linn, C., Nojima, S., and Roelofs, W. (2005a). Antagonist effects of non-host fruit volatiles on discrimination of host fruit by Rhagoletis flies infesting apple (Malus pumila), hawthorn (Crataegus spp.), and flowering dogwood (Cornus florida). Entomol. Exp. Appl. *114*, 97–105.

Linn, C.E., Dambroski, H.R., Feder, J.L., Berlocher, S.H., Nojima, S., and Roelofs, W.L. (2004). Postzygotic isolating factor in sympatric speciation in Rhagoletis flies:

Reduced response of hybrids to parental host-fruit odors. Proc. Natl. Acad. Sci. U. S. A. *101*, 17753–17758.

Linn, C.E., Dambroski, H., Dambroski, H., Nojima, S., Nojima, S., Feder, J.L., Feder, J.L., H, S., H, S., Roelofs, W.L., et al. (2005b). Variability in response specificity of apple, hawthorn, and flowering dogwood-infesting. Entomol. Exp. Appl. 55–64.

Long, T.F., and Murdock, L.L. (1982). Stimulation of blowfly feeding behavior by octopaminergic drugs. Proc. Natl. Acad. Sci. U. S. A. *80*, 4159–4163.

di Maro, A., Dosi, R., Ferrara, L., Rocco, M., Sepe, J., Ferrari, G., and Parente, A. (2011). Free amino acid profile in Malus domestica cv Annurca apples from the Campania region and other Italian vegetables. Aust. J. Crop Sci. *5*, 154–161.

Martin, J.P., Beyerlein, A., Dacks, A.M., Reisenman, C.E., Riffell, J.A., Lei, H., and Hildebrand, J.G. (2011). The neurobiology of insect olfaction: Sensory processing in a comparative context. Prog. Neurobiol. *95*, 427–447.

Matsumoto, M., and Takeda, M. (2002). Changes in brain monoamine contents in diapause pupae of Antheraea pernyi when activated under long-day and by chilling. J. Insect Physiol. *48*, 765–771.

Mendleson TC and Shaw KL (2005). Sexual Behaviour Rapid speciation in an arthropod. Nature *433*, 375–376.

Molnár, B.P., Boddum, T., Hill, S.R., Hansson, B.S., Hillbur, Y., and Birgersson, G. (2018). Ecological and phylogenetic relationships shape the peripheral olfactory systems of highly specialized gall midges (Cecidomiiydae). Front. Physiol. *9*, 1–12.

Monastirioti, M. (1999). Biogenic amine systems in the fruit fly Drosophila melanogaster. Microsc. Res. Tech. *45*, 106–121.

Mora, C., Tittensor, D.P., Adl, S., Simpson, A.G.B., and Worm, B. (2011). How many species are there on earth and in the ocean? PLoS Biol. *9*, 1–8.

Natarajan, N., Ramakrishnan, P., Lakshmanan, V., Palakodeti, D., and Rangiah, K. (2015). A quantitative metabolomics peek into planarian regeneration. Analyst *140*, 3445–3464.

Nathanson J.A (1979). Octopamine Receptors, Adenosine 3’,5’-Monophosphate, and

Neural Control of Firefly Flashing. Science (80-.). *203*, 56–68.

Noguchi, H., and Hayakawa, Y. (1997). Role of dopamine at the onset of pupal diapause in the cabbage armyworm Mamestra brassicae. FEBS Lett. *413*, 157–161.

Nojima, S., Linn, C., Morris, B., Zhang, A., and Roelofs, W. (2003a). Identification of host fruit volatiles from hawthorn (Crataegus spp.) attractive to hawthorn-origin Rhagoletis pomonella flies. J. Chem. Ecol. *29*, 321–336.

Nojima, S., Linn, C., and Roelofs, W. (2003b). Identification of host fruit volatiles from flowering dogwood (Cornus florida) attractive to dogwood-origin Rhagoletis pomonella flies. J. Chem. Ecol. *29*, 2347–2357.

Olsson, S.B., Linn, C.E., Michel, A., Dambroski, H.R., Berlocher, S.H., Feder, J.L., and Roelofs, W.L. (2006). Receptor expression and sympatric speciation: Unique olfactory receptor neuron responses in F1 hybrid Rhagoletis populations. J. Exp. Biol. *209*, 3729–3741.

Olsson, S.B., Linn, C.E., Feder, J.L., Michel, A., Dambroski, H.R., Berlocher, S.H., and Roelofs, W.L. (2009). Comparing peripheral olfactory coding with host preference in the rhagoletis species complex. Chem. Senses *34*, 37–48.

Olsson, S.B., Kesevan, S., Groot, A.T., Dekker, T., Heckel, D.G., and Hansson, B.S. (2010). Ostrinia revisited: Evidence for sex linkage in European Corn Borer Ostrinia nubilalis (Hubner) pheromone reception. BMC Evol. Biol. *10*, 1–12.

Payne, A., and Berlocher, H. (1995). Distribution and Host Plants of the Blueberry Maggot Fly , Rhagoletis mendax (Diptera : Tephritidae) in Southeastern North America Author (s): Jerry A . Payne and Stewart H . Berlocher Source : Journal of the Kansas Entomological Society , Vol . 68 ,. J. Kans. Entomol. Soc. *68*, 133–142.

Powell, T.H.Q., Cha, D.H., Linn, C.E., and Feder, J.L. (2012). On the scent of standing variation for speciation: behavioral evidence for native sympatric host races of rhagoletis pomonella (diptera: tephritidae) in the southern united states. Evolution (N. Y). *66*, 2739–2756.

Powell, T.H.Q., Nguyen, A., Xia, Q., Feder, J.L., Ragland, G.J., and Hahn, D.A. (2020). A rapidly evolved shift in life-history timing during ecological speciation is driven by the transition between developmental phases. J. Evol. Biol. *33*, 1371–1386.

Pratt, G.F. (1994). Evolution of Euphilotes (Lepidoptera: Lycaenidae) by seasonal and host shifts. Biol. J. Linn. Soc. *51*, 387–416.

Prokopy, R.J. (1968a). Visual responses of Apple maggot flies, Rhagoletis pomonella (Diptera : tephritidae) : Orchard Studies. *11*, 403-422.

Prokopy, R.J. (1968b). Influence of photoperiod, temperature, and food on initiation of diapause in the apple maggot. Can. Entomol. *100*, 318–329.

Prokopy, R.J., and Bush, G.L. (1973). Mating behavior of rhagoletis pomonella (diptera: Tephritidae): IV. courtship. Can. Entomol. *105*, 873–891.

Prokopy, R.J., Bennett, E.W., and Bush, G.L. (1971). Mating Behavior in Rhagoletis pomonellaa (Diptera : Tephritidae) 1. Site of assembly. Can. Entomol. *103*, 1405–1409.

Puiroux, J., Moreau, R., and Gourdoux, L. (1990). Variations of biogenic amine levels in the brain of Pieris brassicae pupae during nondiapausing and diapausing development. Arch. Insect Biochem. Physiol. *14*, 57–69.

Ragland, G.J., Fuller, J., Feder, J.L., and Hahn, D.A. (2009). Biphasic metabolic rate trajectory of pupal diapause termination and post-diapause development in a tephritid fly. J. Insect Physiol. *55*, 344–350.

Ramesh, D., and Brockmann, A. (2019). Mass Spectrometric Quantification of Arousal Associated Neurochemical Changes in Single Honey Bee Brains and Brain Regions. ACS Chem. Neurosci. *10*, 1950–1959.

Rangiah, K., and Palakodeti, D. (2013). Comprehensive analysis of neurotransmitters from regenerating planarian extract using an ultrahigh-performance liquid chromatography/mass spectrometry/selected reaction monitoring method. Rapid Commun. Mass Spectrom. *27*, 2439–2452.

Roeder, T. (2005). Tyramine and octopamine: Ruling behavior and metabolism. Annu. Rev. Entomol. *50*, 447–477.

Roeder, T., Degen, J., and Gewecke, M. (1998). Epinastine, a highly specific antagonist of insect neuronal octopamine receptors. Eur. J. Pharmacol. *349*, 171–177.

Roelofs, W., Glover, T., Tang, X.-H., Sreng, I., Robbins, P., Eckenrode, C., Löfstedt, C., Hansson, B.S., and Bengtsson, B.O. (1987). Sex pheromone production and perception in European corn borer moths is determined by both autosomal and sex-linked genes. Proc. Natl. Acad. Sci. *84*, 7585–7589.

Rybak, J., Kuß, A., Lamecker, H., Zachow, S., Hege, H.C., Lienhard, M., Singer, J., Neubert, K., and Menzel, R. (2010). The digital bee brain: Integrating and managing neurons in a common 3D reference system. Front. Syst. Neurosci. *4*, 1–15.

Scheiner, R., Plückhahn, S., Öney, B., Blenau, W., and Erber, J. (2002). Behavioural pharmacology of octopamine, tyramine and dopamine in honey bees. Behav. Brain Res. *136*, 545–553.

Scheiner, R., Baumann, A., and Blenau, W. (2006). Aminergic Control and Modulation of Honeybee Behaviour. Curr. Neuropharmacol. *4*, 259–276.

Scheiner, R., Toteva, A., Reim, T., Søvik, E., and Barron, A.B. (2014). Differences in the phototaxis of pollen and nectar foraging honey bees are related to their octopamine brain titers. Front. Physiol. *5 MAR*, 1–8.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: An open-source platform for biological-image analysis. Nat. Methods *9*, 676–682.

Schluter, D. (2001). Ecology and the origin of species. Trends Ecol. Evol. *16*, 372–380.

Schulz, D.J., Barron, A.B., and Robinson, G.E. (2002). A role for octopamine in honey bee division of labor. Brain. Behav. Evol. *60*, 350–359.

Schwaerzel, M., Monastirioti, M., Scholz, H., Friggi-Grelin, F., Birman, S., and Heisenberg, M. (2003). Dopamine and Octopamine Differentiate between Aversive and Appetitive Olfactory Memories in Drosophila. J. Neurosci. *23*, 10495–10502.

Shanbhag, S.R., Hekmat-Scafe, D., Kim, M.S., Park, S.K., Carlson, J.R., Pikielny, C., Smith, D.P., and Steinbrecht, R.A. (2001). Expression mosaic of odorant-binding proteins in Drosophila olfactory organs. Microsc. Res. Tech. *55*, 297–306.

Singh, A.P., Das, R.N., Rao, G., Aggarwal, A., Diegelmann, S., Evers, J.F., Karandikar, H., Landgraf, M., Rodrigues, V., and VijayRaghavan, K. (2013). Sensory Neuron-Derived Eph Regulates Glomerular Arbors and Modulatory Function of a

Central Serotonergic Neuron. PLoS Genet. *9*.

Smadja, C., and Butlin, R.K. (2009). On the scent of speciation: The chemosensory system and its role in premating isolation. Heredity (Edinb). *102*, 77–97.

Smith, D.C. (1988). Heritable divergence of Rhagoletis pomonella host races by seasonal asynchrony. Nature *336*, 66–67.

Solari, P., Corda, V., Sollai, G., Kreissl, S., Galizia, C.G., and Crnjar, R. (2016). Morphological characterization of the antennal lobes in the Mediterranean fruit fly Ceratitis capitata. J. Comp. Physiol. A Neuroethol. Sensory, Neural, Behav. Physiol. *202*, 131–146.

Sombke, A., Harzsch, S., and Hansson, B.S. (2011). Organization of deutocerebral neuropils and olfactory behavior in the centipede Scutigera coleoptrata (Linnaeus, 1758) (Myriapoda: Chilopoda). Chem. Senses *36*, 43–61.

Sombke, A., Lipke, E., Kenning, M., Müller, C.H.G., Hansson, B.S., and Harzsch, S. (2012). Comparative analysis of deutocerebral neuropils in Chilopoda (Myriapoda): implications for the evolution of the arthropod olfactory system and support for the Mandibulata concept. BMC Neurosci. *13*, 1–17.

Stork, N.E. (2018). How Many Species of Insects and Other Terrestrial Arthropods Are There on Earth? Annu. Rev. Entomol. *63*, 31–45.

Tait, C., Batra, S., Ramaswamy, S.S., Feder, J.L., and Olsson, S.B. (2016). Sensory specificity and speciation: A potential neuronal pathway for host fruit odour discrimination in rhagoletis pomonella. Proc. R. Soc. B Biol. Sci. *283*.

Tait, C., Kharva, H., Schubert, M., Kritsch, D., Sombke, A., Rybak, J., Feder, J.L., and Olsson, S.B. (2021). A reversal in sensory processing accompanies ongoing ecological divergence and speciation in Rhagoletis pomonella. Proc. R. Soc. B *288*.

Terai, Y., Seehausen, O., Sasaki, T., Takahashi, K., Mizoiri, S., Sugawara, T., Sato, T., Watanabe, M., Konijnendijk, N., Mrosso, H.D.J., et al. (2006). Divergent selection on opsins drives incipient speciation in Lake Victoria cichlids. PLoS Biol. *4*, 2244–2251.

Unbehend, M., Kozak, G.M., Koutroumpa, F., Coates, B.S., Dekker, T., Groot, A.T., Heckel, D.G., and Dopman, E.B. (2021). bric à brac controls sex pheromone choice by male European corn borer moths. Nat. Commun. *12*, 1–11.

Via, S. (2001). Sympatric speciation in animals: the ugly duckling grows up. Trends Ecol. Evol. *16*, 381–390.

Vosshall, L.B., Amrein, H., Morozov, P.S., Rzhetsky, A., and Axel, R. (1999). A spatial map of olfactory receptor expression in the Drosophila antenna. Cell *96*, 725–736.

Walsh. B (1867). The apple-worm and the apple maggot. Am. J. Hortic. *2*, 338–343.

Wang, Q., Hanatani, I., Takeda, M., Oishi, K., and Sakamoto, K. (2015). D2-like dopamine receptors mediate regulation of pupal diapause in Chinese oak silkmoth Antheraea pernyi. Entomol. Sci. *18*, 193–198.

Webb, M.L.Z., and Denlinger, D.L. (1998). GABA and picrotoxin alter expression of a maternal effect that influences pupal diapause in the flesh fly, Sarcophaga bullata. Physiol. Entomol. *23*, 184–191.

Wilczynski, W., McClelland, B.E., and Rand, A.S. (1993). Acoustic, auditory, and morphological divergence in three species of neotropical frog. J. Comp. Physiol. A *172*, 425–438.

Wilson, R.I., Turner, G.C., and Laurent, G. (2004). Representation of Odor in the Brain. Sci. STKE *2004*, 366–370.

Wood, T.K. and, and Keese, M. (1990). Host-Plant-Induced Assortative Mating in Enchenopa Treehoppers. Evolution (N. Y). *44*, 619.

Wood, T.K., Olmstead, K.L., and Guttman, S.I. (1990). Insect phenology mediated by host-plant water relations. Evolution (N. Y). *44*, 629–636.

Wu, H., Xu, M., Hou, C., Huang, L.Q., Dong, J.F., and Wang, C.Z. (2015). Specific olfactory neurons and glomeruli are associated to differences in behavioral responses to pheromone components between two Helicoverpa species. Front. Behav. Neurosci. *9*, 1–11.

Younus, F., Chertemps, T., Pearce, S.L., Pandey, G., Bozzolan, F., Coppin, C.W., Russell, R.J., Maïbèche-Coisne, M., and Oakeshott, J.G. (2014). Identification of candidate odorant degrading gene/enzyme systems in the antennal transcriptome of

Drosophila melanogaster. Insect Biochem. Mol. Biol. *53*, 30–43.

Zhang, A., Linn, C., Wright, S., Prokopy, R., Reissig, W., and Roelofs, W. (1999). Identification of a new blend of apple volatiles attractive to the apple maggot, Rhagoletis pomonella. J. Chem. Ecol. *25*, 1221–1232.

Zhang, Q., Lu, Y.X., and Xu, W.H. (2013). Proteomic and metabolomic profiles of larval hemolymph associated with diapause in the cotton bollworm, Helicoverpa armigera. BMC Genomics *14*.

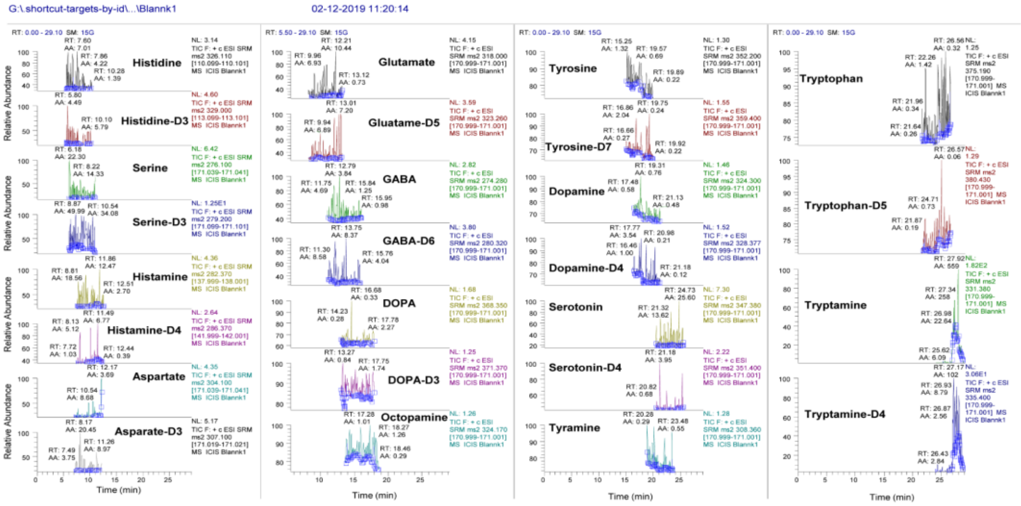
Zhukovskaya, M.I. (2008). Selective regulation of sensitivity to odours of different behavioural significance in the American cockroach, Periplaneta americana. Physiol. Entomol. *33*, 162–166.

Zhukovskaya, M.I. (2012). Modulation by octopamine of olfactory responses to nonpheromone odorants in the cockroach, Periplaneta Americana L. Chem. Senses *37*, 421–429.

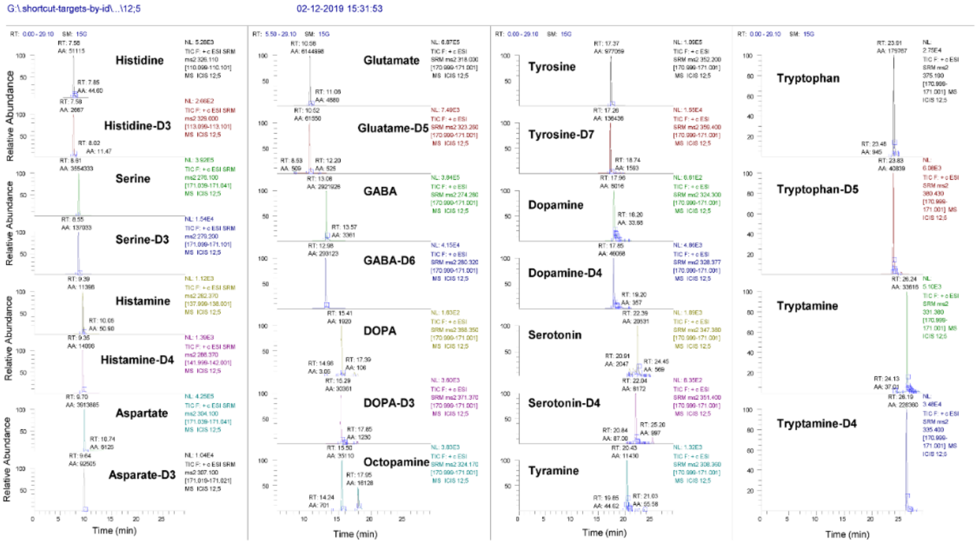
APPENDIX A:

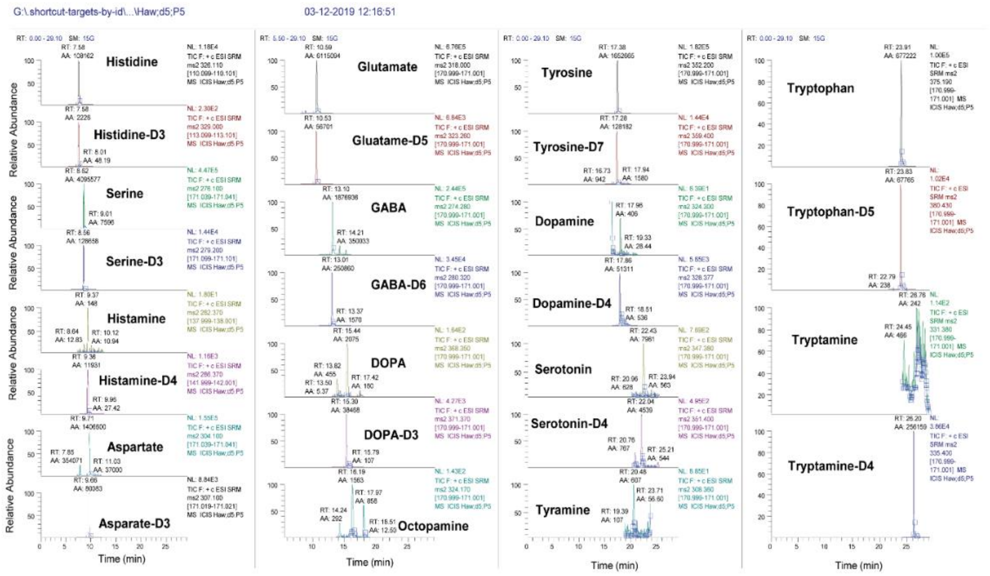
SUPPORTING INFORMATION FOR CHAPTER 3

A)



B)





c)

Figure A.1: Sample chromatograms obtained from LCMS neurochemical analysis.

A) Blank (2% ACN prepared in 0.5% FA), B) Standard 12;5 from calibration curve and C) Pooled Hawthorn brain sample. For each neurochemical compound, the molecular weight and retention time details mention in table s11. Note that the blank chromatograms (A) do not show any peak at respected retention times. In contrast, calibration and sample chromatogram indicate the internal standard and compound peaks at particular retention time points where they are eluting.

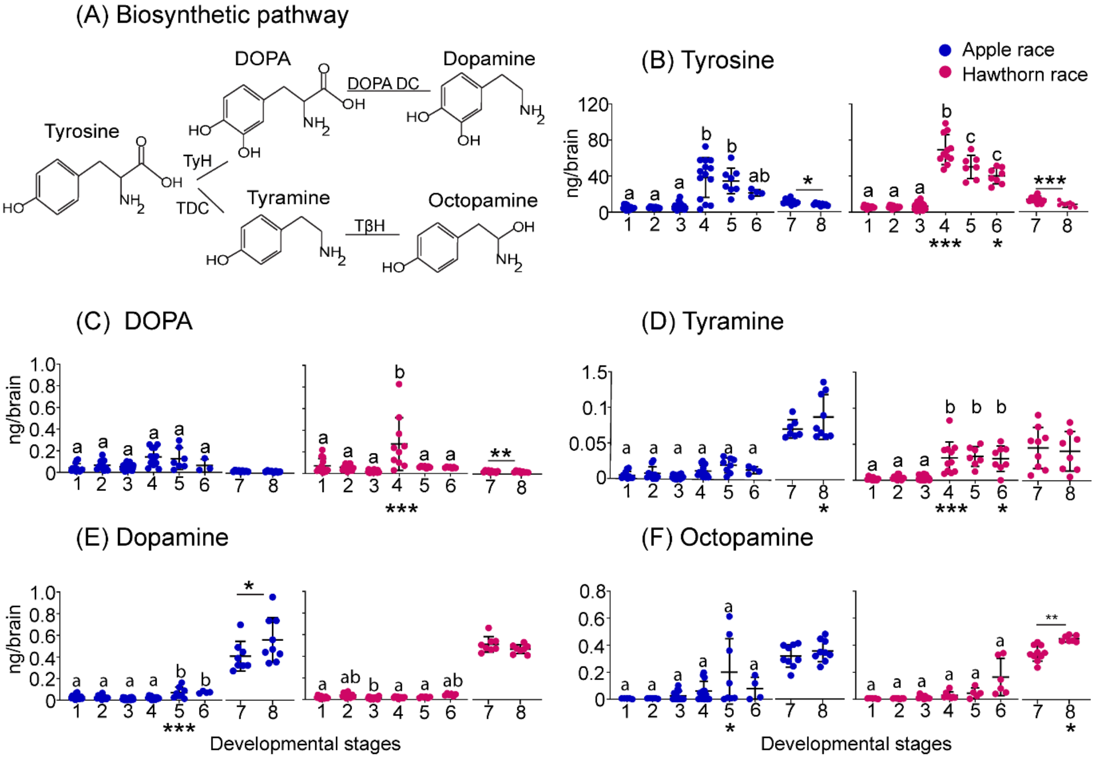


Figure A.2: Quantification of biogenic amines and their precursors across the different stages of development as defined in Figure 1. (A) Biosynthetic pathways for octopamine and dopamine with enzymes, tyrosine hydroxylase (TyH), DOPA decarboxylase (DOPA DC), tyrosine decarboxylase and tyramine beta-hydroxylase (T□H); (B-F) 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 medians 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.

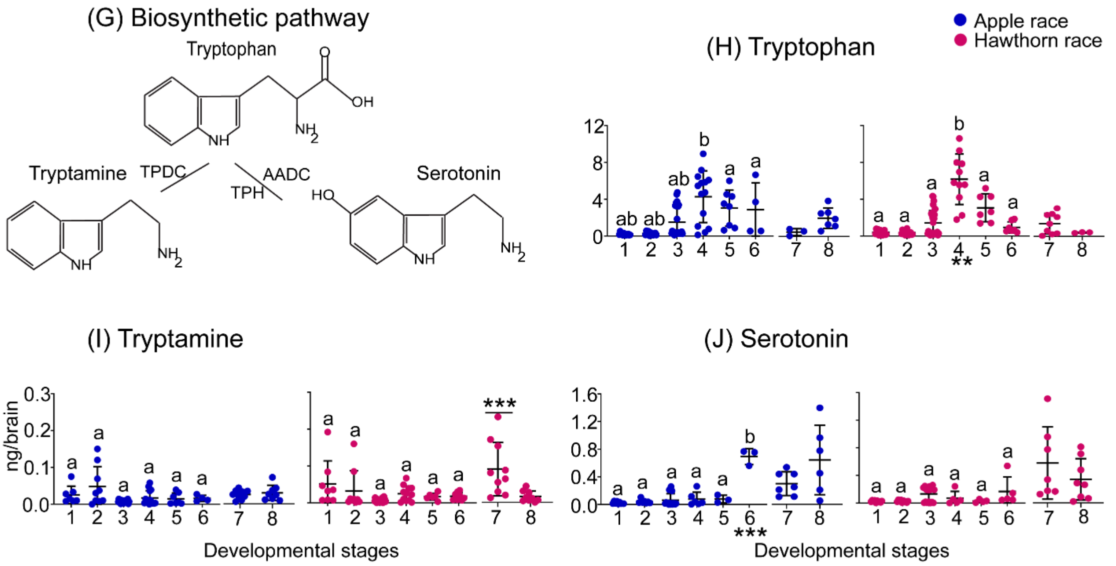


Figure A.3: Quantification of biogenic amines and their precursors across the different stages of development as defined in Figure 1. (G) Biosynthetic pathway for serotonin with enzymes tryptophan decarboxylase (TPDC), tryptophan hydroxylase (TPH) and amino acid decarboxylase (AADC); (H-J) Scatter plots 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 s3.

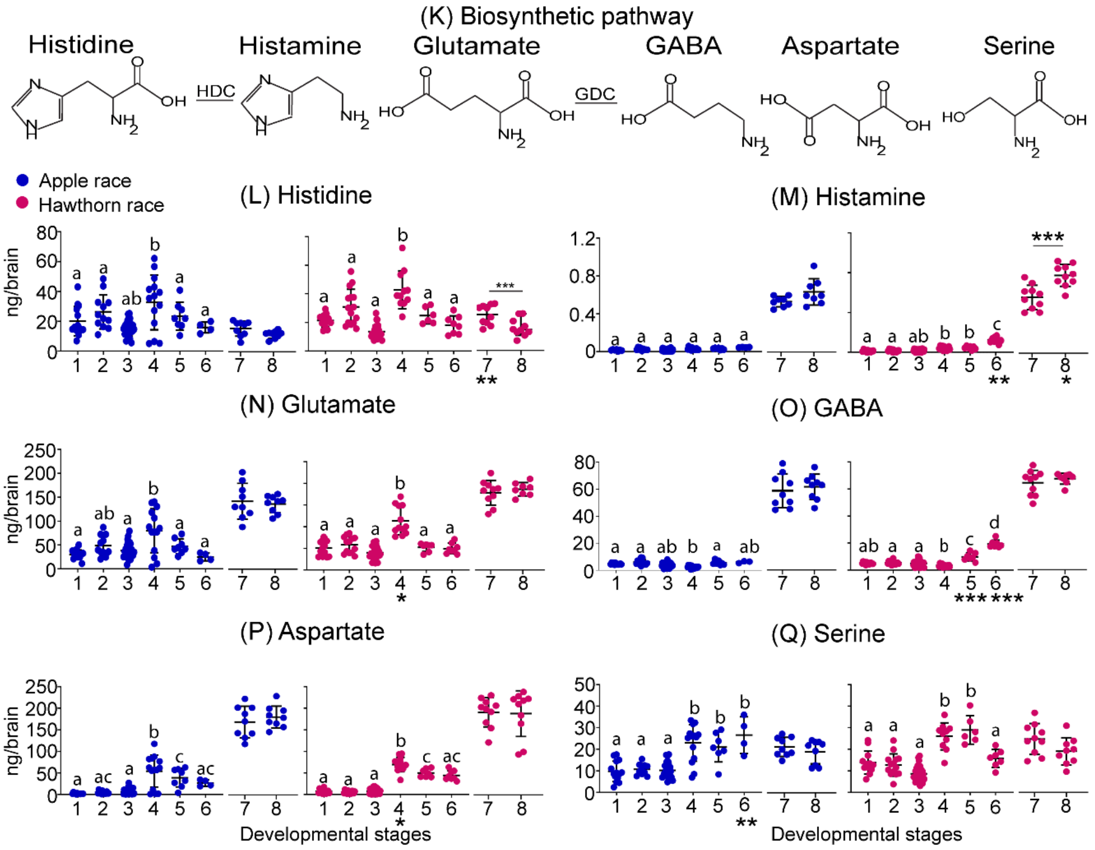


Figure A.4: Quantification of biogenic amines and their precursors across the different stages of development as defined in Figure 1. (K) Biosynthetic pathway for histamine, GABA, aspartate and glutamate with enzymes histidine decarboxylase (HDC) and glutamic acid decarboxylase (GDC); (L-Q) Scatter plots 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.

Table A.1: Apple race developmental stage comparisons for each neurotransmitter.

Histidine	estimate	SE	df	t.ratio	p.value	
Stage 1 - stage 2	6.371	3.407	151.083	1.870	0.7757	
Stage 1 - stage 3	-4.061	3.585	33.874	-1.133	0.9907	
Stage 1 - stage 4	-10.741	3.899	47.071	-2.755	0.2328	
Stage 1 - stage 5	1.367	4.392	66.085	0.311	1.0000	
Stage 1 - stage 6	-4.725	5.428	104.076	-0.871	0.9993	
Stage 2 - stage 3	10.432	3.704	38.220	2.816	0.2135	
Stage 2 - stage 4	-4.370	4.010	51.762	-1.090	0.9939	
Stage 2 - stage 5	5.004	4.491	70.592	1.114	0.9930	
Stage 2 - stage 6	11.096	5.507	107.217	2.015	0.6825	
Stage 3 - stage 4	-14.802	3.030	161.972	-4.884	0.0002	***
Stage 3 - stage 5	5.429	3.670	161.903	1.479	0.9442	
Stage 3 - stage 6	-0.664	4.847	161.618	-0.137	1.0000	
Stage 4 - stage 5	-9.373	3.869	154.609	-2.423	0.3975	
Stage 4 - stage 6	-15.466	5.065	158.895	-3.054	0.1032	
Stage 5 - stage 6	-6.092	5.379	154.487	-1.133	0.9927	
Stage 6 - stage 7	1.202	5.429	126.111	0.221	1.0000	
Stage 7 - stage 8	-3.832	2.015	34.319	-1.902	0.2461	
Serine	**estimate**	**SE**	**df**	**t.ratio**	**p.value**	
Stage 1 - stage 2	1.063	1.949	138.037	0.545	1.0000	
Stage 1 - stage 3	1.032	2.076	22.095	0.497	1.0000	
Stage 1 - stage 4	-12.141	2.225	30.012	-5.456	0.0003	***
Stage 1 - stage 5	10.053	2.565	47.807	3.919	0.0134	*
Stage 1 - stage 6	16.247	3.034	76.310	5.355	0.0001	***
Stage 2 - stage 3	0.031	2.231	28.723	0.014	1.0000	
Stage 2 - stage 4	-11.078	2.370	37.352	-4.673	0.0020	**
Stage 2 - stage 5	-8.990	2.692	55.369	-3.339	0.0602	
Stage 2 - stage 6	-15.185	3.142	82.795	-4.833	0.0004	***
Stage 3 - stage 4	-11.109	1.677	152.999	-6.626	0.0000	***
Stage 3 - stage 5	9.021	2.136	152.892	4.223	0.0024	**
Stage 3 - stage 6	15.215	2.662	151.748	5.716	0.0000	***
Stage 4 - stage 5	-2.087	2.217	144.325	-0.941	0.9985	
Stage 4 - stage 6	4.107	2.770	147.460	1.483	0.9431	
Stage 5 - stage 6	6.194	2.993	141.446	2.070	0.6448	

Stage 6 - stage 7	-5.013	3.391	83.998	-1.478	0.9821	
Stage 7 - stage 8	-1.905	2.140	33.275	-0.890	0.8100	

Histamine	estimate	SE	df	t.ratio	p.value	
Stage 1 - stage 2	0.008	0.005	124.053	1.479	0.9435	
Stage 1 - stage 3	0.004	0.007	22.004	0.600	1.0000	
Stage 1 - stage 4	-0.011	0.008	25.061	-1.502	0.9265	
Stage 1 - stage 5	0.012	0.008	31.253	1.489	0.9328	
Stage 1 - stage 6	0.019	0.009	43.301	2.116	0.6149	
Stage 2 - stage 3	0.004	0.007	18.503	0.535	1.0000	
Stage 2 - stage 4	-0.003	0.007	21.442	-0.455	1.0000	
Stage 2 - stage 5	-0.004	0.008	27.418	-0.504	1.0000	
Stage 2 - stage 6	-0.011	0.009	39.327	-1.244	0.9814	
Stage 3 - stage 4	-0.007	0.004	128.587	-1.765	0.8337	
Stage 3 - stage 5	0.008	0.005	126.947	1.586	0.9111	
Stage 3 - stage 6	0.014	0.006	126.495	2.372	0.4329	
Stage 4 - stage 5	0.001	0.005	123.084	0.119	1.0000	
Stage 4 - stage 6	0.007	0.006	124.226	1.164	0.9907	
Stage 5 - stage 6	0.007	0.007	122.512	1.001	0.9974	
Stage 6 - stage 7	0.547	0.037	65.148	14.904	0.0000	***
Stage 7 - stage 8	0.108	0.049	32.291	2.199	0.1450	

Aspartate	estimate	SE	df	t.ratio	p.value	
Stage 1 - stage 2	1.399	3.992	150.143	0.350	1.0000	
Stage 1 - stage 3	9.246	7.308	14.882	1.265	0.9720	
Stage 1 - stage 4	-40.685	7.475	16.384	-5.443	0.0021	**
Stage 1 - stage 5	29.274	7.953	20.686	3.681	0.0481	*
Stage 1 - stage 6	17.301	8.608	28.021	2.010	0.6836	
Stage 2 - stage 3	-7.847	7.216	14.207	-1.087	0.9904	
Stage 2 - stage 4	-39.286	7.385	15.680	-5.320	0.0029	**
Stage 2 - stage 5	-27.875	7.869	19.922	-3.543	0.0655	
Stage 2 - stage 6	-15.902	8.530	27.180	-1.864	0.7697	
Stage 3 - stage 4	-31.439	3.753	156.071	-8.376	0.0000	***
Stage 3 - stage 5	20.028	4.794	158.099	4.178	0.0028	**
Stage 3 - stage 6	8.055	5.695	154.341	1.414	0.9591	
Stage 4 - stage 5	-11.411	4.654	150.449	-2.452	0.3785	
Stage 4 - stage 6	-23.383	5.811	150.461	-4.024	0.0050	**

Stage 5 - stage 6	-11.973	6.239	149.385	-1.919	0.7455	
Stage 6 - stage 7	140.810	15.459	28.189	9.109	0.0000	***
Stage 7 - stage 8	19.787	12.142	33.940	1.630	0.3761	
Glutamate	estimate	SE	df	t.ratio	p.value	
Stage 1 - stage 2	14.427	7.698	151.146	1.874	0.7731	
Stage 1 - stage 3	8.118	9.807	23.444	0.828	0.9993	
Stage 1 - stage 4	-38.445	10.269	28.316	-3.744	0.0321	*
Stage 1 - stage 5	5.231	11.139	37.501	0.470	1.0000	
Stage 1 - stage 6	-14.688	13.042	61.913	-1.126	0.9922	
Stage 2 - stage 3	6.309	9.901	24.449	0.637	0.9999	
Stage 2 - stage 4	-24.018	10.359	29.410	-2.319	0.4864	
Stage 2 - stage 5	9.196	11.221	38.699	0.820	0.9995	
Stage 2 - stage 6	29.115	13.113	63.205	2.220	0.5418	
Stage 3 - stage 4	-30.327	6.618	159.008	-4.583	0.0006	***
Stage 3 - stage 5	-2.887	7.989	159.672	-0.361	1.0000	
Stage 3 - stage 6	-22.805	10.430	157.563	-2.186	0.5619	
Stage 4 - stage 5	-33.214	8.195	151.828	-4.053	0.0045	**
Stage 4 - stage 6	-53.133	10.774	153.802	-4.932	0.0001	***
Stage 5 - stage 6	-19.918	11.387	151.137	-1.749	0.8420	
Stage 6 - stage 7	138.704	14.722	70.082	9.421	0.0000	***
Stage 7 - stage 8	-5.360	10.449	33.531	-0.513	0.9554	
GABA	estimate	SE	df	t.ratio	p.value	
Stage 1 - stage 2	1.241	0.677	150.344	1.834	0.7968	
Stage 1 - stage 3	-0.631	0.658	48.227	-0.959	0.9979	
Stage 1 - stage 4	2.451	0.736	69.142	3.329	0.0577	*
Stage 1 - stage 5	0.509	0.849	95.475	0.599	1.0000	
Stage 1 - stage 6	0.916	1.129	130.683	0.811	0.9996	
Stage 2 - stage 3	1.872	0.657	48.532	2.851	0.1915	
Stage 2 - stage 4	3.693	0.735	69.593	5.022	0.0002	***
Stage 2 - stage 5	0.733	0.848	96.002	0.864	0.9993	
Stage 2 - stage 6	0.325	1.129	131.041	0.288	1.0000	
Stage 3 - stage 4	1.821	0.577	157.999	3.153	0.0793	
Stage 3 - stage 5	1.139	0.723	157.540	1.575	0.9155	
Stage 3 - stage 6	1.547	1.042	157.327	1.484	0.9429	

Stage 4 - stage 5	2.960	0.786	155.447	3.766	0.0122	*
Stage 4 - stage 6	3.368	1.092	157.989	3.083	0.0958	
Stage 5 - stage 6	0.407	1.149	151.780	0.355	1.0000	
Stage 6 - stage 7	54.622	3.142	48.236	17.385	0.0000	***
Stage 7 - stage 8	3.147	3.162	33.405	0.995	0.7532	
Dopa	estimate	SE	df	t.ratio	p.value	
Stage 1 - stage 2	0.023	0.028	151.202	0.815	0.9996	
Stage 1 - stage 3	0.010	0.026	51.438	0.382	1.0000	
Stage 1 - stage 4	-0.090	0.030	76.858	-3.012	0.1250	
Stage 1 - stage 5	0.084	0.034	99.031	2.437	0.3920	
Stage 1 - stage 6	0.038	0.046	135.843	0.826	0.9996	
Stage 2 - stage 3	0.013	0.028	61.159	0.449	1.0000	
Stage 2 - stage 4	-0.068	0.032	85.070	-2.144	0.5935	
Stage 2 - stage 5	-0.061	0.036	104.808	-1.711	0.8592	
Stage 2 - stage 6	-0.016	0.047	137.512	-0.329	1.0000	
Stage 3 - stage 4	-0.080	0.025	156.137	-3.208	0.0684	
Stage 3 - stage 5	0.074	0.030	154.741	2.424	0.3964	
Stage 3 - stage 6	0.028	0.043	157.566	0.649	1.0000	
Stage 4 - stage 5	-0.006	0.033	152.074	-0.195	1.0000	
Stage 4 - stage 6	-0.052	0.045	157.392	-1.148	0.9919	
Stage 5 - stage 6	-0.046	0.048	153.403	-0.957	0.9983	
Stage 6 - stage 7	-0.060	0.044	164.574	-1.359	0.9928	
Stage 7 - stage 8	-0.004	0.002	32.308	-2.134	0.1639	
Octopamine	estimate	SE	df	t.ratio	p.value	
Stage 1 - stage 2	0.001	0.048	76.506	0.025	1.0000	
Stage 1 - stage 3	0.040	0.058	14.225	0.691	0.9998	
Stage 1 - stage 4	-0.064	0.058	14.302	-1.097	0.9897	
Stage 1 - stage 5	0.178	0.060	16.767	2.959	0.2045	
Stage 1 - stage 6	0.046	0.067	23.801	0.689	0.9999	
Stage 2 - stage 3	-0.039	0.058	14.225	-0.670	0.9999	
Stage 2 - stage 4	-0.062	0.058	14.302	-1.077	0.9911	
Stage 2 - stage 5	-0.176	0.060	16.767	-2.939	0.2111	
Stage 2 - stage 6	-0.045	0.067	23.801	-0.671	0.9999	
Stage 3 - stage 4	-0.024	0.030	79.895	-0.778	0.9997	
Stage 3 - stage 5	0.138	0.037	88.385	3.766	0.0148	*

Stage 3 - stage 6	0.006	0.046	86.032	0.134	1.0000	
Stage 4 - stage 5	0.114	0.035	85.911	3.216	0.0737	
Stage 4 - stage 6	-0.017	0.047	85.383	-0.371	1.0000	
Stage 5 - stage 6	-0.131	0.048	82.623	-2.729	0.2309	
Stage 6 - stage 7	0.266	0.053	52.013	5.005	0.0007	***
Stage 7 - stage 8	0.035	0.028	31.031	1.261	0.5941	
Tyrosine	estimate	SE	df	t.ratio	p.value	
Stage 1 - stage 2	0.214	3.506	143.844	0.061	1.0000	
Stage 1 - stage 3	3.727	3.743	20.949	0.996	0.9961	
Stage 1 - stage 4	-31.550	3.951	27.630	-7.984	0.0000	***
Stage 1 - stage 5	29.089	4.564	43.701	6.373	0.0000	***
Stage 1 - stage 6	14.414	5.361	72.441	2.689	0.2531	
Stage 2 - stage 3	-3.513	4.144	30.704	-0.848	0.9992	
Stage 2 - stage 4	-31.336	4.333	38.561	-7.232	0.0000	***
Stage 2 - stage 5	-28.875	4.898	55.046	-5.895	0.0000	***
Stage 2 - stage 6	-14.200	5.649	82.935	-2.514	0.3463	
Stage 3 - stage 4	-27.823	3.130	154.271	-8.889	0.0000	***
Stage 3 - stage 5	25.362	3.965	149.990	6.397	0.0000	***
Stage 3 - stage 6	10.688	4.769	158.934	2.241	0.5228	
Stage 4 - stage 5	-2.461	3.909	150.955	-0.630	1.0000	
Stage 4 - stage 6	-17.135	4.933	154.834	-3.474	0.0314	*
Stage 5 - stage 6	-14.674	5.298	148.207	-2.770	0.2041	
Stage 6 - stage 7	-8.167	5.179	105.731	-1.577	0.9691	
Stage 7 - stage 8	-3.043	1.010	33.180	-3.012	0.0243	*
Dopamine	estimate	SE	df	t.ratio	p.value	
Stage 1 - stage 2	-0.003	0.007	129.585	-0.443	1.0000	
Stage 1 - stage 3	-0.018	0.007	39.654	-2.775	0.2294	
Stage 1 - stage 4	0.010	0.007	52.027	1.430	0.9519	
Stage 1 - stage 5	0.037	0.008	68.581	4.577	0.0012	**
Stage 1 - stage 6	0.036	0.010	98.808	3.554	0.0274	*
Stage 2 - stage 3	0.015	0.007	47.039	2.201	0.5569	
Stage 2 - stage 4	0.007	0.008	59.031	0.979	0.9976	
Stage 2 - stage 5	-0.040	0.008	74.580	-4.738	0.0006	***
Stage 2 - stage 6	-0.039	0.010	102.045	-3.740	0.0152	*

Stage 3 - stage 4	-0.008	0.006	138.978	-1.355	0.9699	
Stage 3 - stage 5	0.055	0.007	138.998	7.962	0.0000	***
Stage 3 - stage 6	0.054	0.009	138.996	5.900	0.0000	***
Stage 4 - stage 5	0.047	0.007	132.607	6.437	0.0000	***
Stage 4 - stage 6	0.046	0.010	137.264	4.815	0.0002	***
Stage 5 - stage 6	-0.001	0.010	133.701	-0.117	1.0000	
Stage 6 - stage 7	0.326	0.038	51.695	8.569	0.0000	***
Stage 7 - stage 8	0.137	0.048	29.463	2.853	0.0372	*
Tyramine	estimate	SE	df	t.ratio	p.value	
Stage 1 - stage 2	0.003	0.003	135.180	1.027	0.9968	
Stage 1 - stage 3	0.003	0.005	17.670	0.561	1.0000	
Stage 1 - stage 4	-0.006	0.005	19.819	-1.376	0.9555	
Stage 1 - stage 5	0.013	0.005	24.772	2.620	0.3205	
Stage 1 - stage 6	0.007	0.006	38.618	1.260	0.9795	
Stage 2 - stage 3	0.001	0.005	18.217	0.134	1.0000	
Stage 2 - stage 4	-0.003	0.005	20.397	-0.694	0.9998	
Stage 2 - stage 5	-0.010	0.005	25.406	-1.972	0.7068	
Stage 2 - stage 6	-0.004	0.006	39.351	-0.694	0.9999	
Stage 3 - stage 4	-0.004	0.003	141.612	-1.509	0.9360	
Stage 3 - stage 5	0.010	0.003	143.022	3.336	0.0482	*
Stage 3 - stage 6	0.005	0.004	141.109	1.110	0.9938	
Stage 4 - stage 5	0.007	0.003	136.402	2.082	0.6366	
Stage 4 - stage 6	0.001	0.004	137.728	0.156	1.0000	
Stage 5 - stage 6	-0.006	0.004	135.929	-1.348	0.9710	
Stage 6 - stage 7	0.049	0.009	67.221	5.461	0.0001	***
Stage 7 - stage 8	0.019	0.010	29.919	1.772	0.3062	
Serotonine	estimate	SE	df	t.ratio	p.value	
Stage 1 - stage 2	0.014	0.030	85.493	0.457	1.0000	
Stage 1 - stage 3	-0.061	0.048	29.979	-1.273	0.9766	
Stage 1 - stage 4	0.042	0.044	21.584	0.961	0.9972	
Stage 1 - stage 5	0.042	0.054	43.400	0.786	0.9997	
Stage 1 - stage 6	0.591	0.054	42.806	10.982	0.0000	***
Stage 2 - stage 3	-0.028	0.045	24.148	-0.631	0.9999	
Stage 2 - stage 4	-0.047	0.049	32.698	-0.963	0.9976	
Stage 2 - stage 5	-0.029	0.055	45.984	-0.524	1.0000	

Stage 2 - stage 6	-0.578	0.055	45.380	-10.512	0.0000	***
Stage 3 - stage 4	-0.019	0.031	90.632	-0.605	1.0000	
Stage 3 - stage 5	0.000	0.040	90.045	0.009	1.0000	
Stage 3 - stage 6	0.549	0.040	91.956	13.743	0.0000	***
Stage 4 - stage 5	-0.018	0.042	86.815	-0.433	1.0000	
Stage 4 - stage 6	0.531	0.046	90.905	11.592	0.0000	***
Stage 5 - stage 6	0.549	0.052	89.988	10.537	0.0000	***
Stage 6 - stage 7	-0.276	0.119	90.703	-2.318	0.6121	
Stage 7 - stage 8	0.351	0.170	23.979	2.066	0.1930	

Trytophan	estimate	SE	df	t.ratio	p.value	
Stage 1 - stage 2	0.039	0.374	153.277	0.104	1.0000	
Stage 1 - stage 3	-3.202	0.845	15.303	-3.790	0.0517	*
Stage 1 - stage 4	1.360	0.829	14.162	1.642	0.8673	
Stage 1 - stage 5	2.218	0.874	17.431	2.539	0.3782	
Stage 1 - stage 6	2.397	0.941	23.160	2.547	0.3611	
Stage 2 - stage 3	-1.321	0.831	14.321	-1.590	0.8875	
Stage 2 - stage 4	-3.163	0.847	15.469	-3.734	0.0566	*
Stage 2 - stage 5	-2.179	0.876	17.606	-2.488	0.4042	
Stage 2 - stage 6	-2.357	0.943	23.357	-2.500	0.3859	
Stage 3 - stage 4	-1.842	0.331	155.833	-5.563	0.0000	***
Stage 3 - stage 5	0.858	0.406	156.640	2.113	0.6141	
Stage 3 - stage 6	1.036	0.535	155.789	1.937	0.7345	
Stage 4 - stage 5	-0.984	0.419	153.907	-2.347	0.4485	
Stage 4 - stage 6	-0.806	0.553	154.418	-1.457	0.9495	
Stage 5 - stage 6	0.178	0.582	153.568	0.306	1.0000	
Stage 6 - stage 7	-1.898	1.014	59.751	-1.872	0.8779	
Stage 7 - stage 8	1.508	0.662	22.000	2.277	0.1342	

Tryptamine	estimate	SE	df	t.ratio	p.value	
Stage 1 - stage 2	0.011	0.013	117.630	0.871	0.9993	
Stage 1 - stage 3	0.015	0.015	29.367	0.982	0.9970	
Stage 1 - stage 4	-0.021	0.015	27.358	-1.448	0.9423	
Stage 1 - stage 5	-0.020	0.016	39.291	-1.230	0.9830	
Stage 1 - stage 6	-0.016	0.018	55.095	-0.864	0.9992	

Stage 2 - stage 3	0.032	0.014	22.894	2.307	0.4987
Stage 2 - stage 4	0.026	0.014	24.745	1.805	0.8008
Stage 2 - stage 5	0.031	0.016	34.570	1.979	0.7036
Stage 2 - stage 6	0.027	0.018	50.649	1.508	0.9315
Stage 3 - stage 4	-0.007	0.009	121.931	-0.733	0.9999
Stage 3 - stage 5	0.001	0.012	123.901	0.104	1.0000
Stage 3 - stage 6	0.005	0.014	122.157	0.384	1.0000
Stage 4 - stage 5	-0.005	0.011	117.781	-0.481	1.0000
Stage 4 - stage 6	-0.001	0.014	119.058	-0.087	1.0000
Stage 5 - stage 6	0.004	0.016	116.644	0.274	1.0000
Stage 6 - stage 7	0.012	0.019	85.467	0.643	1.0000
Stage 7 - stage 8	0.006	0.017	32.294	0.348	0.9853

Table A.2: Hawthorn race developmental stage comparisons for each neurotransmitter.

Histidine	estimate	SE	df	t.ratio	p.value	
Stage 1 - stage 2	9.276	3.347	150.263	2.771	0.2033	
Stage 1 - stage 3	-8.142	3.637	35.672	-2.238	0.5348	
Stage 1 - stage 4	-18.568	4.222	57.470	-4.398	0.0026	**
Stage 1 - stage 5	1.402	4.916	79.211	0.285	1.0000	
Stage 1 - stage 6	-4.555	4.666	62.243	-0.976	0.9977	
Stage 2 - stage 3	17.418	3.458	29.777	5.037	0.0011	**
Stage 2 - stage 4	-9.292	4.068	51.231	-2.284	0.5002	
Stage 2 - stage 5	7.874	4.784	73.875	1.646	0.8858	
Stage 2 - stage 6	13.831	4.527	56.897	3.055	0.1196	
Stage 3 - stage 4	-26.710	3.223	160.624	-8.287	0.0000	***
Stage 3 - stage 5	9.544	4.179	155.893	2.284	0.4926	
Stage 3 - stage 6	3.587	3.907	134.569	0.918	0.9988	
Stage 4 - stage 5	-17.166	4.439	154.574	-3.867	0.0086	**
Stage 4 - stage 6	-23.123	4.184	160.902	-5.527	0.0000	***
Stage 5 - stage 6	-5.956	4.705	152.912	-1.266	0.9821	
Stage 6 - stage 7	7.499	4.607	75.357	1.628	0.9579	
Stage 7 - stage 8	-9.468	1.968	35.419	-4.811	0.0002	***
Serine	**estimate**	**SE**	**df**	**t.ratio**	**p.value**	
Stage 1 - stage 2	-1.418	1.868	141.230	-0.759	0.9998	
Stage 1 - stage 3	-5.356	2.122	23.491	-2.524	0.3726	
Stage 1 - stage 4	-10.034	2.449	39.893	-4.098	0.0094	**
Stage 1 - stage 5	13.044	2.784	53.839	4.686	0.0011	**
Stage 1 - stage 6	0.213	2.726	46.666	0.078	1.0000	
Stage 2 - stage 3	3.938	2.050	20.828	1.921	0.7351	
Stage 2 - stage 4	-11.452	2.386	36.864	-4.799	0.0014	**
Stage 2 - stage 5	-14.463	2.729	51.030	-5.300	0.0001	***
Stage 2 - stage 6	-1.632	2.671	43.962	-0.611	1.0000	
Stage 3 - stage 4	-15.391	1.854	152.411	-8.301	0.0000	***
Stage 3 - stage 5	18.401	2.342	144.086	7.858	0.0000	***
Stage 3 - stage 6	5.570	2.284	129.639	2.439	0.3880	
Stage 4 - stage 5	3.010	2.480	144.963	1.214	0.9871	
Stage 4 - stage 6	-9.821	2.425	150.453	-4.049	0.0045	**

Stage 5 - stage 6	-12.831	2.630	139.104	-4.879	0.0002	***
Stage 6 - stage 7	9.510	3.033	54.417	3.135	0.1522	
Stage 7 - stage 8	-5.639	2.156	34.588	-2.615	0.0603	

Histamine	estimate	SE	df	t.ratio	p.value	
Stage 1 - stage 2	0.003	0.005	122.000	0.652	1.0000	
Stage 1 - stage 3	0.006	0.007	16.911	0.862	0.9987	
Stage 1 - stage 4	-0.024	0.007	20.330	-3.422	0.0818	
Stage 1 - stage 5	0.023	0.008	26.656	2.984	0.1684	
Stage 1 - stage 6	0.099	0.008	25.619	12.871	0.0000	***
Stage 2 - stage 3	-0.003	0.007	16.913	-0.427	1.0000	
Stage 2 - stage 4	-0.021	0.007	20.332	-3.008	0.1759	
Stage 2 - stage 5	-0.020	0.008	26.657	-2.602	0.3267	
Stage 2 - stage 6	-0.096	0.008	25.621	-12.485	0.0000	***
Stage 3 - stage 4	-0.019	0.004	128.300	-4.546	0.0007	***
Stage 3 - stage 5	0.017	0.005	130.521	3.306	0.0532	
Stage 3 - stage 6	0.093	0.005	131.989	18.023	0.0000	***
Stage 4 - stage 5	-0.001	0.005	123.136	-0.274	1.0000	
Stage 4 - stage 6	0.074	0.005	125.213	14.531	0.0000	***
Stage 5 - stage 6	0.076	0.006	122.734	13.533	0.0000	***
Stage 6 - stage 7	0.491	0.032	42.550	15.121	0.0000	***
Stage 7 - stage 8	0.239	0.046	34.818	5.245	0.0000	***

Aspartate	estimate	SE	df	t.ratio	p.value	
Stage 1 - stage 2	-1.449	3.765	148.725	-0.385	1.0000	
Stage 1 - stage 3	2.659	7.098	13.362	0.375	1.0000	
Stage 1 - stage 4	-51.190	7.497	16.510	-6.828	0.0002	***
Stage 1 - stage 5	30.027	7.997	20.931	3.755	0.0409	*
Stage 1 - stage 6	25.495	7.969	20.489	3.199	0.1244	
Stage 2 - stage 3	-4.108	7.065	13.108	-0.581	1.0000	
Stage 2 - stage 4	-52.639	7.466	16.231	-7.051	0.0001	***
Stage 2 - stage 5	-31.476	7.968	20.624	-3.950	0.0275	*
Stage 2 - stage 6	-26.943	7.940	20.187	-3.393	0.0868	
Stage 3 - stage 4	-48.531	3.757	158.024	-12.917	0.0000	***
Stage 3 - stage 5	27.367	4.838	159.305	5.657	0.0000	***

Stage 3 - stage 6	22.835	4.833	160.420	4.725	0.0003	***
Stage 4 - stage 5	-21.163	4.729	149.953	-4.476	0.0009	***
Stage 4 - stage 6	-25.696	4.726	152.108	-5.437	0.0000	***
Stage 5 - stage 6	-4.532	5.133	149.299	-0.883	0.9992	
Stage 6 - stage 7	138.073	14.720	23.154	9.380	0.0000	***
Stage 7 - stage 8	1.989	11.920	34.600	0.167	0.9983	

Glutamate	estimate	SE	df	t.ratio	p.value	
Stage 1 - stage 2	10.268	7.187	150.078	1.429	0.9560	
Stage 1 - stage 3	-9.792	9.444	20.556	-1.037	0.9945	
Stage 1 - stage 4	-54.054	10.433	29.450	-5.181	0.0008	***
Stage 1 - stage 5	-4.787	11.563	40.664	-0.414	1.0000	
Stage 1 - stage 6	-5.993	11.470	38.221	-0.523	1.0000	
Stage 2 - stage 3	20.060	9.447	20.566	2.123	0.6138	
Stage 2 - stage 4	-43.786	10.436	29.456	-4.196	0.0102	*
Stage 2 - stage 5	15.055	11.566	40.666	1.302	0.9742	
Stage 2 - stage 6	16.261	11.473	38.224	1.417	0.9527	
Stage 3 - stage 4	-63.846	6.777	160.688	-9.420	0.0000	***
Stage 3 - stage 5	5.005	8.655	160.956	0.578	1.0000	
Stage 3 - stage 6	3.799	8.599	158.982	0.442	1.0000	
Stage 4 - stage 5	-58.841	8.814	151.686	-6.676	0.0000	***
Stage 4 - stage 6	-60.047	8.771	155.796	-6.846	0.0000	***
Stage 5 - stage 6	-1.206	9.594	151.276	-0.126	1.0000	
Stage 6 - stage 7	130.702	13.177	45.848	9.919	0.0000	***
Stage 7 - stage 8	0.599	10.508	32.803	0.057	0.9999	

GABA	estimate	SE	df	t.ratio	p.value	
Stage 1 - stage 2	0.408	0.646	146.484	0.633	1.0000	
Stage 1 - stage 3	-0.125	0.631	42.598	-0.199	1.0000	
Stage 1 - stage 4	2.123	0.739	66.294	2.872	0.1752	
Stage 1 - stage 5	4.543	0.855	82.223	5.316	0.0001	***
Stage 1 - stage 6	14.067	0.864	75.191	16.289	0.0000	***
Stage 2 - stage 3	0.534	0.630	42.857	0.847	0.9993	
Stage 2 - stage 4	2.532	0.738	66.702	3.429	0.0446	*
Stage 2 - stage 5	-4.135	0.854	82.641	-4.843	0.0004	***
Stage 2 - stage 6	-13.659	0.863	75.552	-15.831	0.0000	***

Stage 3 - stage 4	1.998	0.589	152.002	3.391	0.0405	*
Stage 3 - stage 5	4.669	0.742	138.780	6.293	0.0000	***
Stage 3 - stage 6	14.192	0.756	120.712	18.766	0.0000	***
Stage 4 - stage 5	6.667	0.792	152.142	8.423	0.0000	***
Stage 4 - stage 6	16.191	0.806	157.617	20.090	0.0000	***
Stage 5 - stage 6	9.524	0.887	150.513	10.742	0.0000	***
Stage 6 - stage 7	44.577	2.813	31.803	15.844	0.0000	***
Stage 7 - stage 8	0.119	3.172	32.632	0.038	1.0000	
Dopa	estimate	SE	df	t.ratio	p.value	
Stage 1 - stage 2	-0.015	0.026	149.242	-0.586	1.0000	
Stage 1 - stage 3	-0.057	0.026	50.511	-2.214	0.5476	
Stage 1 - stage 4	-0.197	0.032	84.637	-6.206	0.0000	***
Stage 1 - stage 5	-0.007	0.035	90.981	-0.184	1.0000	
Stage 1 - stage 6	-0.001	0.036	85.102	-0.021	1.0000	
Stage 2 - stage 3	0.042	0.025	45.009	1.691	0.8628	
Stage 2 - stage 4	-0.212	0.031	80.206	-6.860	0.0000	***
Stage 2 - stage 5	-0.009	0.035	87.452	-0.252	1.0000	
Stage 2 - stage 6	-0.014	0.035	81.611	-0.414	1.0000	
Stage 3 - stage 4	-0.254	0.026	154.879	-9.845	0.0000	***
Stage 3 - stage 5	0.051	0.031	137.263	1.654	0.8851	
Stage 3 - stage 6	0.057	0.031	123.811	1.812	0.8085	
Stage 4 - stage 5	-0.203	0.034	153.546	-5.965	0.0000	***
Stage 4 - stage 6	-0.198	0.035	157.037	-5.727	0.0000	***
Stage 5 - stage 6	0.006	0.037	151.827	0.156	1.0000	
Stage 6 - stage 7	-0.049	0.034	105.827	-1.440	0.9865	
Stage 7 - stage 8	-0.006	0.002	33.142	-3.582	0.0057	**
Octopamine	estimate	SE	df	t.ratio	p.value	
Stage 1 - stage 2	0.001	0.045	78.237	0.013	1.0000	
Stage 1 - stage 3	0.014	0.060	16.474	0.230	1.0000	
Stage 1 - stage 4	-0.007	0.060	16.162	-0.112	1.0000	
Stage 1 - stage 5	0.011	0.065	21.600	0.172	1.0000	
Stage 1 - stage 6	0.092	0.064	19.306	1.441	0.9400	
Stage 2 - stage 3	-0.013	0.057	13.286	-0.234	1.0000	
Stage 2 - stage 4	-0.011	0.062	18.191	-0.172	1.0000	

Stage 2 - stage 5	-0.091	0.060	16.016	-1.511	0.9168	
Stage 2 - stage 6	-0.006	0.056	12.984	-0.109	1.0000	
Stage 3 - stage 4	0.007	0.037	81.053	0.192	1.0000	
Stage 3 - stage 5	-0.003	0.046	84.353	-0.057	1.0000	
Stage 3 - stage 6	0.078	0.045	86.910	1.741	0.8437	
Stage 4 - stage 5	0.005	0.043	79.358	0.104	1.0000	
Stage 4 - stage 6	0.085	0.042	82.551	2.021	0.6781	
Stage 5 - stage 6	0.080	0.048	81.878	1.680	0.8719	
Stage 6 - stage 7	0.246	0.048	37.764	5.110	0.0009	***
Stage 7 - stage 8	0.102	0.029	34.099	3.478	0.0073	**
Tyrosine	estimate	SE	df	t.ratio	p.value	
Stage 1 - stage 2	-0.162	3.203	143.955	-0.050	1.0000	
Stage 1 - stage 3	0.893	3.666	20.373	0.244	1.0000	
Stage 1 - stage 4	-59.843	4.264	33.650	-14.033	0.0000	***
Stage 1 - stage 5	41.483	4.747	44.530	8.739	0.0000	***
Stage 1 - stage 6	32.016	4.690	39.213	6.827	0.0000	***
Stage 2 - stage 3	-1.054	3.614	19.252	-0.292	1.0000	
Stage 2 - stage 4	-60.004	4.220	32.347	-14.218	0.0000	***
Stage 2 - stage 5	-41.644	4.707	43.212	-8.847	0.0000	***
Stage 2 - stage 6	-32.178	4.650	37.989	-6.920	0.0000	***
Stage 3 - stage 4	-58.950	3.207	149.014	-18.380	0.0000	***
Stage 3 - stage 5	40.590	3.906	143.094	10.391	0.0000	***
Stage 3 - stage 6	31.124	3.864	123.510	8.055	0.0000	***
Stage 4 - stage 5	-18.360	4.053	146.710	-4.530	0.0007	***
Stage 4 - stage 6	-27.826	4.027	155.429	-6.909	0.0000	***
Stage 5 - stage 6	-9.467	4.361	147.819	-2.171	0.5731	
Stage 6 - stage 7	-25.148	4.438	57.724	-5.667	0.0001	***
Stage 7 - stage 8	-5.046	1.015	34.743	-4.971	0.0001	***
Dopamine	estimate	SE	df	t.ratio	p.value	
Stage 1 - stage 2	0.018	0.006	130.316	2.791	0.1962	
Stage 1 - stage 3	-0.012	0.007	43.805	-1.692	0.8622	
Stage 1 - stage 4	0.003	0.008	59.563	0.382	1.0000	
Stage 1 - stage 5	0.003	0.009	76.067	0.290	1.0000	

Stage 1 - stage 6	0.024	0.009	73.094	2.670	0.2620	
Stage 2 - stage 3	0.030	0.007	43.931	4.242	0.0056	**
Stage 2 - stage 4	0.021	0.008	59.756	2.660	0.2716	
Stage 2 - stage 5	0.015	0.009	76.288	1.683	0.8705	
Stage 2 - stage 6	-0.006	0.009	73.300	-0.707	0.9999	
Stage 3 - stage 4	-0.009	0.007	138.767	-1.340	0.9722	
Stage 3 - stage 5	0.015	0.008	136.845	1.764	0.8341	
Stage 3 - stage 6	0.036	0.008	133.070	4.352	0.0015	**
Stage 4 - stage 5	0.006	0.008	132.109	0.664	0.9999	
Stage 4 - stage 6	0.027	0.009	136.874	3.165	0.0782	
Stage 5 - stage 6	0.022	0.009	132.595	2.284	0.4930	
Stage 6 - stage 7	0.425	0.037	43.356	11.619	0.0000	***
Stage 7 - stage 8	-0.039	0.050	29.556	-0.783	0.8613	
Tyramine	estimate	SE	df	t.ratio	p.value	
Stage 1 - stage 2	0.000	0.003	135.973	0.140	1.0000	
Stage 1 - stage 3	0.003	0.005	17.571	0.746	0.9997	
Stage 1 - stage 4	-0.026	0.005	22.760	-5.287	0.0011	**
Stage 1 - stage 5	0.022	0.005	28.946	4.152	0.0116	*
Stage 1 - stage 6	0.024	0.005	30.015	4.598	0.0035	**
Stage 2 - stage 3	-0.003	0.004	15.570	-0.674	0.9999	
Stage 2 - stage 4	-0.025	0.005	20.545	-5.343	0.0013	**
Stage 2 - stage 5	-0.021	0.005	26.577	-4.168	0.0124	*
Stage 2 - stage 6	-0.024	0.005	27.641	-4.622	0.0038	**
Stage 3 - stage 4	-0.022	0.003	143.647	-8.128	0.0000	***
Stage 3 - stage 5	0.018	0.003	146.146	5.285	0.0000	***
Stage 3 - stage 6	0.021	0.004	146.802	5.827	0.0000	***
Stage 4 - stage 5	-0.004	0.003	137.031	-1.151	0.9916	
Stage 4 - stage 6	-0.001	0.004	139.614	-0.367	1.0000	
Stage 5 - stage 6	0.003	0.004	136.606	0.692	0.9999	
Stage 6 - stage 7	0.021	0.008	46.521	2.679	0.3735	
Stage 7 - stage 8	-0.005	0.010	31.490	-0.436	0.9718	
Serotonine	estimate	SE	df	t.ratio	p.value	
Stage 1 - stage 2	0.006	0.027	89.821	0.221	1.0000	
Stage 1 - stage 3	0.056	0.043	20.501	1.318	0.9669	

Stage 1 - stage 4	-0.015	0.050	31.267	-0.304	1.0000	
Stage 1 - stage 5	-0.023	0.051	36.331	-0.448	1.0000	
Stage 1 - stage 6	0.140	0.050	31.763	2.776	0.2376	
Stage 2 - stage 3	-0.050	0.044	21.172	-1.152	0.9876	
Stage 2 - stage 4	-0.009	0.050	31.693	-0.180	1.0000	
Stage 2 - stage 5	0.029	0.052	36.655	0.558	1.0000	
Stage 2 - stage 6	-0.134	0.051	32.172	-2.616	0.3121	
Stage 3 - stage 4	0.041	0.038	92.342	1.074	0.9951	
Stage 3 - stage 5	-0.079	0.041	94.984	-1.947	0.7267	
Stage 3 - stage 6	0.083	0.040	91.104	2.063	0.6493	
Stage 4 - stage 5	-0.038	0.042	89.791	-0.903	0.9989	
Stage 4 - stage 6	0.124	0.047	93.061	2.676	0.2551	
Stage 5 - stage 6	0.162	0.045	94.856	3.616	0.0230	*
Stage 6 - stage 7	0.277	0.111	64.520	2.503	0.4824	
Stage 7 - stage 8	-0.083	0.166	24.891	-0.500	0.9584	

Trytophan	estimate	SE	df	t.ratio	p.value	
Stage 1 - stage 2	-0.029	0.360	153.272	-0.081	1.0000	
Stage 1 - stage 3	0.769	0.823	13.797	0.934	0.9971	
Stage 1 - stage 4	-4.620	0.857	16.158	-5.391	0.0024	**
Stage 1 - stage 5	1.955	0.893	18.913	2.188	0.5752	
Stage 1 - stage 6	1.105	0.903	19.616	1.224	0.9800	
Stage 2 - stage 3	-0.798	0.820	13.621	-0.973	0.9959	
Stage 2 - stage 4	-4.649	0.854	15.969	-5.441	0.0022	**
Stage 2 - stage 5	-1.984	0.891	18.711	-2.226	0.5520	
Stage 2 - stage 6	-1.134	0.900	19.412	-1.260	0.9753	
Stage 3 - stage 4	-3.850	0.357	157.125	-10.774	0.0000	***
Stage 3 - stage 5	1.185	0.454	158.822	2.609	0.2837	
Stage 3 - stage 6	0.336	0.477	159.967	0.705	0.9999	
Stage 4 - stage 5	-2.665	0.462	154.036	-5.774	0.0000	***
Stage 4 - stage 6	-3.514	0.483	155.391	-7.283	0.0000	***
Stage 5 - stage 6	-0.849	0.513	153.991	-1.655	0.8854	
Stage 6 - stage 7	0.238	0.866	33.672	0.275	1.0000	
Stage 7 - stage 8	-1.082	0.640	22.000	-1.690	0.3522	

Tryptamine	estimate	SE	df	t.ratio	p.value	
Stage 1 - stage 2	-0.012	0.012	115.536	-1.017	0.9970	
Stage 1 - stage 3	-0.037	0.014	23.247	-2.665	0.3017	
Stage 1 - stage 4	0.026	0.015	28.290	1.732	0.8391	
Stage 1 - stage 5	-0.040	0.017	39.914	-2.389	0.4358	
Stage 1 - stage 6	-0.039	0.016	34.460	-2.423	0.4184	
Stage 2 - stage 3	0.025	0.014	21.537	1.836	0.7826	
Stage 2 - stage 4	0.013	0.015	26.479	0.930	0.9980	
Stage 2 - stage 5	0.027	0.016	38.035	1.684	0.8644	
Stage 2 - stage 6	0.027	0.016	32.662	1.695	0.8576	
Stage 3 - stage 4	-0.012	0.009	123.758	-1.232	0.9853	
Stage 3 - stage 5	-0.002	0.012	123.974	-0.192	1.0000	
Stage 3 - stage 6	-0.002	0.012	120.855	-0.141	1.0000	
Stage 4 - stage 5	-0.014	0.012	116.620	-1.140	0.9921	
Stage 4 - stage 6	-0.013	0.012	120.676	-1.140	0.9921	
Stage 5 - stage 6	0.001	0.013	115.855	0.054	1.0000	
Stage 6 - stage 7	0.074	0.017	52.353	4.442	0.0042	**
Stage 7 - stage 8	-0.074	0.017	34.526	-4.423	0.0005	***