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The Impeccable Timing of the Apple Maggot Fly, *Rhagoletis pomonella*
(Diptera: Tephritidae), and its Implications for Ecological Speciation

by

Monte Arthur Mattsson

A thesis submitted in partial fulfillment of the
requirements for the degree of

Master of Science
in
Biology

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Portland State University
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Abstract

Speciation is the process by which life diversifies into discrete forms, and understanding its underlying mechanisms remains a primary focus for biologists. Increasingly, empirical studies are helping explain the role of ecology in generating biodiversity. Adaptive radiations are often propelled by selective fitness tradeoffs experienced by individuals that invade new habitats, resulting in reproductive isolation from ancestral conspecifics and potentially cladogenesis. Host specialist insects are among the most speciose organisms known and serve as highly useful models for studying adaptive radiations. We are just beginning to understand the pace and degree with which these insects diversify. The apple maggot, *Rhagoletis pomonella*, is a well-studied insect whose eastern and southern populations are models for ecological speciation. Recently (40–65 ya), the fly has invaded the Pacific Northwestern United States through human-transported apples infested with larvae. There, populations of *R. pomonella* have rapidly colonized two novel hawthorn hosts whose fruiting times bracket apple's (early-season native *Crataegus douglasii* and introduced *C. monogyna*, which fruits late in the season). The recent introduction might initiate host shifts, providing opportunities to examine the pace and mechanistic means with which host races (an evolutionary stage preceding speciation) become established. Here, I demonstrate that host-associated populations at a site in southwest Washington are partially allochronically isolated from one another, and life cycles temporally match with natal host fruit ripening times in sympatry. If spatially widespread, these temporal barriers could result in reproductive isolation and possibly cladogenesis. Implications of these findings reach

beyond academic import, as *R. pomonella* is expanding not only its host range, but its geographic range is encroaching upon central Washington, the site of a multi-billion dollar per year apple-growing industry.

For my parents, Jim & Laura Mattsson, whose charisma to explore the world and listen to music was the greatest heirloom.

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Chapter 1:

Introduction

The theory of natural selection proposed by Charles Darwin and Alfred Russel Wallace revolutionized our understanding of the genesis of species (Darwin & Wallace 1858; Darwin 1859). A contemporary of these scientists, Benjamin Walsh, formulated a lesser known, but perhaps equally important postulate in 1864. Walsh, a British entomologist living in the United States, developed a framework in attempt to explain the immense diversity of herbivorous insects. The speciation mechanism he proposed described a situation where a subpopulation of a herbivore specialist species begins to oviposit (lay eggs) in a host plant species other than its own, and within a “sufficient number” of generations, the laws of inheritance reinforce this subpopulation’s fidelity for that host such that it becomes a ‘phytophagic variety’ distinct from its ancestors. Over time, this phytophagic variety progresses into a distinct species (Walsh 1864, 1867). Contemporary evidence suggests that Walsh was right in his hypothesis. The insights of Darwin, Wallace, and Walsh were of great magnitude but still, more than 150 years later, scientists continue to identify key behavioral, physiological, and genetic circumstances that lead to speciation (Coyne & Orr 2004; Nosil 2012; Feder et al. 2012).

‘Species’ must be defined in order to postulate anything regarding speciation, yet a consistent definition that can be applied to all organisms remains elusive (de Queiroz 1998). One reason for this might be that the concept of ‘species’ is too blunt given the array of life histories that exist among living organisms. For instance, a species is not always a fixed entity, but rather resides along a ‘species continuum’ due to the gradual

temporal nature of the speciation process (Nosil 2012). Still, it is clear that discrete species have diverged from common ancestors, and therefore cladogenesis happens. Thus, many species concepts have been proposed for categorizing life at this level (Coyne & Orr 2004). Arguably, the Biological Species Concept applies most broadly when discussing sexually reproducing organisms, stating: “species are groups of interbreeding natural populations that are reproductively isolated from other such groups” (Mayr 1963; Coyne & Orr 2004). The Biological Species Concept serves to frame the work presented herein.

One way in which speciation results is from prolonged reproductive isolation between previously interbreeding populations. Understanding how reproductive isolation develops is therefore a central interest to the study of speciation (Funk et al. 2009). The most obviously observed process to date has been allopatric speciation due to vicariance, where a physical barrier gradually or suddenly appears and divides a single population, thereby preventing allelic exchange, and the two groups thereafter follow distinct evolutionary paths. Allopatric speciation remains among the most widespread and widely accepted mode of speciation of plants and animals (Mayr 1970; Futuyma & Mayer 1980; Bush 1994).

Sympatric speciation, on the other hand, remains less well understood despite many advances toward describing its importance in generating biodiversity (Via 2001; Schluter 2009; Nosil 2012). Sympatric speciation is a process by which individual demes are formed (evolve) in the absence of physical barriers, a concept that dates to at least Charles Darwin and Benjamin Walsh. While Darwin only hinted at the possibility of

speciation in the absence of geographic barriers (Darwin 1859:113-114), Walsh specified his hypothesis more definitively (Walsh 1864, 1867). After witnessing the phytophagous true fruit fly *Rhagoletis pomonella* (the apple maggot fly) undergo a dietary shift in the 1860s from feeding almost exclusively on hawthorn fruits to begin infesting apple fruits in the Hudson River Valley of New York, Walsh identified the plausibility for races (which he termed ‘varieties’) of host-specialist insects forming when provided with novel ecological opportunities. In his exemplar case, new opportunity was a novel habitat, domestic apple trees, which were introduced to the region for the first time by European colonists some 400 ya. Apple trees grew in sympatry with the fly’s ancestral host hawthorn (*Crataegus* spp.; Walsh 1867) and thereby provided *R. pomonella* an unoccupied dietary niche to colonize. The subsequent expansion of the apple race’s distribution as far west as Manitoba, Canada was documented by apple growers over the following 50 ya (Bush 1969). The appearance of this unique race was simultaneously a scourge to apple farmers and the beginnings of a prominent model system in evolutionary biology.

Walsh’s observations and predictions, while prescient, lacked mechanistic detail and required definitive tests to demonstrate their plausibility and prevalence in nature. Now, a body of evidence has accumulated suggesting that sympatric speciation, wherein populations become ecologically and reproductively isolated in the absence of geographic barriers, may also be an important phenomenon in some taxa, especially host-associated phytophagous insects (Bush 1994; Schluter 2001; Funk et al. 2002; Berlocher & Feder 2002; Nosil 2012).

Ernst Mayr (1963) argued that new species could only arise in allopatry, else the homogenizing effects of even the most minimal gene flow between populations in parapatry or sympatry will prohibit the level of genetic fixation required for independent species to emerge. In this view, any two or more distinct species whose current ranges overlap, yet are morphologically or otherwise phylogenetically similar, must have recently experienced a prolonged interval of complete physical isolation from one another in order to develop reproductive isolation. This viewpoint endured as a tenet of evolutionary biology, though not without opponents, until relatively recently (Bush 1969, 1975; Futuyma & Mayer 1980; Felsenstein 1981; Futuyma & Peterson 1985; Rice & Salt 1990; Coyne & Orr 2004; Schluter 2009). Theoretical opposition to the ‘allopatry only’ school of thought might have begun in earnest with Maynard Smith (1966), who mathematically modeled how a polymorphic trait based on one allele could arise in a population through divergent selection (i.e., dark coloration provides crypsis and fitness to individuals that stay low in forest canopy whereas light coloration benefits those that remain high in forest canopy) and stably persist between populations despite some level of gene flow, given sufficient fitness benefits and habitat preferences encoded by the alternative alleles. However, Smith also pointed out that the existence of stable polymorphisms does not in and of itself constitute speciation, rather, they merely set the stage for reproductive isolation to arise between genotypes given stochastic changes of the environment.

Since Smith’s seminal paper, a volley of theoretical and empirical demonstrations have alternatively supported and refuted the plausibility of sympatric speciation as a

viable means of species diversification. However, a persistent theme emerges in cases supporting its existence: divergent selection acting upon multiple genomic regions. For example, divergent selection for habitat preference/fidelity between two populations in turn pleiotropically selects for positive assortative mating. That is, two or more separate genomic loci (i.e., one promoting habitat preference and another promoting assortative mating) can covary and are capable of overcoming the homogenizing effects of recombination (Via 2001; Rice & Hostert 1993; Feder & Forbes 2007).

Field and laboratory investigations of host-specialist phytophagous insects have generated a large body of evidence supporting instances of sympatric speciation via host race formation, where ‘host races’ are defined as “conspecific populations that are partially reproductively isolated due to host-association in sympatry” (Diehl & Bush 1984). Examples of host race formation can be found across disparate orders of insects, including the Coleoptera (Nishida et al. 1997; Funk 1998; Wadsworth et al. 2013), Hemiptera (Carroll et al. 1998; Via 1999, 2000; Wood et al. 1999), Lepidoptera (Groman & Pellmyr 2000; Emelianov et al. 2001), Phasmatoidea (Nosil 2007), Diptera (Craig et al. 2001; Berlocher & Feder 2002), Orthoptera (Harrison 1985; Sword et al. 2005), and Hymenoptera (Stireman et al. 2006; Forbes et al. 2009). In nearly all cases, reproductive isolation between/among host races appears to be due to the inextricable linkage between habitat/host preference and mate preference (Funk et al. 2002).

Seasonal Biorhythms and Their Implications for Evolution

Selection pressures from seasonal changes promote adaptive traits of organisms that enhance survival through periods of intense environmental stress, such as winter (Tauber et al. 1986; Wolda 1988; Denlinger 2002; Dingle & Drake 2007). Temperate regions in particular experience drastic fluctuations in nutrient availability, solar energy, humidity, and water availability. Biotic responses to seasonal variations include cyclical fluctuations in mate density, predator density, protective habitat availability, and inter- and intraspecific competition. Unlike rare events that recur outside of the generation length of a typical organism (e.g., volcanoes, earthquakes, catastrophic floods, catastrophic meteor impacts), the predictability of annual seasonal cycling is reflected in the periodicity of heritable life cycle events of organisms, such as the programmatic nature of annual insect diapause (Tauber et al. 1986; Storey & Storey 2012).

Phenology is the study of cyclical changes of living things, and in a broad sense, the phenologies of organisms are sculpted in response to the oscillatory nature of seasonal changes. For example, flower and fruit production of perennial plants typically occur during spring and summer when solar radiation is abundant, followed by dormant periods in fall and winter when environments become relatively cold and dark (low energy input). In animals, common adaptations to seasonal changes include (singly or in combination): migration to favorable environments (Dingle & Drake 2007), resource provisioning, polyphenism (Tauber et al. 1986), and metabolic and (or) reproductive dormancy (Denlinger 2002; Košťál 2006).

In this thesis I examine how nuanced polymorphisms of adaptations that promote seasonal survival and reproduction of temperate herbivorous insects can serve as raw material upon which natural selection can act to initiate cladogenesis (i.e., speciation). I accomplish this through detailed investigations of the diapause syndrome and other phenological life history components of one specific insect, the host-specialist true fruit fly, *Rhagoletis pomonella* Walsh (Diptera: Tephritidae).

The benefits of understanding insect evolution are far reaching. For instance, managers and scientists seeking to protect natural and agricultural resources require a fundamental understanding of the biology of both beneficial and pest insects, and their evolutionary responses to human-induced environmental changes (Parmesan 2006; Bale & Hayward 2010; Stoeckli et al. 2012). Also, basic research aimed at developing foundational theories of evolutionary biology seeks to understand diversification of life on Earth, of which the contribution of insects cannot be understated (Darwin 1859; Marie Curie SPECIATION Network 2012).

One million insect species have been described (Chapman 2009) and estimates of total numbers of insect species (including undescribed) range from 3 to 100 million (Gaston 1991), with a more tenable recent estimate of ≈ 5 million (Gaston 1991; Chapman 2009; Mora et al. 2011). Regardless of exact numbers, the species richness of Class Insecta indisputably constitutes the vast majority of animal species richness on Earth (Groombridge 1992). Interestingly, most insect species are either parasites or parasitoids (Price 1977; Windsor 1998). Of particular interest here are the highly speciose host-specialist phytophagous insects, who appear to be predisposed to biodiversification,

presumably as a result of specialized parasitic associations with host plants at the genus level and particularly the species level (Mitter et al. 1988; Thompson 1988; Hawkins 1993; Aluja & Mangan 2008). In addition, parasitic insects often exclusively attack certain life stages of hosts (e.g., fruits or flowers in the case of phytophagous forms, and either eggs, larvae, pupae, or adults, for entomophagous forms) and therefore face selection pressures for coordinating their life cycles to exploit seasonally ephemeral resources. Accordingly, survival is contingent upon synchronized developmental phenologies among organisms.

***Rhagoletis pomonella* and Ecological Sympatric Speciation**

Species of *Rhagoletis* (Diptera: Tephritidae) are host-specialist fruit flies found throughout Holarctic and Neotropical ecosystems (Bush 1966). In temperate and subtropical North America, the genus is organized into roughly five sibling species complexes based on genetic and (or) morphological homologies (Fig. 1.1). Bush (1966) hypothesized that members of the *Rhagoletis pomonella* species complex in particular have recently speciated through ecological specialization following shifts to novel hosts in sympatry, initially resulting in novel host races (Walsh's 'phytophagic varieties') that eventually became further reproductively isolated into distinct species (Bush 1966). The four species within the *R. pomonella* complex are *R. pomonella*, *R. mendax*, *R. zephyria*, and *R. cornivora*, whose respective hosts are apple/hawthorn (*Malus domestica* and *Crataegus* spp.), blueberry (*Vaccinium* spp.), snowberry (*Symphoricarpos* spp.), and dogwood (*Cornus* spp.). These fly species have little to no interspecific gene flow

despite extensive geographic range overlap. Following Bush's hypothesis, these species first passed through a host race period before becoming reproductively isolated species.

Mechanistic explanations of speciation within the *R. pomonella* complex have been tested using the recently established host races of *Rhagoletis pomonella* Walsh in the eastern United States (McPherson et al. 1988; Feder et al. 1988, 1993, 1994; Bush 1994; Feder 1998). *Rhagoletis pomonella* is endemic to eastern North America and Mexico, where it historically attacked native hawthorn species, such as downy hawthorn (*Crataegus mollis* [Torr. & Gray] Scheele), but expanded its host range ~160 ya to include non-native apple (*Malus domestica* Borkh.) following its introduction by European colonists 350-400 ya (Walsh 1867). Numerous studies examining the genomic, behavioral, and phenological identities of this species support the designation of partially reproductively isolated apple and hawthorn host races (Smith 1988; Feder et al. 1988; McPherson et al. 1988; Feder et al. 1994).

The biological life history of *R. pomonella* is central to its predisposition for sympatric ecological speciation. Most notably, it typically produces only one generation per year (i.e., it is univoltine). Its annual life cycle is as follows: adults emerge in summer and feed on insect honeydew, bird feces, yeast, and plant leachates for approximately 7 – 10 days before becoming reproductively mature. Adults then mate on or near natal host fruits, after which females oviposit under the skin of the ripe fruit (N.B.: total adult life span is estimated to be ≤ 30 days [Dean & Chapman 1973]). The eggs hatch within 3 to 7 days (depending on temperature) and the larvae consume and store nutrients from the fruit for 3 – 5 weeks. When the fruit later abscises (falls) from the tree, larvae emerge

from the fruit, burrow 1 – 5 cm into the ground beneath their host plant, and form puparia. The pupae enter a facultative diapause to enhance winter survival. As temperatures and day lengths increase during the following spring and summer, adult flies develop and eclose (emerge from soil as adults) to repeat their reproductive cycle (Dean & Chapman 1973; Boller & Prokopy 1976; Fig. 1.2).

Two traits are critical for the establishment and maintenance of the distinct apple and hawthorn host races of flies in the eastern US. First, because apples ripen 2 – 3 weeks before hawthorn fruits, diapause phenologies are shifted in order to synchronize adult eclosion with fruit ripening phenologies of their respective hosts (Smith 1988; Feder et al. 1993; Dambroski & Feder 2007). Because of a short adult life span, the shift in diapause phenology decreases mating opportunities among conspecifics. The second trait is strong natal host preference (Prokopy et al. 1971, 1972; Linn et al. 2003) and avoidance of non-natal hosts (Forbes & Feder 2007). The reinforcement of reproductive isolation due to a genetically based preference for hosts translates into positive assortative mating because the flies mate directly on, or very near, host fruits. The combined effects of allochronic isolation due to differences in host plant phenology, and assortative mating due to strong host fidelity, has led to partially reproductively isolated host races with a potential to become new species. It is reasonable to conclude that the current apple and hawthorn host races thereby represent incipient species, offering insight into the more ancient origins of congeneric species within the *R. pomonella* complex.

***Rhagoletis pomonella* in the Pacific Northwestern United States**

Historically absent from the Pacific Northwestern (PNW) United States, a 1979 report by a homeowner in Portland, OR indicated that their backyard apple tree was infested with *R. pomonella* (AliNiazee & Penrose 1981). The mode of establishment was likely through human transported apples infested with larvae. The insect quickly dispersed throughout Washington and Oregon west of the Cascade Range, in addition to penetrating the Columbia River Gorge toward fruit growing regions of central Washington and Oregon (Fig. 2.2).

The host breadth of *R. pomonella* also expanded to native black hawthorn (*Crataegus douglasii* Lindl.) as well as introduced English ornamental hawthorn (*Crataegus monogyna* Jacq.), thus begging the question: Is host race formation once again underway? If so, there are both practical and theoretical considerations. The species is a scourge that threatens the extremely valuable commercial apple industry in central Washington and central Oregon, and malleability in host acceptance could provide a means of ‘host hopping’ more easily into fruit orchards of Washington. On the other hand, the experimental framework constitutes an unprecedented opportunity to examine an important evolutionary system at an early stage of lineage divergence. For analysis from an evolutionary perspective, it is critical to establish that a single population was introduced and subsequently radiated into novel dietary niches, i.e., that *R. pomonella* in the PNW form a monophyletic group.

There were reports of *R. pomonella* in the PNW prior to the 1979 instance, but it is unlikely that these populations became established. For instance, a single lot of apples

shipped from Portland, OR to California was found to contain *R. pomonella* larvae as early as 1947 (AliNiazee & Westcott 1986). However, the host tree from which the apples were harvested was identified and monitored with yellow sticky traps, along with several adjacent properties in 1948 (the following year). Only two adult apple maggot flies were found, yet it remains unknown whether they were truly apple maggot flies, or perhaps instead adult snowberry maggot flies (*R. zephyria* Snow), which are morphologically cryptic with *R. pomonella*—the subtle differences in genitalia and wing shape (Yee et al. 2011) were not known at that time. To further complicate matters, snowberry bushes were found growing across the street from the purported host apple tree (AliNiazee & Westcott 1986). The fly subsequently remained undetected until the late 1970s, despite extensive programs following its 1947 discovery designed to monitor it and other pests of agriculture in the area, such as *R. indifferens* Curran (cherry fruit fly) and *R. completa* Cresson (walnut husk fly) (Dowell 1988).

Alternatives to the hypothesis of introduction via infested apples exist. For instance, it is possible that an undetected native population parasitized black hawthorn (*C. douglasii*) prior to the introduction of *M. domestica*, and that the current apple-infesting populations shifted from this native hawthorn population. However, a recent wide-range geographic survey of the western US found that host plant ranges are often contiguous, whereas *R. pomonella* populations are not (Hood et al. 2013). Most interestingly, native *C. douglasii* remain uninfested at numerous sites spanning much of the Pacific Northwest despite the availability of suitable habitat. The same survey

revealed that rates of infestation (# pupae per fruit) were highest near Portland, Oregon, the putative site of introduction, and decreased with radial distance from Portland.

In addition to the historical and geographic evidence, there is genetic evidence in support of the single introduction hypothesis. McPheron (1987) analyzed allozymes from both eastern and western apple maggots, and found very little genetic variation among populations of Oregon and Washington relative to eastern populations, suggesting a recent, single introduction (founder effect).

Rhagoletis pomonella was found throughout western Oregon, Washington, and northern California by the early 1980s (Dowell 1988). Although the rapid rate and geographic extent of the area to which this species has spread might seem surprising, it is not. A single apple can harbor at least 10 maggots (personal observation) and a single female can oviposit >200 eggs during its brief lifetime (Dean and Chapman 1973). Thus, the transport of only 20 apples containing ~100 females (given a 50:50 sex ratio) could in one year easily seed a new population of twenty thousand individuals. Also, human facilitated transport of infested apples by automobile provides ample dispersal opportunities. Finally, the dispersal capabilities of individual flies on the wing during a single season are at least 0.3 km (Maxwell & Parsons 1968), but individual flights have been documented to be as far as 4.5 km (Sharp 1978).

The studies I present herein build upon previous investigations suggesting that host race formation is again taking place in this species, similar to the classic hawthorn to apple host shift scenario previously observed in the eastern US. Behavioral experiments testing black hawthorn-, apple-, and ornamental hawthorn-associated fly populations

from western Washington both in the field and laboratory have determined that each of the newly established populations associated with distinct host plants respond to the fruit volatiles emanating from its natal host, while avoiding non-natal fruit odors (Linn et al. 2012; Sim et al. 2012).

My goal in the present work was to complement these behavioral studies to further examine mechanisms driving host race formation, as host preference behavior alone may only cause low levels of reproductive isolation. If a second, genetically underpinned trait, such as difference in life history timing, were identified, a more confident conclusion regarding the mechanisms responsible for host race formation could be reached.

Specifically, I tested for temporal isolation among populations as a result of shifts in diapause phenologies through a series of field- (Chapter 2), and laboratory- (Chapter 3) based assays. Chapter 4 discusses a related phenomenon involving the phenological variability of parasitoid wasps attacking the separate populations of *R. pomonella*, a situation in which host race formation of a phytophagous insect might transcend trophic levels to affect trophically higher entomophagous taxa.

Fig. 1.1 Phylogenetic relationships among *Ragoletis* species and closely related flies within Tephritidae, as published by Smith et al. 2005. Symbols beside species names indicate general global distribution (■ = Palearctic; ● = Nearctic; ▲ = Neotropical). Nearctic species complexes are named for one species within the group and are indicated with brackets. The tree was constructed from mitochondrial DNA characters (see Smith et al. 2005 for details).

Fig. 1.2 The univoltine life cycle of *Rhagoletis pomonella* consists of a ~30 day adult reproductive stage that begins when adults eclose in summer. Following a 7 – 10 day preoviposition period, adults mate directly upon (or very near) ripe host fruits. Eggs are laid into fruits where larvae then feed on the fruit pulp for 3 – 5 weeks. When fruits abscise (fall), larvae emerge and burrow into the soil and form puparia in which they overwinter in a facultative diapause (illustrations by M.M.).

Chapter 2:
Rapid and Repeatable Shifts in Life History Timing of *Rhagoletis pomonella*
(Diptera: Tephritidae) Following Colonization of Novel Host Plants in the Pacific
Northwestern United States

Abstract

Host shifts of phytophagous insect specialists to novel plants can result in divergent ecological adaptation, generating reproductive isolation and potentially new species. *Rhagoletis pomonella* fruit flies in eastern North America underwent a host shift ~160 ya from native downy hawthorn (*Crataegus mollis*) to introduced, domesticated apple (*Malus domestica*). Divergent selection on diapause phenology related to the earlier fruiting time of apples versus downy hawthorns resulted in partial allochronic reproductive isolation between the fly races. Here, I test for how rapid and repeatable shifts in life history timing are driving ecological divergence of *R. pomonella* in the Pacific Northwestern US. The fly was introduced into the region via larval-infested apples 40-65 ya and now attacks native black hawthorn (*Crataegus douglasii*) and introduced ornamental hawthorn (*Crataegus monogyna*), in addition to early- and late-maturing apple varieties in the region. To investigate the life history timing hypothesis, I used a field-based experiment to characterize the host-associated eclosion and flight activity patterns of adults, and the feeding times of larvae at a field site in Vancouver, Washington. I also assessed the degree to which differences in host fruiting time generate allochronic isolation among apple-, black hawthorn-, and ornamental hawthorn-

associated fly populations. I conclude that host-associated fly populations are temporally offset 24.4% to 92.6% in their seasonal distributions. My results imply that *R. pomonella* possesses the capacity for rapid and repeatable shifts in diapause life history to match host fruiting phenology, which can generate ecologically-based reproductive isolation, and potentially biodiversity in the process.

Introduction

Ecological speciation is initiated when divergent adaptation to novel habitats generates reproductive isolation (Schluter, 2001; Rundle & Nosil, 2005). Phytophagous insect specialists may be particularly prone to speciate ecologically when they shift and differentially adapt to attacking new host plants (Bush, 1969; Harrison, 1985; How *et al.*, 1993; Abrahamson *et al.*, 1994; Horner *et al.*, 1999; Via, 1999; Wood *et al.*, 1999; Groman & Pellmyr, 2000; Via *et al.*, 2000; Drès & Mallet, 2002; Berlocher & Feder, 2002; Funk *et al.*, 2002). When divergent selection pressures between alternate host plants are strong, migrants between habitats and hybrids of mixed ancestry will suffer reduced fitness, generating ecologically-based reproductive isolation that can potentially initiate speciation even in the face of gene flow (Bush, 1969; Feder *et al.*, 1988; Berlocher & Feder, 2002; Drès & Mallet, 2002; Funk *et al.*, 2002; Sim *et al.*, 2012; Nosil, 2012).

Apple- and downy hawthorn-infesting populations of the fruit fly *Rhagoletis pomonella* (Walsh) (Diptera: Tephritidae) have been hypothesized to represent an example of incipient ecological speciation with gene flow (Berlocher & Feder, 2002). Sometime in the mid-1850s, *R. pomonella* shifted in the eastern United States (US) from its native host, downy hawthorn, *Crataegus mollis* ([[Torr.](#) & [A. Gray](#)] Scheele), to form a new ‘host race’ on domesticated apple trees (*Malus domestica* [Borkh.]) after apples were introduced from Europe ~400 ya (Walsh, 1867; Bush, 1966; Feder *et al.*, 1988; McPherson *et al.*, 1988). Host races are hypothesized to be the initial stage of ecological speciation, representing partially reproductively isolated populations that owe their

isolation to divergent host or habitat-associated adaptations (Diehl & Bush, 1984; Drès & Mallet, 2002).

One key ecological adaptation reproductively isolating and genetically differentiating the *R. pomonella* host races is the relationship between host fruiting phenology and fly diapause (Fig. 2.1). Phytophagous insects must time their life cycle to coincide with environmental conditions when plant resources are plentiful for feeding, mating, and oviposition (Tauber *et al.*, 1986; Denlinger, 2002). Life history timing is particularly important for univoltine insects like *Rhagoletis* that are host specialists, where short-lived adults reproduce once a year and then die, and their offspring feed and develop on only one or a few specific plants. In this case, the seasonal window when hosts are present and in optimal condition can be limited. Mismatches between the timing of host and parasite life cycle events can therefore have disastrous fitness consequences for a univoltine specialist insect, as it will be active at a time when both its host is absent and climatic conditions are potentially unfavorable for its survival (Tauber *et al.*, 1986; Denlinger, 2002).

Fruit on apple varieties favored by *R. pomonella* ripen on average 3-4 weeks earlier than those on downy hawthorn (Feder *et al.*, 1993, 1994) (Fig. 2.1). Because *R. pomonella* live for a maximum of perhaps one month in nature, apple and downy hawthorn flies must differentially time their life histories to match the difference in the fruiting phenologies of their respective host plants. They accomplish this through variation in the timing of the overwintering pupal diapause. Apple flies have been shown to differ genetically from downy hawthorn flies in the intensity to which they initiate and

terminate (break) diapause. Most notably, apple flies eclose as adults 10 days earlier on average than downy hawthorn flies to coincide with the earlier fruiting time of apples (Feder *et al.*, 1993, 1994, 1995; Feder, 1995; Egan *et al.*, 2015). Due to the limited lifespan of adults, the difference in eclosion time results in partial allochronic mating isolation between the apple and downy hawthorn host races (Feder *et al.*, 1993, 1994). In addition, a degree of postzygotic isolation also likely exists due the diapause timing of hybrids being less well-adapted to exploiting apple or hawthorn fruit resources as those of parental genotypes (Smith, 1988).

In the current study, I examine the rapidity and repeatability of the evolution of diapause life history timing in a unique circumstance involving host-associated populations of *R. pomonella* in the Pacific Northwest (PNW) region of the US. *Rhagoletis pomonella* is native to the eastern US. However, the fly was discovered infesting an apple tree in the yard of a homeowner in the Portland, Oregon (OR) area in 1979 (AliNiazee & Penrose, 1981). It is possible that *R. pomonella* was present earlier, as a specimen taken from near Rowena, OR in 1951 was identified as being an apple maggot fly (AliNiazee & Westcott, 1986). However, *R. pomonella* was probably not established at that time, as no other apple maggot fly was collected from the area until 1984 (AliNiazee & Westcott, 1986; Dowell, 1988). In the 1980s, *R. pomonella* was found (had spread) north and south in Oregon and Washington (WA) on the western side of the Cascade Mountains and into the Columbia River Gorge and other passages in the Cascades, encroaching on the commercial apple-growing region of central WA (Dowell, 1988; see arrows in Fig. 2.2A), where it is now a quarantine pest threatening the \$2.25

billion annual apple industry of the state (Mertz *et al.*, 2013). Genetic, behavioral, and distributional data support the hypothesis that the fly was recently introduced into the PNW via the human transport of larval-infested apples (McPherson, 1987; Dowell, 1988; Hood *et al.*, 2013; Linn *et al.* 2012; Sim *et al.* 2012; Sim, 2013).

In addition to apple, *R. pomonella* also currently infests native black hawthorns, primarily *C. douglasii* (Lindl.), but also occasionally *C. suksdorfii* ([Sarg.](#)) [Kruschke](#) at low levels, and introduced ornamental hawthorn (*Crataegus monogyna* [Jacq.]) (AliNiazee & Westcott, 1987; Tracewski *et al.*, 1987; Dowell, 1988; Yee, 2008; Yee *et al.*, 2012; Hood *et al.*, 2013; Sim, 2013). Thus, the possibility exists that *R. pomonella* has shifted from apple within the last 65 years and is in the process of ecologically differentiating on novel black and ornamental hawthorn hosts in the PNW on an even more recent timescale than what occurred in the opposite direction in the eastern US.

Interestingly, the fruiting phenologies of hawthorns in the PNW bracket that of apple. Fruit on ornamental hawthorns in the PNW, like those of downy hawthorn in the eastern US, ripen later than sweeter apple varieties most favorable for *R. pomonella* larval development and survivorship. In contrast, the native *C. douglasii* (black hawthorn hereafter) is an early fruiting host that precedes ornamental hawthorns and most of even the earliest fruiting apple varieties in the PNW. The native *C. suksdorfii*, another “black hawthorn” species in the PNW, is relatively rarely attacked by *R. pomonella* (Yee *et al.* 2012) and generally has a fruiting phenology comparable to that of *C. douglasii*, although it can vary earlier or later locally (MM, pers. obs.). The existence of a variety of hawthorn-associated forms of *R. pomonella* in the PNW, coupled with presence of the

apple race in the eastern US, therefore suggests that the fly has repeatedly shifted its diapause life history to attack novel host plants with different fruiting times. Moreover, the documented introduction of *R. pomonella* into the PNW within the last 40-65 years provides a historical context to infer that diapause life history shifts can occur rapidly. Finally, the different hawthorn-associated populations of *R. pomonella* in the PNW imply that the fly can readily change the timing of its life history to attack novel plants that fruit not only earlier in the field season, as is the case for the downy hawthorn to apple shift in eastern US, but also later, as well.

Here, I conduct a series of field-based studies to test the hypothesis that variation in host plant fruiting phenology is a rapid and repeated driver of diapause life history adaptation generating reproductive isolation among host-associated populations of *R. pomonella* in the PNW. To accomplish this, I first characterize the fruit ripening times of black hawthorn, apple, and ornamental hawthorn trees at field site in Vancouver, WA where these alternative host plants co-occur sympatrically. I then compare these seasonal windows of fruit resource availability with adult eclosion, flight activity, and oviposition times of the associated black hawthorn-, apple-, and ornamental hawthorn-infesting *R. pomonella* populations at the site. Taken together, the results allow me to estimate the degree of temporal overlap and premating allochronic isolation generated by differences in diapause timing across multiple life history stages of the fly, potentially contributing to the formation of new hawthorn-associated forms of *R. pomonella* in the PNW.

Materials and Methods

Life history

Rhagoletis pomonella are univoltine and adults live ~30 days (Dean & Chapman, 1973; Boller & Prokopy, 1976). After mating on or near host fruit, females oviposit just below the outer skin surface of ripe fruit on trees. After eggs hatch, larvae feed within host fruit for 2-5 weeks and undergo three larval instars. When fruits abscise from trees, larvae emerge from the fruit, burrow 1-5 cm into the ground, form puparia, and then enter a facultative pupal diapause in which they overwinter. The following summer flies have terminated diapause and eclose as adults, completing their univoltine life cycle (Dean & Chapman, 1973; Boller & Prokopy, 1976; Bush, 1994).

Fruit ripeness

To determine the temporal windows and seasonal overlap of host resources for mating and oviposition, I characterized the fruit ripening phenologies of black hawthorn (*C. douglasii*), ornamental hawthorn, and early- and late-maturing varieties of apples, the primary hosts of *R. pomonella* in the PNW, at a field site where the trees co-occur in the Burnt Bridge Creek Greenway (45° 37.9' N; 122° 37.1' W) in Vancouver, Washington, USA in the summer of 2013 (Fig. 2.2). I quantified fruit ripening by measuring the size and firmness of fruits from nine different black hawthorn trees, seven ornamental hawthorn trees, three early-maturing apple trees, and seven late-maturing apple trees. Data from the early- and late-maturing apple varieties were considered separately in all subsequent statistical analyses. A similar intraspecific bimodality was

not observed for either black or ornamental hawthorn trees.

Previous research has indicated that fruit firmness is an important indicator of the susceptibility of host fruit to attack by *R. pomonella*. For example, Messina & Jones (1990) found that populations of *R. pomonella* in Utah do not begin infesting black hawthorn fruits until penetration resistance drops below 60 kg per cm². Whether the physical resistance of the fruit prohibits oviposition, or reduced firmness (hereafter “softness”) simply coincides with rises in concentrations of sugars, tolerable pH levels, or other variables of host fruit acceptability, remains unknown. Regardless, excessively firm fruit present a biological (or mechanical) obstacle to oviposition, as females must perforate the skin of fruit to lay eggs without damaging their ovipositors. In this regard, Dean & Chapman (1973) showed that softer apple fruits require approximately 10 sec for females to successfully oviposit, as opposed to 3 to 4 times longer for hard fruit. I therefore focused on fruit softness and size as metrics of fruit ripeness in the current study.

To gauge fruit ripeness, black hawthorn fruits were collected from trees three times per week from 4 June to 17 July. Ornamental hawthorn fruits were sampled from one to two times per week from 3 July to 19 September. Five to seven fruits were collected from each hawthorn tree on each sample date (approximately 60 fruit total per hawthorn host species per sampling date; Table A.1). Because apples were less numerous than hawthorn fruits, a slightly different sampling method was used to prevent the depletion of apples on source trees. For apples, five fruits were collected per tree every seven to ten days from 19 June to 19 September (Table A.1). Equal numbers of fruits

were collected randomly from each tree on each sampling date to reduce bias in the analysis and generate a sample of the spectrum of ripeness at the time of collection, as fruits on a single tree can ripen at unequal rates. To accomplish this, I repeatedly generated random numbers between 0 and 360 and treated a tree's canopy as a circle. The random number represented the degree from north (clockwise) at which the fruit first seen facing the trunk at eye-level (1.7 m) was picked. Apples were sampled in a similar manner except that I also randomized the horizontal (distance from tree trunk) and vertical (distance from lowest to highest reachable canopy) location of harvesting by generating numbers from one to five and dividing the height and width of trees into 20% subsections. The maximum height sampled in an apple tree was 6.8 m.

The size and firmness of fruits were recorded within 2 h of sampling. For size, the transverse diameter of the widest portion of the fruit halfway between the calyx and peduncle was measured using a ruler to the nearest 0.5 mm (hawthorn) or 0.5 cm (apple). Oviposition and/or larval feeding are impeded by excessive firmness of fruits (Dean & Chapman, 1973). Thus, a penetrometer (Firmness Tester FT-02[®] for hawthorn; FT-327[®] for apple, QA Supplies, LLC, Norfolk, VA) affixed with either an one mm probe (for hawthorns) or an 11 mm probe (for apples) was used to measure the resistance of a fruit's surface to penetration. A single firmness measurement per fruit was taken at the widest portion of the fruit halfway between the calyx and peduncle on the rosiest (ripest) portion of the fruit. In keeping with methods employed in previous studies (Messina & Jones, 1990; Stoeckli *et al.*, 2011), the apple skin was removed before taking the measurement. I used the pressure required to penetrate fruit for statistical analysis, which was calculated

by dividing the penetrometer's measure of firmness by the cross-sectional area of the probe used, resulting in a final measurement with units in kg per cm². Early and late in the season, fruit firmness often was above and below, respectively, the measurement capabilities of the penetrometer. In such cases, fruit firmness was recorded as either the highest (1000 g for hawthorn and 13 kg for apple) or lowest (100 g for hawthorn and 0.5 kg for apple) possible value. Hawthorn fruit measurements were discontinued when all fruits' penetration resistances were below the measurement capabilities of the penetrometer, which coincided with the time when essentially all fruit on trees was wrinkled, shrunken, or rotten. *Rhagoletis pomonella* do not oviposit into abscised fruit and rarely use fruit on trees that is beginning to rot (Dean & Chapman, 1973; Boller & Prokopy, 1976). Fruit measurements for early apples were discontinued when all fruit had abscised from trees and for late apples and ornamental hawthorn on 26 Sep 2013.

Field eclosion time

To characterize the eclosion times of adult *R. pomonella* in the field at the Vancouver, Washington study site, I constructed 1.0 m length X 1.0 m width X 0.75 m height tents made of white tulle netting (mesh diameter ≤ 1 -mm) on the ground beneath the canopy of host trees to intercept eclosing flies. Within each tent, an unbaited 11.5 cm x 15.25 cm Pherocon[®] No Bait AM Yellow Sticky Trap (Trece, Inc., Adair, OK) was hung from the inside-top of the tent to attract and capture newly eclosing adult flies. A total of 27 tents were distributed among the same host trees used in the fruit ripeness study (Fig. 2.2). One tent was placed beneath each host tree with the exception that two

tents were placed beneath one of the seven ornamental hawthorn trees. Tents were constructed beneath the portion of the tree's canopy with the most dense foliage and/or flowers on 27 May 2013. It was assumed that the highest number of fruit had fallen in those areas the previous year and therefore contained the highest densities of overwintering *R. pomonella* pupae. Tents were checked three times per week from 30 May through 22 September 2013. Adult flies were removed from traps, counted, sexed, placed into 22 mL condiment cups (SOLO[®] Cup Company, Lake Forest, IL), and frozen at -80 °C within 24 hours of collection. To account for potential shading effects on soil temperature associated with the tents, daily minimum and maximum soil temperatures were recorded using Digital Dual Sensor[®] Thermometers (Forestry Suppliers, Inc., Jackson, MS) for three of the tents (1 in each of three areas denoted in Fig. 2.2). Each thermometer had two sensors attached to separate 1 m cables enabling soil temperatures to be recorded at a depth of 2 cm both inside and outside of the eclosion tents.

Flight activity pattern

Adult flight activity was monitored at the Vancouver, WA site to further assess the extent to which any observed differences in eclosion time coincided with variation in fruit maturation, as well as fly mating and oviposition, to quantify host-associated allochronic isolation. Adult flight activity was measured by trapping flies in host tree canopies using Pherocon[®] No Bait AM Yellow Sticky Traps. A single sticky trap affixed with a vial containing 15 g of ammonium carbonate was hung in the canopy of each of the 26 host trees used in the fruit ripeness and eclosion studies directly above the eclosion

tent. The sticky traps in the tree canopies were monitored on the same days and in the same manner as those used to determine eclosion time in the ground tents.

Larval infestation of fruit

To characterize the abundance of fly larvae during the field season, additional samples of fruit were collected from the canopy of each of the 26 study trees at the Vancouver, WA site in the same manner as described above for the ripeness study. Black hawthorn fruits were collected on 12 July, 18 July, 12 August; early- and late-apple varieties on 18 July, 31 July, and 14 August; and ornamental hawthorn fruits on 6 and 26 September (Table 2.1). Fruits were returned to the laboratory (Biology Department, Portland State University, Portland, OR) within 2 h of picking and placed into separate wire baskets made of 1.0 cm mesh-size hardware cloth. The baskets were positioned above plastic collecting trays and held at a 15:9 (light: dark) photoperiod at 22–23°C. Emerging larvae and puparia were counted and removed from the collecting trays every other day until none was found in the tray for an eight week period.

Environmental data

Abiotic conditions might directly influence adult eclosion, resulting in a plastic, rather than genetic, basis for eclosion phenotypes. To assess environmental correlations with eclosion phenologies, we compiled ambient air temperature and precipitation data from the website Weather Underground (http://www.wunderground.com/history/airport/KVUO/2013/3/25/MonthlyHistory.html?req_city=NA&req_state=NA&req

[statename=NA](#)) for Pearson Field Airport (airport code KVUO), Vancouver, WA, located ~5 km from the study site.

Data analysis

Individual measures of fruit firmness and size were divided by the respective maximums recorded in the study (firmness = 1000 g for hawthorn, 13 g for apple; size = 9.9 mm, 6.9 cm, 6.6 cm and 10.5 mm for black hawthorn, early apple, late apple, and ornamental hawthorn respectively) to generate standardized metrics of relative ripeness for comparison among host species. Relative values for fruit firmness were then subtracted from 1 to produce measures of fruit softness that increased with time in accord with fruit ripening. Relative median fruit softness and mean fruit size data were then compared across sample dates for statistical significance between host species using paired t-tests.

Eclosion time was analyzed using a linear mixed effects model, where ‘eclosion date’ was considered as the response variable, ‘host’ a fixed explanatory variable, and ‘individual tent’ a random explanatory variable. Repeated measures analysis of variance (ANOVA) was performed to determine if mean eclosion times ($\pm$ the standard error in days) varied among host populations. I used post-hoc Tukey’s Honestly Significant Difference multiple pairwise tests to determine which of the host populations differed significantly in eclosion time. One-way ANOVA was conducted to determine if mean dates of flight activity varied among host populations. To ensure that the assumptions of ANOVA were not violated, the normality of residuals was tested using qq-plots and

homoscedasticity using Brown-Forsyth tests. Pairwise estimates of allochronic isolation (AI) were calculated between host populations from the field eclosion curves and activity patterns of adult flies using the formula of Feder *et al.* (1993):

$$1 - \left(\frac{\sum x_i y_i}{\sqrt{\sum x_i^2 \cdot \sum y_i^2}} \right) \cdot 100 \quad (\text{equation 1}),$$

(equation 1) where x_i and y_i are the proportions of the total numbers of flies present on host x or y on day i , assuming a mean adult life span of 30 days (Dean & Chapman, 1973; Boller & Prokopy, 1977). Newly eclosed adult *R. pomonella* are not sexually mature, but rather require seven to ten days to develop mature eggs (Dean & Chapman, 1973). I therefore considered the period of adult sexual activity to be 10-30 days post-eclosion. Spearman's rank correlation coefficients were used to test for significant relationships among cumulative fly eclosion (data from all tents were pooled for each host-associated population), larval abundance, fruit ripeness (size and firmness), ambient air and soil temperature (minimum, mean, and maximum), and rainfall. Daily eclosion was correlated with the preceding days' temperature data. All data analyses were performed using R (R Core Team, 2013).

Results

Fruit ripeness

The time when fruit ripened, as quantified by fruit softness and size, differed significantly among host species (Figs. 2.3 & S2.1, Table 2.2). However, comparisons of fruit size between early apple versus ornamental hawthorn, and late apple versus

ornamental hawthorn, were not different and represent exceptions to the otherwise different fruiting phenologies (Figs. 2.3 & A.1, Table 2.2). Softness and size were strongly positively correlated (Fig. 2.4), therefore the term “ripeness” is henceforth used to imply both variables unless otherwise noted.

Black hawthorns ripened earliest, beginning in the last week of June and ending in the second week of July, when fruit became wrinkled and rotten. Early-apple was chronologically second, beginning to ripen in the first week of July and concluding by the first week of August. The size of late-apples increased steadily beginning in late June, but did not ripen until later in August, at which time they rapidly softened. Ornamental hawthorn fruit began increasing in size latest in the season, in the first week of August, but started softening prior to late apple fruits. Ornamental hawthorn fruit reached peak ripeness in the first week of September (Fig. 2.3).

Field eclosion

Adult eclosion times differed significantly among flies infesting black hawthorn, early-fruited apple, late-fruited apple, and ornamental hawthorn ($F_{3,14} = 15.48$, $P < 0.0001$, Fig. 2.5). Eclosion times differed significantly between all pairwise comparisons of host populations, with the exception of black hawthorn versus early apple flies (Table 2.3).

The eclosion times of host populations generally matched the chronological sequence of fruit ripening. Adult flies were not captured in all the eclosion tents, although fruits from all trees in the study were infested with larvae (see below). Mean date of

eclosion for the 53 flies captured within the six black hawthorn eclosion tents producing adults was 23 June (± 3.1 days s.e.); for the 115 flies captured from the three early apple tents, 26 June (± 2.9); for the 52 flies captured from the five late apple tents, 5 July (± 1.8); and for the 53 flies captured from four ornamental hawthorn tents, 17 July (± 1.8) (Table A.2). Estimates of premating allochronic isolation between black hawthorn-associated flies and early-apple-, late-apple-, and ornamental hawthorn-associated flies were 24.4%, 55.7%, and 92.6%, respectively. Isolation of early-apple flies from late-apple and ornamental hawthorn flies was estimated to be 43.9% and 87.1%, respectively, and between late-apple and ornamental hawthorn flies, 59.3% (Table 2.4).

Each host population displayed a significant positive relationship between eclosion time and both of the metrics of increasing fruit ripeness measured in the study (fruit softness: black hawthorn $S = 1098.47$, $r_{s,13} = 0.96$; early apple $S = 233.86$, $r_{s,7} = 0.95$; late apple $S = 402.56$, $r_{s,9} = 0.83$; ornamental hawthorn $S = 1548.29$, $r_{s,15} = 0.90$; increasing mean fruit diameter: black hawthorn $S = 63.67$, $r_{s,13} = 0.89$; early apple $S = 2.53$, $r_{s,7} = 0.98$; late apple $S = 5.59$, $r_{s,9} = 0.97$, $P = 0.002$; ornamental hawthorn $S = 78.10$, $r_{s,15} = 0.90$; all $P < 0.0001$ unless noted otherwise; Fig. 2.3). The differences between mean eclosion time and host fruit ripening increased from earlier to later fruiting host plants (Fig. 2.3).

Mean maximum soil temperatures were an average of 2.2°C cooler inside than outside tents during the day and 0.4°C warmer at night. Although these temperature differences were significant (Table A.3), the lower maximum (during daytime) versus higher minimum (during nighttime) temperature differences resulted in roughly the same

thermal accumulation and were consistent among hosts, thus were unlikely to have affected fly eclosion curves.

Tent soil temperature was not significantly correlated with cumulative eclosion time of host populations (black hawthorn $S = 307.87$, $r_{s,13} = 0.45$, $P = 0.09$; early apple $S = 47.48$, $r_{s,7} = 0.60$, $P = 0.08$; late apple $S = 265.21$, $r_{s,9} = -0.21$, $P = 0.54$; ornamental hawthorn $S = 1038.32$, $r_{s,15} = -0.27$, $P = 0.29$). However, ambient air temperature was related to cumulative eclosion for the black hawthorn ($S = 199.28$, $r_{s,13} = 0.64$, $P = 0.01$) and early apple ($S = 24.61$, $r_{s,7} = 0.79$, $P = 0.01$) populations, but not for the late apple ($S = 116.01$, $r_{s,9} = 0.47$, $P = 0.142$) or ornamental hawthorn ($S = 498.51$, $r_{s,15} = 0.39$, $P = 0.12$) populations. Only the black hawthorn population showed a slight, though significant relationship between cumulative adult eclosion and precipitation (black hawthorn $S = 843.61$, $r_{s,13} = -0.51$, $P = 0.05$; early apple $S = 181.28$, $r_{s,7} = -0.51$, $P = 0.16$; late apple $S = 335.66$, $r_{s,9} = -0.53$, $P = 0.10$; ornamental hawthorn $S = 546.47$, $r_{s,15} = 0.33$, $P = 0.20$).

Flight activity pattern

Mean flight activity times of adult flies differed significantly among host populations ($F_{3,21} = 13.01$, $P = 5.07 \times 10^{-5}$; Fig. 2.5) and chronologically matched host-fruit ripening times (Fig. 2.6). Flight activity times differed in three out of six pairwise comparisons (Table 2.3). Mean flight activity on black hawthorn trees was 10 July (± 2.5 days s.e.); on early-apple trees, 15 July (± 3.3); for late-apples, 25 July (± 3.4); and for ornamental hawthorns, 3 August (± 3.3). I found a consistent difference of 17-20 days

between mean eclosion and flight activity times for all host-populations.

Allochronic isolation due to differences in flight activity times was observed among hosts, but was less pronounced than estimates from eclosion time. Black hawthorn flies were 14.7%, 31.3%, and 48.3% allochronically isolated from early-apple, late-apple, and ornamental hawthorn flies, respectively, based on their flight activity patterns. Early-apple flies were 11.5% and 36.6% isolated from late-apple and ornamental hawthorn flies, respectively, and late-apple flies were 18.9% allochronically isolated from ornamental hawthorn flies (Table 2.4).

Larval abundance

Abundances of larvae in black hawthorn, early-apple, late-apple, and ornamental hawthorn fruit followed the sequence of fruit maturation, adult eclosion times, and the activity patterns of flies (Fig. 2.6). The highest densities of larvae reared from black hawthorn and ornamental hawthorn fruit were from 18 July (0.112 larvae per fruit), and 26 September (0.385 larvae per fruit) collections, respectively. For early and late apple varieties, highest infestation levels occurred from 31 July (8.794 larvae per fruit) and 14 August (2.973 larvae per fruit) collections, respectively (Table 2.1). While the time interval between peak adult eclosion and peak fly activity on trees was relatively constant across host populations (range 17 to 20 d), there was an increasing difference between activity time and maximum larval abundance between early- versus late-fruiting hosts that was most pronounced for ornamental hawthorn (Figs. 2.3 & 2.6). One possible explanation for the difference is that eggs hatch and/or larvae develop more slowly in

ornamental hawthorn fruits. It is also possible that ornamental hawthorn adults simply take longer to develop and reach sexual maturity and have a longer lifespan than early-season flies, and therefore oviposit later than early-season populations.

Discussion

I found evidence that the life histories of *R. pomonella* populations infesting different host plants are temporally offset from one another in sympatry at a field site in Vancouver, WA in accord with differences in the fruiting phenologies of their respective hosts. The differences in fruiting time generate partial allochronic mating isolation among fly populations that could contribute to the formation of host races in the PNW. The observed shifts in life history timing likely arose rapidly: an apple-specialized race of *R. pomonella* was introduced to the PNW just 40-65 ya (Dowell, 1988; Sim, 2013). Black hawthorn and ornamental hawthorn are novel hosts whose ripening schedules are earlier and later, respectively, than apples, suggesting temporal changes in life history are not limited to shifting toward earlier phenologies, as seen for *R. pomonella* populations in the eastern US.

It is still too early to conclude, however, that host races exist on black hawthorn, apple, and ornamental hawthorn in the PNW until additional studies show that genetic differences exist among these populations and confirm that the observed life history differences are heritable. In the eastern US, eclosion time differences generating allochronic mating isolation between apple and downy hawthorn flies have been associated with genetic differences between the host races (Feder *et al.*, 1993, 1997;

Filchak *et al.*, 2000; Egan *et al.*, 2015). Thus, it is probable that similar genetic differences exist in the PNW, given that apple flies were introduced from the East. However, the eclosion differences reported in the current study were derived from flies experiencing non-uniform environmental conditions in the field. Consequently, rearing flies under standardized conditions in the laboratory could alter our conclusions. For instance, black hawthorn flies experience a prolonged period of elevated temperatures prior to winter, and thus may require fewer days for development following the onset of warmer temperatures in the spring. Conversely, the opposite could be true for ornamental hawthorn flies. However, this explanation is unlikely, as previous studies have found concordance in eclosion times between non-controlled field- and controlled laboratory-reared flies, implying that the results obtained here reflect genetically based diapause differences in nature (Dambroski & Feder, 2007; Lyons-Sobaski & Berlocher, 2009).

Even if a portion of the observed life history variation is subsequently shown to be plastic and environmentally induced, however, the differences would still generate allochronic mating isolation among host-associated populations at the Vancouver site, facilitating the potential evolution of other ecological adaptations among the flies, including host discrimination and feeding performance (survivorship) differences. In this regard, apple and downy hawthorn flies in the eastern US also display behavioral differences in host fruit odor discrimination (Linn *et al.*, 2003; Dambroski *et al.*, 2005; Forbes & Feder, 2006). These differences are important because *R. pomonella* mate only on or near the fruit of their respective host plants (Prokopy *et al.*, 1971, 1972). As a result, differences in host discrimination generate prezygotic reproductive isolation

between apple and downy hawthorn flies (Feder *et al.*, 1994). Similar behavioral differences in fruit volatile discrimination affecting host choice have been found among black hawthorn-, apple- and ornamental hawthorn-origin flies in the PNW (Linn *et al.*, 2012; Sim *et al.*, 2012), including at the Vancouver, WA site, consistent with the existence of host races in the region, as in the eastern US.

In addition to the issues of genetics and environmental effects, verification of host races also requires showing that the pattern of allochronic isolation among black hawthorn, apple, and ornamental hawthorn flies at Vancouver, WA in 2013 is consistent across years and other sites in the PNW. The black hawthorn stand examined in the current study was planted from nursery-reared saplings between 2000 and 2006, as part of Burnt Bridge Creek Greenway (BBCG) riparian restoration project (Robert Goughnour, *personal communication*). I found no other native black hawthorn growing in the immediate vicinity of the stand of over 30 trees at BBCG, aside from a single uninfested *C. suksdorfii* tree ~0.6 km away. Although the *C. douglasii* at BBCG were planted, their fruiting phenologies, fruit productivity, and infestation rates were very similar to those of other *C. douglasii* located at multiple nearby sites (e.g., Oak Island at Sauvie Island, OR [45° 42.8 N, 122° 49.1 W]; Washington State University Campus, Vancouver, WA [45° 44.0 N, 122° 37.6 W]; Legacy Salmon Creek Medical Center, Vancouver, WA [45° 43.2 N, 122° 39.0 W]; Springwater Corridor Trail near 99W in southeast Portland, OR [45° 27.7 N, 122° 38.1 W], MM, pers. obs.). In addition, Tracewski *et al.* (1987), in a more coarse grained temporal analysis, reported peak *R. pomonella* flight activity and larval infestation of fruits to be 3-4 weeks earlier for black

hawthorn than apple and ornamental hawthorn at a site in the Columbia River Gorge (St. Cloud Park, Skamania, WA) and 3-4 weeks earlier for apple than ornamental hawthorn at sites in the Portland and Vancouver area. In a six-year study (1981-1986), AliNiazee & Westcott (1987) detected consistent differences in fly activity among apple, ornamental hawthorn, and in one case, black hawthorn populations across western Oregon. Thus, existing evidence suggests that the pattern I observed at the Vancouver site may hold to some degree at other locations, particularly with regard to ornamental hawthorn. However, local variability may also exist for black hawthorn and apple, requiring more fine-grained seasonal analysis across the PNW to resolve if these hosts' and associated fly phenologies are consistent throughout the region.

It is also possible that population differentiation occurs on a local, site-by-site scale. That is, gene flow among host-associated populations at one site (e.g., black hawthorn $\leftrightarrow$ early apple $\leftrightarrow$ late apple $\leftrightarrow$ ornamental hawthorn) might be reduced, but still exceed gene flow between populations associated with a specific host between sites (e.g., black hawthorn flies at site A $\leftrightarrow$ black hawthorn flies at site B). This gets at the issue of: what causes greater reproductive isolation, allochronic and behavioral isolation or spatial isolation? These are important issues to resolve for determining the spatial reach of the present findings.

Another unresolved question concerns why estimates of allochronic isolation based on eclosion time were greater than those based on flight activity (Table 2.4). It is possible that post-eclosion migration among hosts could reduce the effects of diapause timing on the temporal divergence of fly populations. In this regard, early- and late-

fruiting apple varieties could serve as a conduit for gene flow among hosts and decrease the consequences of divergent diapause selection on allochronic mating isolation. It is also possible that the increased temporal overlap in flight activity was a consequence of the methods I used to trap flies in trees. In particular, the sticky traps, being yellow in color and baited with ammonium carbonate, are generally attractive to *R. pomonella* (Moericke *et al.*, 1975; Rull & Prokopy, 2000; Pelz-Stelinski *et al.*, 2005; Yee & Goughnour, 2011). Adult flies often forage for food across relatively large distances (0.6 km or more; Bourne *et al.*, 1934; Maxwell & Parsons, 1968; Feder *et al.*, 1994) before returning to host trees to mate and oviposit upon reaching sexual maturity. Thus, the baited yellow sticky traps, which are chemotactically and visually attractive to western *R. pomonella* (Yee & Goughnour, 2011), may have resulted in higher proportions of non-natal flies being trapped in trees than is normally the case. Sampling of flies by other means (i.e., by aspiration directly from host plants) is therefore needed to help discern the cause for the increased temporal overlap of fly populations based on flight activity. I note, however, that levels of AI derived from adult flight activity are nevertheless comparable to those estimated between apple and downy hawthorn flies in the eastern US (Feder *et al.*, 1994, 1998), and, thus, may be sufficient to facilitate host race formation in the PNW.

In conclusion, I documented that seasonal differences in eclosion, flight activity, and larval abundance times exist among host-associated populations of *R. pomonella* at a field site in Vancouver, Washington, USA. These life history differences generate allochronic isolation, similar to the apple and downy host races of the fly in the eastern

US. However, in contrast to the eastern US, flies in the PNW appear to have switched from apple to two novel hawthorn species within the last 40-65 years and shifted their life histories to exploit later, as well as earlier, fruiting hosts. Additional studies are required: 1) to confirm the genetic basis for the eclosion time differences; 2) to determine how environmental cues such as temperature, moisture, and photoperiod, act in concert with gene expression to affect the onset, cessation, and—ultimately—the length of diapause in *R. pomonella* (Prokopy, 1968; Filchak *et al.*, 1999); and 3) to discern the exact stage(s) in the life cycle in which environmental cues influence the diapause schedule of the fly. Previous work indicates that larval and pupal stages of *R. pomonella* respond to temperature and photoperiod cues, whereas the egg and adult stages do not (Prokopy, 1968; Feder *et al.*, 1997). Diapause life history adaptation to match host phenology is common in phytophagous insects (Tauber *et al.*, 1986). Consequently, diapause life history may often rapidly evolve to ecologically adapt phytophagous insects to novel or changing temporal host resources (Funk *et al.*, 2002; Bradshaw & Holzapfel, 2008; Wadsworth *et al.*, 2013). Such shifts can have repercussions for the timing of reproduction that result in allochronic isolation among host-associated populations that can potentially initiate speciation regardless of geographic context (in the face of gene flow or in allopatry in its absence). Given that there are more phytophagous insect specialists than any other life-form (Bush, 1975; Price, 1977), diapause life history adaptation may be a frequent contributor to the great diversity of life on Earth.

Table 2.1. Host species' larval infestation times, as well as infestation rates based on numbers of larvae per fruit.

<u>Host</u>	<u>Date Picked</u>	<u># Fruit</u>	<u># Larvae</u>	<u># Larvae per Fruit</u>
Black haw	12-Jul	7081	318	0.045
Black haw	18-Jul	8152	910	0.112
Black haw	12-Aug	942	57	0.061
Early apple	18-Jul	40	280	7.000
Early apple	31-Jul	63	554	8.794
Early apple	14-Aug	30	162	5.400
Late apple	18-Jul	99	236	2.384
Late apple	31-Jul	141	360	2.553
Late apple	14-Aug	147	437	2.973
Ornamental haw	6-Sep	2294	248	0.108
Ornamental haw	26-Sep	3516	1354	0.385

Table 2.2. Paired t-tests comparing percent maximum (a) median fruit softness; and (b) mean size (diameter) through the sampling period. Host species with the earlier ripening time is listed first under 'Host Pair': BH = black hawthorn, EA = early apple, LA = late apple. Significant *P*-values ($\alpha = 0.05$) are in bold.

(a) Median Softness

Host Pair	Mean % Difference	<i>t</i> -statistic	# Sample Dates	<i>P</i>
BH v. EA	30.51	-2.19	6	0.0398
BH v. LA	66.63	-3.62	4	0.0181
BH v. OH	85.83	-20.60	3	0.0012
EA v. LA	40.63	-4.78	8	0.0010
EA v. OH	38.39	-3.30	4	0.0229
OH v. LA	41.65	-3.38	8	0.0059

(b) Mean Size

Host Pair	Mean % Difference	<i>t</i> -statistic	# Sample Dates	<i>P</i>
BH v. EA	18.90	9.71	6	0.0001
BH v. LA	28.42	13.94	4	0.0004
BH v. OH	24.52	16.64	3	0.0018
EA v. LA	11.37	8.01	8	<0.0001
EA v. OH	12.90	2.20	4	0.0575
LA v. OH	1.76	0.97	8	0.1830

Table 2.3 Pairwise comparisons of (a) mean eclosion times; and (b) flight activity times between host-associated fly populations. (BH = black hawthorn; EA= early apple; LA = late apple; OH= ornamental hawthorn). The population with the earlier phenology is listed first under 'Hosts'. Difference = number of days between mean dates of (a) eclosion or (b) flight activity for indicated host comparisons. Significant *P*-values ($\alpha = 0.05$) are in bold.

(a) Eclosion

Hosts	Difference (Days)	s.e.	<i>z</i> -value	<i>P</i>
BH - EA	1.88	3.73	0.504	0.958
BH - LA	12.04	3.45	3.493	0.003
BH - OH	24.25	3.89	6.236	< 0.001
EA - LA	10.16	3.79	2.684	0.036
EA - OH	22.37	4.19	5.337	< 0.001
LA - OH	12.21	3.94	3.099	0.010

(b) Activity

Hosts	Difference (Days)	s.e.	<i>z</i> -value	<i>P</i>
BH - EA	5.65	5.11	1.106	0.682
BH - LA	15.43	3.83	4.026	< 0.001
BH - OH	26.00	4.02	6.462	< 0.001
EA - LA	9.79	5.28	1.855	0.243
EA - OH	20.36	5.41	3.760	0.001
LA - OH	10.57	4.24	2.496	0.059

Table 2.4 Estimates of percent reproductive isolation among host populations as a result of (a) adult eclosion times, (b) flight activity times, (c) behavioral responses recorded in laboratory wind tunnels (Linn et al. 2012), (d) behavioral responses recorded in the field (Sim et al. 2012), (e) combined estimate using eclosion and laboratory behavioral isolation, and (f) combined estimate using eclosion and field behavioral isolation. Data collected from co-occurring populations of *R. pomonella* in southwestern Washington State. (See text for calculations)

(a) Eclosion

	Black hawthorn	Early apple	Late apple
Early apple	24.4%	—	—
Late apple	55.7%	43.9%	—
Ornamental hawthorn	92.6%	87.1%	59.3%

(b) Activity

	Black hawthorn	Early apple	Late apple
Early apple	14.7%	—	—
Late apple	31.3%	11.5%	—
Ornamental hawthorn	48.3%	36.6%	18.9%

Fig. 2.1 Allochronic premating isolation resulting from differences in eclosion times (idealized here for clarity) of (a) downy hawthorn- and (b) apple-associated populations of *R. pomonella* in the eastern US. Panel (c) illustrates a hypothetical shift resulting from novel colonization of a temporally adjacent, earlier fruiting host. Eclosion of adults strongly correlates with natal fruit ripening (quantified using fruit diameter and flesh softness), followed by mating and oviposition (‘Adult Activity’) and larval development within fruit. Flies reach sexual maturity 7–10 days post-eclosion, helping to explain why eclosion curves slightly precede fruit maturation. Modified from Bush (1969).

Fig. 2.2 (A) *Rhagoletis pomonella* were first detected in Portland, Oregon in 1979 (AliNiazee & Penrose, 1981) and have spread north and south in Washington and Oregon, as well as into the Columbia River Gorge (gray arrows) toward Washington's fruit growing regions (shaded area). (B) The Burnt Bridge Creek Greenway, Vancouver, WA including surrounding bodies of water. Sample sites were located in three general areas, indicated by boxes 1–3. Panel (C) details each of the three boxed areas of (B) to show the specific arrangement of eclosion tents. Inset photograph depicts one of the eclosion tents.

Fig. 2.3 Cumulative adult eclosion (bold line), relative host fruit softness (filled triangles) and mean fruit diameter (open circles) plotted against time. Spearman's rank correlation coefficients are given for cumulative eclosion versus median fruit softness (r_{soft}) and mean fruit diameter (r_{diam}), where $*$ = $P < 0.0001$. For standard errors and interquartile ranges of fruit ripening data see supplementary Fig. A.1.

Fig. 2.4 Percent maximum fruit softness and size were strongly correlated for each host species [Spearman's rank correlation coefficients (r_s) and significance level (** = $P < 0.0001$; * = $P < 0.001$) shown in lower right of each graph].

Fig. 2.5 Cumulative (a) eclosion curves; and (b) flight activity times for black hawthorn (BH), early apple (EA), late apple (LA), and ornamental hawthorn (OH) flies at the Vancouver, WA study site. Sample sizes (numbers of flies) for generating the eclosion curves were $n = 53, 115, 52,$ and 53 for BH, EA, LA, and OH, respectively; and for activity curves $n = 1108, 539, 2074,$ and $873,$ respectively. Populations not sharing a superscript letter in common indicate a significant difference in mean eclosion or activity time (see Table 2.3 for significance levels). Numbers along the 50% stippled line of the y-axis indicate the difference (in days) between median eclosion or activity times between host populations.

Fig. 2.6 Mean dates of adult eclosion (diamonds) and flight activity (triangles), and maximum larval abundance (circles) for host populations at the Vancouver, WA site. Black and gray horizontal lines indicate ranges of dates when host fruits were most rapidly growing or softening, respectively.

Chapter 3:
**Effects of Pre-winter Conditions on Mortality Rates and Diapause Phenologies of
Host-associated Populations of *Rhagoletis pomonella* (Diptera: Tephritidae) in
southwest Washington State**

Abstract

The apple maggot fly, *Rhagoletis pomonella* (Diptera: Tephritidae), is a host-specialist true fruit fly endemic to eastern North America that shifted 160 ya from its ancestral host hawthorn to the derived host apple in sympatry after apples were introduced into its range 400 ya. The host shift occurred in the absence of geographic barriers, providing insights into ecologically driven speciation. In *R. pomonella*, strong host fidelity and phenological shifts (coupled with short life span) are the primary traits upon which selection acted to initiate reproductive isolation between early fruiting apple- and late fruiting hawthorn populations. The fly was introduced to Portland, Oregon, US 40-65 years ago from the eastern US via transported apples infested with larvae. Introduced flies were likely apple-specialists, but have since broadened their host range to additionally infest early fruiting native black hawthorn (*Crataegus douglasii*) and late fruiting introduced ornamental hawthorn (*C. monogyna*) in the region. Seasonal distributions of the separate host-associated populations of *R. pomonella* have rapidly become offset at a field site where hosts co-occur in western Washington: eclosion now mirrors offset ripening times of host fruits. Whether divergent phenologies are maintained by environmental or genetic mechanisms is not known. In the present study, I manipulated the pre-winter environment under standardized laboratory conditions to test

three hypotheses: (1) ‘Thermal accumulation’: Pre-winter thermal accumulation modulates post-winter eclosion timing; (2) ‘Selection’: Mortality increases when pre-winters are longer or shorter than host-specific intervals in nature; (3) ‘Diapause depth’: each population is adapted to assume various depths of pupal diapause in accord with its seasonal distribution and to prevent premature eclosion. Mean numbers of days to eclosion were unaffected by pre-winter treatments, remaining fixed within all host populations (45.3, 49.2, 54.1, and 64.8 for black hawthorn-, early-apple-, late-apple-, and ornamental hawthorn-associated flies, respectively). Mortality rates were also unaffected by treatments, though consistently lower for hawthorn-origin (~32%) than apple-origin (~50%) flies.

Introduction

Host-specialist phytophagous insects are among the most speciose organisms, hence excellent models for studying adaptive radiations (Bush 1975; Price 1977; Mitter et al. 1988; Abrahamson et al. 1994; Wood et al. 1999; Drès & Mallet 2002; Funk et al. 2002; Emelianov et al. 2004; Nosil 2007; Wadsworth et al. 2013; Powell et al. 2014). Certain lineages of specialized insect herbivores are undergoing rapid evolution that can be studied within ecological time-scales and provide insight into specific roles that ecology can play in the speciation process in not only these, but a diverse array of taxa (Funk et al. 2009). The narrow dietary habits of host-specialists, wherein feeding is often restricted to a single host genus or species, are maintained by adaptations that confer host recognition and fidelity. However, when ecological specializations restrict host breadth, reproductively isolated subpopulations have the potential to arise when individuals shift to exploit alternative hosts that are geographically and (or) phylogenetically adjacent (Sheldon & Jones 2001; Carrol et al. 2005; Michel et al. 2010). Human-mediated alterations in the geographic distribution of insects or plants can also influence evolutionary trajectories of organisms. This is because relocation can provide opportunities for parasitic insects to colonize and specialize on novel host plants, reducing opportunities for gene flow with populations remaining on the ancestral hosts (Feder 1995; Funk 1998; Via et al. 2000; Nosil et al. 2005; Schwarz et al. 2005; Ghalambor et al. 2007; Funk et al. 2009).

Rhagoletis pomonella (Diptera: Tephritidae) is a host-specific insect whose eastern and southern populations are well-studied models for ecological speciation

through host race formation (Feder et al. 1993; Bush 1994; Lyons-Sobaski & Berlocher 2009; Powell et al. 2013, 2014; Egan et al. *in press*). Host races are conspecific populations that are partially reproductively isolated due to adaptations to alternative hosts in sympatry. Host races are hypothesized to be at the phase along the continuum between panmictic population and reproductively isolated species (Bush 1975; Diehl & Bush 1984; Drès & Mallet 2002; Feder et al. 2012).

In the case of *R. pomonella*, a novel host race recently evolved in the eastern United States when a shift occurred in the mid-1800s from the native downy hawthorn host (*Crataegus mollis* [Torr. & A. Gray] Scheele) to introduced domesticated apple (*Malus domestica* Borkh.) (Walsh 1867; Bush 1994). The shift from hawthorn to apple hosts was accomplished through divergent selection upon two life history characteristics of the species: host fidelity and timing of reproduction. Following eclosion, adults require 7–10 days to reach sexual maturity, at which time they locate host fruits using visual and chemical stimuli (Prokopy et al. 1971, 1972). The apple and hawthorn races prefer volatiles emanating from natal host fruits, while avoiding those from non-natal hosts. Since mating occurs directly on or near host fruits, this creates a scenario where habitat and reproduction are inextricable, and mating is therefore assortative (Dean & Chapman 1973; Boller & Prokopy 1976; Linn et al. 2003; Forbes & Feder 2006).

The second characteristic that reproductively isolates apple and hawthorn races is divergent eclosion phenologies. On average, apple-origin flies eclose ~10 d before hawthorn-origin flies at sites of sympatry, because apples ripen 3–4 weeks before hawthorn fruits (Feder et al. 1993). Adults live just 30 d in nature, so the result is reduced

opportunities for gene flow between flies infesting the alternative hosts. In sum, behavioral (olfactory preference and avoidance coupled with host fidelity) and allochronic isolation are two prezygotic isolating mechanisms that reduce gene flow between host races to ~6% per generation (Feder et al. 1994).

Rhagoletis pomonella has recently (40–65 ya) invaded the Pacific Northwestern (PNW) United States, probably via the human-mediated transport of larval-infested apples (AliNiazee & Penrose 1981; Sim 2013). In the PNW, populations of *R. pomonella* have rapidly colonized two local hawthorn hosts: native black hawthorn (*Crataegus douglasii* [Lindl.]) and non-native ornamental hawthorn (*C. monogyna* [Jacq.]) (AliNiazee & Westcott 1986; Tracewski et al. 1987; Yee 2008). Field and laboratory assays indicate that *R. pomonella* populations in the PNW prefer and avoid natal and non-natal host fruit volatiles, respectively (Sim et al. 2012; Linn et al. 2012), suggesting selective divergence in host recognition behavior has evolved rapidly.

In addition, the fruiting times of the two hawthorns bracket that of apple, where black hawthorn ripens earliest in the season, and ornamental hawthorn latest. In a recent field investigation at a site of host sympatry in southwestern Washington (Fig. 3.1), I found that populations of *R. pomonella* infesting black hawthorn and early-ripening apple varieties eclose early in the summer, and are partially allochronically isolated from populations infesting late-maturing apple varieties as well as ornamental hawthorn from 24.4%–92.6%, coincident with the offset fruiting times of their hosts (Fig. 3.2; Mattsson et al., *in review*). The mechanistic means for shifting life-history timing during the early stages of population divergence is not completely understood, but it is likely that

selection upon pupal diapause plays a central role in shaping the alternative phenologies in this univoltine species (Tauber et al. 1986).

Insect diapause can be a dynamic, genetically based trait whose onset, duration and cessation are orchestrated by abiotic cue(s) detected by the organism prior to the diapause episode itself (Tauber et al. 1986; Denlinger 2002). However, pupal diapause in *R. pomonella* is facultative in eastern apple and hawthorn races. For example, when flies are artificially subjected to extended periods of elevated temperatures ($\geq 26^{\circ}\text{C}$) and long photoperiods (17h:7h L:D), many pupae forego diapause and develop directly into a second generation of adults during the same year (Prokopy 1968; Feder et al. 1997; Dambroski & Feder 2007). In nature, this bivoltinism is somewhat inconsequential for late-season (i.e., downy hawthorn-infesting) flies that rarely experience such conditions prior to winter's onset. The pupae of early-season flies (i.e., apple-infesting flies in the east), on the other hand, are subjected to relatively longer photoperiods and higher temperatures, thus being at risk of foregoing diapause and eclosing at a time when neither habitat (host fruit) nor mates are available, a fatal consequence resulting in the reduction of that genotype's frequency in the population. Dambroski & Feder (2007) found that the apple race is less likely than the hawthorn race to develop directly into adults when subjected to extended pre-winter conditions, supporting their hypothesis that apple race pupae enter a 'deeper' diapause than hawthorn race pupae. The authors also tested populations along a latitudinal cline and found that southern populations of both fly races were more recalcitrant to direct development, in accordance with increased pre-winter temperatures and milder winters in the south.

In southwest Washington, it is not known whether the divergent eclosion phenologies I observed among populations infesting alternative hosts are maintained primarily by environmental or genetic mechanisms. This distinction has important evolutionary implications, for if the discrete eclosion phenologies are genetically polymorphic, it suggests that the offset phenologies have progressed beyond a phase of environmentally induced phenotypic plasticity and are now heritable. If this is the case, reproductive isolation among host populations and shifts toward both earlier (black hawthorn) and later (ornamental hawthorn) phenologies have evolved rapidly following introduction 40–65 ya. Flies infesting black hawthorn and early-maturing apple varieties eclose about the same time during early summer, and two weeks before flies that infest late-maturing apple varieties and three weeks before flies infesting ornamental hawthorn in the field (Mattsson et al. *in review*). Here, I propose three testable hypotheses to begin to explain how allochronic isolation is maintained among these populations despite the potential for gene flow:

I: Thermal Accumulation Hypothesis

It is possible that the asynchronous eclosion schedules among co-occurring populations of *R. pomonella* are due to non-uniform thermal environments experienced during certain stages of development. Specifically, all individuals within the species may require roughly an equivalent amount of thermal energy to complete development from pupa to adult. The early pupation times of flies associated with black hawthorn and early-ripening apple varieties might allow them to transduce thermal energy during their first

summer to advance development, requiring only a brief period of elevated temperatures the following spring to complete development and eclose as adults. Conversely, late-season individuals associated with late-ripening apple and ornamental hawthorn have little thermal energy available prior to the onset of winter, and therefore require a longer period of development the following summer. Thus, eclosion schedules are established and maintained by environmental inputs, enabling adult insects to exploit alternative host species that happen to be available at times coincident with their staggered eclosion schedules.

I hypothesize that if fly populations typically experiencing extended pre-winter periods in nature are artificially subjected to an abbreviated pre-winter interval (comparable to that experienced by late-season populations), they will require a longer period to eclose following the overwinter period of pupal diapause. Alternatively, when individuals from late-season populations are subjected to extended pre-winters (comparable to those experienced by early-season populations), they will eclose more rapidly following pupal overwintering. If these reciprocal transplants do not modulate post-diapause time intervals to eclosion, it suggests that genetic, rather than environmental, mechanisms underlie the asynchronous phenologies. Such genetic differences would imply that host-associated adaptations are causing partial allochronic reproductive isolation, which may initiate host race formation.

II: Selection Hypothesis

Insects prepare for diapause during ‘prediapause’ or ‘diapause induction’ periods (*sensu* Tauber et al. [1986] pp. 47–66). Critical preparations include upregulation of heat shock proteins (Storey & Storey 2012) and the storage of nutrients and lipid energy reserves to ensure survival and cold-hardiness during stressful abiotic conditions such as winter (Košťál 2006; Hahn & Denlinger 2007). Physiological variation in preparation rates appears to have been a fulcrum that facilitated the host shift from hawthorn to apple in eastern *R. pomonella*. For example, Egan et al. (2015) performed an experiment where individuals from the eastern downy hawthorn race were exposed to either a 32 d or 7 d period of 26°C following pupation but prior to an extended, laboratory simulated 4°C ‘winter’ (pre-winter time intervals were selected because they simulate durations naturally experienced by apple and hawthorn flies, respectively, at a site near Grant, Michigan). Survivorship following winter plummeted for hawthorn race individuals experiencing the 32 d pre-winter conditions typical of the apple race. Over 100 independent gene regions responded to the single selection event imposed in the experiment, indicating the strength of selection associated with pre-winter developmental rates of pupae.

The selection hypothesis should therefore also describe patterns in the recently introduced PNW populations currently developing new host affiliations. I predict that early-pupating populations, such as black hawthorn and early-apple flies, should suffer increased mortality if subjected to shorter-than-natural pre-winter intervals because they do not have time to make necessary physiological preparations for stressful winter

conditions. Conversely, populations pupating later in the season (late-apple and ornamental hawthorn flies) should suffer increased mortalities as a result of long pre-winter treatments more typical of early season individuals, due to increased metabolic rates hastening the depletion of energy reserves and water, typical of poikilothermic organisms exposed to elevated temperatures (Košťál 2006; Hahn & Denlinger 2007; Storey & Storey 2012).

III: Diapause Depth Hypothesis

In the eastern US, the apple race of *R. pomonella* ecloses earlier than the hawthorn race, has been shown to undergo ‘deep diapause’, and is relatively more resistant, though not immune, to bivoltinism when reared in environments with elevated temperatures and summer-like photoperiods (Feder et al. 1997; Dambroski & Feder 2007). Therefore I hypothesize that populations in the PNW associated with black hawthorn and early season apple will also have reduced incidence of direct developing forms, having been selected against for 45–60 generations. Late season apple and ornamental hawthorn-associated flies experience less severe selection against direct development, and therefore will have higher incidences of ‘shallow diapausing’ pupae in their populations.

In the current study, I manipulated pre-winter environments to investigate how environmental conditions prior to winter’s onset affect three life history parameters of four host-associated populations of *R. pomonella* in southwest Washington: (1) post-

winter adult eclosion times (thermal accumulation hypothesis); (2) pupal mortality (selection hypothesis); (3) and direct development of adults (diapause depth hypothesis).

Materials and Methods:

Sample Collection

From June through September of 2013 I collected fruit infested with *R. pomonella* larvae from black hawthorn, apple, and ornamental hawthorn trees growing within 100 m of the Burnt Bridge Creek Greenway (BBCG), Vancouver, Washington, USA (45° 37.9' N; 122° 37.1' W; Fig. 3.1), and from an additional ornamental hawthorn tree located near Mount Tabor Park in Portland, Oregon, USA (45°30.63'N, 122°35.23'W). Hawthorn fruit were collected evenly from around the canopy perimeter while standing either at ground level or from a 1.0 m stepladder (researcher height = 1.85 m). I collected 346–573 fruit from each of seventeen black hawthorn trees on 12 and 18 July, and also 942 fruits total on 12 August. For ornamental hawthorn, I collected 99–771 fruits from each of eight ornamental hawthorn trees on 6 September and 26 September.

I collected apples evenly around the canopy perimeter using an extendable fruit-picking apparatus at heights ranging from 2.0–6.5 meters. Apple trees at the site belonged to two distinct groups: an early maturing variety whose fruit ripened more than one month before the second, late maturing variety, and flies from each were analyzed separately. On 18 July, 31 July, and 14 August I collected 10–63 apples from each of three early-ripening, and 10–43 apples from each of seven late-ripening apple trees. Fruits were returned to the laboratory within 2 h of collection and spread atop baskets

built from 1 cm mesh-size galvanized steel hardware cloth positioned in plastic collecting trays. In the laboratory, I collected larvae and pupae emerging from fruit every other day using a soft paintbrush and subdivided them into five Petri dishes containing moist vermiculite, and each Petri dish was assigned to a specific pre-winter treatment group (see next section). Total numbers of pupae reared from each fruit collection date are presented in Table 3.1.

Pre-winter Treatments

Pupae were subjected to four pre-winter intervals: intervals that were equivalent to natural intervals for a given population, and those equivalent to intervals experienced by sympatric populations infesting other host plant species in close proximity. Experimental pre-winter treatment intervals roughly matched empirical field measurements recorded at the BBCG in 2013 (Table 3.2, Fig. 3.2). Black hawthorn-associated flies oviposit mid to late July; early-apple flies from the end of July through early August; late-apple flies during mid to late August; and ornamental hawthorn flies from the middle to end of September (Mattsson et al. *in review*; Table 3.2). Larvae require 2–5 weeks to molt through three instars before leaving fruit to pupate in the soil (Dean & Chapman 1973). Ambient and soil temperatures in the region begin to drop below 5°C beginning mid October (Bates 1975; Weather Underground™ website). Thus, assuming a 24 d (3.5 week) larval development period, *R. pomonella* pupae from black hawthorn-, early apple-, late apple-, and ornamental hawthorn-associated populations experience, roughly, 63, 51, 32, and 1 d pre-winter intervals, respectively (Table 3.2).

Experimental pre-winter intervals were rounded to 60 d, 45 d, 30 d, and 15 d, respectively, in order to have even distribution between treatments (Fig. 3.2). The fifth treatment group was not overwintered, but rather used to test predictions of the diapause depth hypothesis (arrows ascending from host names in Fig. 3.2). All other abiotic variables were held at standard laboratory conditions, which included a 15L:9D L:D photoperiod and 22–23°C ambient temperature.

Following pre-winter treatments, Petri dishes (with the exception of those belonging to the non-overwintering group) were placed into a refrigerator (photoperiod = 0L:24D; temperature = 4–6°C) for 25 weeks to simulate natural PNW overwintering conditions (Bates 1975; Zheng et al. 1993). The non-overwintering group was held at (15D:9L; 22–23°C) for the 580 d duration of the experiment (total numbers of pupae entering each treatment are summarized in Table 3.3).

After the 25 wk overwintering period, I transferred contents of Petri dishes into 480 ml beverage containers (SOLO[®] Cup Company, Lake Forest, IL) covered with paper towels that were kept moist and secured with rubber bands for the remainder of the study. Eclosion containers were haphazardly distributed and rotated weekly in a room with 15L:9D photoperiod and 22–23°C. I aspirated eclosing adults from containers every other day, determined their sex, and transferred them into 1.5 ml micro centrifuge tubes which were then placed at –80°C.

Eggs, larvae and possibly pupae of *Rhagoletis pomonella* are attacked by multiple genera of endoparasitoid and ectoparasitoid Hymenopteran wasps from the families Braconidae, Pteromalidae, and Eulophidae, which consume their hosts and diapause over

winter within the flies' puparia (Gut & Brunner 1994; Forbes et al. 2010). To tabulate mortality of *R. pomonella* during this experiment, each wasp that eclosed was assumed to represent a single mortality of *R. pomonella*, as a wasp always kills its host and only one wasp successfully survives per puparium (Dean & Chapman 1973; Hood et al. 2012). Eclosing wasps were aspirated and stored at -80°C . I monitored the eclosion containers for 300 days.

Data analysis:

I. Thermal Accumulation Experiment

One-way ANOVA (if the data were homoscedastic as determined by Brown-Forsythe tests, $\alpha = 0.05$) or Welch's ANOVA (when data were heteroscedastic) tests were used to compare mean number of days to eclosion (DTE) following removal from overwintering conditions, among treatments both within and among populations, using each individual eclosing fly as a replicate. When mean DTE were significantly different ($P \leq 0.05$), Tukey's Honestly Significant Difference (HSD) post-hoc analysis was conducted to determine the degree of differences between pre-winter treatment pairs either within or between populations.

II. Selection Experiment

I first grouped fates of individuals into general 'survival' or 'mortality' categories (Fig. 3.3) using the numbers of flies and/or wasps eclosing following the winter treatment. I then sieved and sorted vermiculite from each eclosion container to track and

tabulate fates of all pupae and adults. I dissected pupae using a stereomicroscope to determine if the organism remained viable, and if so, what life stage it was in. Survival and mortality fates were then further subcategorized as follows: individuals were considered survivors when (1) eclosion followed over-wintering period, (2) a viable larva remained within the puparium, or (3) a viable pre-adult remained within the puparium. I assigned mortality fates when (1) a wasp depredated the pupa, evidenced by either an eclosing wasp, or a viable or nonviable wasp within the puparium, (2) the puparium contained a desiccated adult or was otherwise nonviable, (3) the adult eclosed prior to the overwinter period, or (4) neither pupal case nor adult were found (Fig. 3.3).

The last of these ‘mortality fates’ requires further explanation. When searching through vermiculite to determine fates, appearances of pupae ranged from the typical ovoid shape and light tan color, to flattened, shrunken, black and desiccated. It is likely that the latter were occasionally overlooked during the sieving process, due to their appearance being nearly indistinguishable from the surrounding vermiculite substrate. I further investigated this phenomenon by comparing whether numbers of what I will call ‘unaccounted for pupae’ occurred randomly among treatments and populations, or rather increased in association within specific pre-winter treatments or host populations. One-way ANOVA or Welch’s ANOVA tests were used to compare percentages of unaccounted for individuals for each treatment both within and among populations, using each eclosion container as a replicate.

To estimate rates of treatment-induced mortality for testing the selection hypothesis, it was necessary to exclude mortality fates not induced by treatments, such as

predation by wasps or pre-winter eclosion forced by environmental conditions. I determined that treatment-induced mortalities included total numbers of individual flies found dead within puparia plus unaccounted individuals (black boxes with white type in Fig. 3.3). Once mortality fates were determined for all pupae within each container, treatment-induced mean mortality rates (number of treatment-induced mortalities ÷ initial number of pupae) for each treatment were compared within and among populations using either one-way ANOVA or Welch's ANOVA tests, using each container as a replicate.

III. Diapause Depth Experiment

I calculated relative numbers of non-diapausing (direct developing) flies for each eclosion container by dividing the number of flies that eclosed in the non-wintering treatment by the total numbers of pupae that entered the treatment (after subtracting the number of wasp-induced mortalities). Data were normal and homoscedastic, so I used a one-way ANOVA to compare the percentages of non-diapausing flies among host populations, followed by post hoc analyses using Tukey's HSD pairwise tests, using each eclosion container as a replicate.

Results

I. Thermal Accumulation Experiment

Contrary to predictions of the thermal accumulation hypothesis, pre-winter treatments had no effect on mean DTE following winter within black hawthorn-, early apple-, or late apple-associated *R. pomonella* (one-way ANOVA, black hawthorn $F_{3,455} =$

0.283, $P = 0.838$; early apple $F_{3,318} = 0.07$, $P = 0.976$; late apple $F_{3,374} = 0.694$, $P = 0.556$; Fig. 3.4). A treatment effect was detected within ornamental hawthorn-associated flies (one-way ANOVA, $F_{3,563} = 3.693$, $P = 0.012$), where the 45-day pre-winter treatment group eclosed 4 d earlier, on average, than the 60-day group (Tukey HSD, $P = 0.048$), but at the similar times as the 15-day ($P = 0.065$) and 30-day ($P = 0.984$) groups (Fig. 3.4). One possible explanation for the difference between 45- and 60-day groups is that a single fly within the 45-day group eclosed just 17 d following removal from overwintering conditions. It is highly unusual for *R. pomonella* to eclose this rapidly (Prokopy 1968; Dambroski & Feder 2007), and the cause of single instance of rapid eclosion is unknown.

Overwhelmingly, no thermal accumulation effects due to pre-winter treatments were detected among treatments within host-associated populations, thus, I pooled treatments within each population to compare mean DTE among populations. This allowed me to test another hypothesis, the ‘phenology matching hypothesis’, which predicts that eclosion schedules are a genetically programmed adaptive trait to ensure that the flies’ reproductive stage coincides with their natal host fruiting phenologies. Fruits of each host ripen at separate, but partially overlapping times in summer and fall, as found in a previous field investigation (Mattsson et al. *in review*). Mean days to eclosion ($\pm$ standard error in days) differed among all populations (Welch’s ANOVA, $F_{3,871.7} = 261.3$, $P < 0.0001$). For black hawthorn flies, mean DTE was 45.3 (± 0.51 ; $n = 428$ flies); for early-apple flies, 49.2 (± 0.76 ; $n = 288$); for late-apple flies, 54.1 (± 0.71 ; $n = 343$); and

for ornamental hawthorn flies, 64.8 (± 0.53 , $n = 549$). Pairwise tests revealed statistically significant differences among all populations (Tukey HSD, all $P < 0.001$; Fig. 3.5).

II. Selection Experiment

There were no relationships between pre-winter treatment length and mortality rates within populations (one-way ANOVAs; black hawthorn $F_{3,40} = 2.005$, $P = 0.129$; early apple $F_{3,70} = 1.256$, $P = 0.296$; late apple $F_{3,74} = 1.632$, $P = 0.188$; ornamental hawthorn $F_{3,47} = 1.552$, $P = 0.214$). Since treatments did not affect mortality within a given population, data were pooled within populations to analyze inter-population mortality rates, which revealed that mortality rates among populations differed (Welch's ANOVA; $F_{3,128} = 14.248$, $P < 0.0001$; Fig. 3.6). Percent mortality was not statistically different between black hawthorn (33.3%) and ornamental hawthorn (31.6%) (Tukey HSD, $P = 0.98$). Early and late apple flies also had similar mortality rates to one another, 50.2% and 50.9%, respectively ($P = 0.99$). Thus, apple flies suffered >1.5 times higher mortality than hawthorn flies ($P < 0.0001$ for all hawthorn versus apple comparisons).

Unaccounted for pupae

Unaccounted for pupae (no evidence remaining of pupae entering the treatment) ranged from 0.0% to 83.3% per eclosion container, but did not differ among populations when treatments were pooled (mean = 20.8% per eclosion container, Welch's ANOVA, $F_{3,125.5} = 2.38$, $P = 0.077$, Fig. 3.7A). However, the proportion of unaccounted for pupae were different among treatments when populations were pooled (Welch's ANOVA,

$F_{3,132.2} = 19.76$, $P < 0.0001$; Fig. 3.7B). The percentages of unaccounted for pupae increased as pre-winter intervals increased, with the 60-day treatment having 12.7% ($P = 0.001$) and 20.1% ($P < 0.0001$) more than the 30-day and 15-day treatment groups, respectively. Also, the 45-day treatment group had 15.2% ($P < 0.0001$) more unaccounted for pupae than the 15-day group. Similar trends emerge when pairwise comparisons are made between treatments within populations. For each population, percentages of unaccounted for pupae for the 60-day groups exceeded that of the 15-day groups, with the exception of ornamental hawthorn population, where the percentage unaccounted for pupae did not differ among treatments (data not shown).

It is clear that prolonged exposure before the onset of winter presents these organisms with stresses that can lead to death and disintegration of puparia. However, correlations between increasing percentages of unaccounted for pupae and increasing pre-winter treatment length were confounded by the mortality response within the non-overwintering treatment used in the diapause depth experiment (Fig. 3.7B). It appears that exposure alone does not directly cause mortality, but rather that there is an interaction between pre-winter length and exposure to cold temperatures. The non-overwintering group received up to 545 days of exposure to standard laboratory conditions, compared with the 60 d maximum for the other treatment groups, yet the proportion of unaccounted pupae for the non-overwintering group was most similar to the 15-day treatment group (Fig. 3.7B).

III. Diapause Depth Experiment

The four populations had unequal proportions of flies developing directly into adults within the non-overwintering treatment (one-way ANOVA $F_{3, 51} = 2.98$, $P = 0.04$), although not in the manner predicted by the diapause depth hypothesis. The inequality was driven primarily by the ornamental hawthorn population, which had ~20% fewer non-diapausing flies than the late apple population (Tukey HSD $P = 0.02$), but similar to black hawthorn ($P = 0.63$) and early apple ($P = 0.44$) populations (Fig. 3.8). Specifically, mean percentages ($\pm$ standard error) of non-diapausing flies belonging to the black hawthorn, early apple, late apple, and ornamental hawthorn populations were 21.8% (± 5.2), 22.8% (± 4.9), 32.7% (± 4.8), and 12.6% (± 4.8), respectively.

Each population's eclosion curve in the diapause depth experiment was bimodal within the 30 to 90 DTE range (Fig. 3.9). An initial group of flies developed directly into adults and eclosed 35–45 d after pupation, followed by a second distinct eclosion peak 57–90 d post-pupation. Among the individuals foregoing diapause, the black hawthorn population had the greatest proportion of flies eclose 35–45 d following pupation (44/61 individuals, or 71.2%), while the late season ornamental hawthorn population had the fewest (9/33, or 27.3%, Fig. 3.9). Regarding shallow diapausing forms (those eclosing 57–90 days post-pupation), the black hawthorn population had relatively few (10/61, or 16.4%) while the early apple, late apple, and ornamental hawthorn populations had high representations of shallow diapausing forms (51.6%, 54.3%, 54.5%, respectively). Finally, in addition to the presence of the aforementioned phenotypes, there were several instances of individuals eclosing 90–282 d post-pupation with no detectable pattern,

although these occurrences were most prevalent within the black hawthorn and ornamental hawthorn populations (Fig. 3.9).

Discussion

The thermal accumulation experiment aimed to establish whether the temporally disjunct eclosion phenologies of *R. pomonella* infesting alternative hosts at a site in southwest Washington are (1) the result of genetically-based life history shifts that have evolved rapidly since its recent introduction, or (2) caused by plastic responses to seasonal environmental differences. It appears the former is true: eclosion phenologies within each of four host populations in southwest Washington and Oregon were unaffected by shortened or prolonged pre-winter intervals in the thermal accumulation experiment. In addition, each population eclosed at fixed times that would have been appropriate for exploiting their natal host fruits in nature.

Nevertheless, it is not well understood what physiological mechanism(s) enable univoltine insects such as *R. pomonella* to synchronize annual eclosion with host fruit availability. One hypothesis is that species-specific thermal energy requirements must be met in order to eclose after pupae break diapause. Indeed, accumulated degree-days are used in agriculture to predict and manage schedules of crops and their associated pests, including *R. pomonella* (Reissig et al. 1979; Jones et al. 1989; Herms 2004). Predictive degree-day models are generated by recording adult insect eclosion times over multiple years and subsequently regressing those estimates against accumulated thermal units above a given threshold to establish whether there are consistent interannual patterns of

insect eclosion. However, it remains uncertain whether thermal units (degree-days) themselves directly cause eclosion, or instead correlate with an unidentified parameter. Here, the numbers of post-winter degree-days (base temperature = 6.4°C, *sensu* Reissig 1979) corresponding to median cumulative eclosion were nearly identical between field estimates recorded 2013 and laboratory estimates from this study for each population (Fig. 3.10). This is a useful discovery not only for agricultural practitioners needing to manage this introduced pest species that threatens expansion into multi-billion dollar fruit crops in the region (Yee et al. 2012; Mertz 2013), but also for providing insight into the physiological basis of diapause phenology. Each population examined in this study appears to possess a unique eclosion phenotype that correlates with unique numbers of degree-days, potentially a basis for causing allochronic isolation among conspecifics. Allochronic isolation is a known precursor for generating unique ecotypes and potentially distinct species (Feder & Filchak 1999; Groman & Pellmyr 2000).

The selection experiment sought to examine a mechanism of selection at the physiological level. Specifically, do early versus late populations differ in their rates of winter preparation? The answer appears to be ‘no’, based on the fact that extended or shortened pre-winter intervals had no effect on mortality rates within any of the populations examined (data not shown).

However, the 22–23°C standard non-winter temperature used in this experiment might not represent natural conditions in southwest Washington, where summer temperatures can fluctuate between 14°C and 37°C in a 24 h period. An example of how various temperatures can affect survivorship was provided by Feder et al. (2010), who

performed an experiment using eastern hawthorn flies and found that unnaturally long pre-winter intervals had minimal affect on survivorship when temperatures were mild (18° and 22°C), but mortality increased disproportionately for long versus short pre-winter intervals when temperatures were 26°C. Thus, a follow-up experiment is needed testing a broader range of pre-winter temperatures, as well as temperature fluctuations, in order to tease apart the variable tolerances to seasonally unique pre-winter temperatures that might exist among early and late pupating populations in the PNW.

Foundational evidence suggests that it was the apple race of *R. pomonella* that was introduced to the PNW, as inferred through genetic (McPherson 1987; Sim 2013), geographic (Dowell 1988; Hood et al. 2013), and behavioral (Sim et al. 2012; Linn et al. 2012) evidence. That populations associated with black and ornamental hawthorns in the PNW exhibit enhanced survivorship compared with their apple-infesting progenitors, where the opposite would be expected, is therefore puzzling. One explanation might be that fruits of the western and eastern hawthorn species have similar nutrient composition, and digestive specializations for catabolizing these nutrients were still present in larvae within the recently evolved (~165 ya) apple race (see Bush 1994) that were introduced to the PNW. Therefore, the flies readily acclimatized to western hawthorn fruits following introduction. This hypothesis finds partial support in that, in the east, both the hawthorn race and the apple race still have higher egg-to-pupa survivorship when larvae are reared in hawthorn (ancestral) fruits rather than apples (Prokopy et al. 1988).

It remains possible that selection caused by unnatural pre-winter intervals does not reveal itself through mortality within a single generation, but rather requires the

observation of multiple generations to appreciate the strength of selection imposed by this environmental variable. For instance, I did not follow any eclosing individuals through to their next reproductive cycle, so caution is urged when interpreting these results without further testing of multiple generations following a single episode of selection.

The results of the diapause depth experiment conflict with previous studies' findings. Using eastern apple and downy hawthorn races, Dambroski & Feder (2007) previously identified the bimodal trend of eclosion phenotypes observed in the current study. They referred to flies from the first and second eclosion peaks as 'non-diapausing' and 'shallow-diapausing' forms, respectively. Those authors found support for the hypothesis that the early pupating apple population should have fewer non-diapausing individuals, since individuals that eclose prematurely would find an environment devoid of ripe host fruit. Furthermore, both southern apple and southern hawthorn populations had fewer non-diapausing individuals than their northern counterparts, presumably due to the exact same mechanism of selection.

In contrast, I observed the opposite trend. The earliest population to eclose (black hawthorn) had the highest prevalence of non-diapausing flies. Perhaps these early season flies are shifting toward bivoltine habits afforded by early eclosion and the availability of late season hosts (late apple and ornamental hawthorn). Such shifts toward bivoltinism have been identified in other insect taxa spanning climatic clines (Roff 1980). In the southern US, green hawthorn (*C. viridis*) and western mayhaw (*C. opaca*) are two hosts infested by *R. pomonella* that are highly temporally disjunct, yet flies exhibit similar eclosion phenologies when reared under standard laboratory conditions, suggesting

bivoltine forms might be present and infest the early and late season hosts (Powell et al. 2014).

Two critical parameters remain to be examined before definitive conclusions can be made regarding the degree of reproductive isolation among populations. First, direct genetic tests are needed to properly conclude if phenotypes are driven by alternative allele frequencies among host-associated populations. Second, it is important to establish the spatial expanse of populations that are adapted to alternative hosts. After establishing these points, an analysis could combine them by examining whether genetic differences among host-associated populations within a site are consistent across the spatial distribution of these populations; that is: are the genetic differences local or region-wide. The initial site of introduction for the species in the PNW was Portland, OR, so testing populations radiating from this location would be one appropriate means for analyzing this phenomenon. Preliminary evidence of phenological specializations existing on a broader geographic scale was provided by AliNiazee & Westcott (1987), who documented unique flight activity times of black hawthorn, apple, and ornamental hawthorn populations across the northern Willamette Valley of Oregon from 1981–86; their figures suggest patterns similar to those detected here, and previously at BBCG in southwest Washington in 2013 (Mattsson et al. *in review*).

In conclusion, it appears that host specific adaptations have rapidly arisen in newly established populations of this species in western Washington. Because the approximate date of introduction is known (AliNiazee & Penrose 1981; Dowell 1988), and the species is univoltine, we can equate the 40–65 years since introduction with the

number generations that it took for these life history specializations to arise. Phenological overlap exists among populations associated with the alternative hosts in the region, providing opportunities for gene exchange between early- and late-season groups via mid-season populations. However, the present study offers strong evidence that these populations may indeed represent true host races, as it seems clear that genetic mechanisms underscore fixed eclosion phenologies, one of the primary criteria for host race designation put forth by Drès & Mallet (2002). Looking forward, insights obtained from detailed examinations of life history parameters of host-specialist insects will continue to inform and shape our understanding of the interplay between ecology and evolution of species.

Table 3.1 Numbers of pupae reared from host fruits during times of peak *R. pomonella* activity.

<u>Host</u>	<u>Date picked</u>	<u># Fruit</u>	<u># Pupae</u>	<u># Pupae per fruit</u>
Black haw	12-Jul	7081	318	0.045
Black haw	18-Jul	8152	910	0.112
Black haw	12-Aug	942	57	0.061
Early apple	18-Jul	40	280	7.000
Early apple	31-Jul	63	554	8.794
Early apple	14-Aug	30	162	5.400
Late apple	18-Jul	99	236	2.384
Late apple	31-Jul	141	360	2.553
Late apple	14-Aug	147	437	2.973
Ornamental haw	6-Sep	2294	248	0.108
Ornamental haw	26-Sep	3516	1354	0.385

Table 3.2 Estimates of prewinter intervals experienced by alternative host populations in nature based on field observations collected in 2013. Pupation dates were calculated as (mean oviposition date + 24 days).

Host Population	Mean Oviposition Date	Pupation Date	Winter (5°C) Onset	Prewinter Interval
Black hawthorn	21-Jul	14-Aug	15-Oct	62
Early apple	1-Aug	25-Aug	15-Oct	51
Late apple	20-Aug	13-Sep	15-Oct	32
Ornamental hawthorn	20-Sep	14-Oct	15-Oct	1

Table 3.3 Numbers of pupae entering pre-winter treatments

<u>Population</u>	<u>Treatment</u>	<u># Pupae</u>	<u># Containers</u>	<u>Median # Pupae per Container (Range)</u>
Black hawthorn	non-overwintering	218	9	23.0 (4 – 64)
	15 day	237	11	21.0 (2 – 63)
	30 day	197	11	14.0 (2 – 63)
	45 day	264	11	23.0 (4 – 63)
	60 day	232	11	15.0 (3 – 63)
Early apple	non-overwintering	148	15	10.0 (4 – 15)
	15 day	165	16	9.5 (4 – 23)
	30 day	211	22	9.5 (2 – 22)
	45 day	185	18	9.5 (5 – 24)
	60 day	174	18	9.0 (3 – 24)
Late apple	non-overwintering	132	16	8.5 (5 – 12)
	15 day	145	18	8.0 (4 – 18)
	30 day	218	20	8.0 (4 – 63)
	45 day	219	20	8.0 (6 – 70)
	60 day	233	12	8.0 (3 – 90)
Ornamental haw	non-overwintering	313	15	14.0 (1 – 47)
	15 day	372	14	24.0 (1 – 51)
	30 day	274	13	18.0 (5 – 45)
	45 day	304	12	20.5 (3 – 49)
	60 day	278	12	16.5 (4 – 47)

Fig. 3.1 (A) *Rhagoletis pomonella* was introduced to Portland, OR, USA sometime in the mid-1900s and spread north and south into Washington and Oregon (gray arrows), as well as eastward into the Columbia River Gorge toward the fruit growing regions of central Washington (shaded area). (B) Burnt Bridge Creek Greenway, Vancouver, WA, USA where majority of the infested fruits were sampled for this study. One additional ornamental hawthorn tree was sampled at an off-map site, as indicated in the lower right. General locations of host species are represented by symbols, but due to scaling and resolution constraints there are not individual symbols for each and every tree sampled.

Fig. 3.2 Populations of *R. pomonella* existing on alternative hosts have disjunct pupation times at a site in southwest Washington (see text for details): bold vertical arrows descending from host names are pointing at their unique pupation times on the horizontal bold arrow, which represents time moving left to right. The four other faint arrows radiating from each host represent experimental reciprocal transplants, where pupae reared from each host were subjected to pre-winter intervals experienced by conspecifics. The non-overwinter treatment, however, did not endure winter treatment. Horizontal bars below the time arrow indicate the exact pre-winter intervals used in the experiment.

Fig. 3.4 Mean numbers of days to eclosion ($\pm$ 95% confidence intervals) from the 15 day, 30 day, 45 day and 60 day pre-winter treatments, from populations associated with (a) black hawthorn; (b) early apple; (c) late apple; and (d) ornamental hawthorn hosts. Treatments not sharing letters are statistically different at $\alpha = 0.05$ (n = numbers of flies eclosing for that treatment).

Fig. 3.5 Mean numbers of days to eclosion ($\pm$ 95% confidence intervals) for host-associated populations after pooling treatments. Populations not sharing letters are statistically different at $\alpha = 0.05$ (n = numbers of flies eclosing within that population).

Fig. 3.6 Mean percent mortality ($\pm$ 95% confidence intervals) for host-associated populations of *R. pomonella* due to treatment-induced effects in the selection experiment (see text for details). Populations not sharing letters had statistically different mortality at $\alpha = 0.05$ (n = numbers of eclosion containers).

Fig. 3.7 Percentages of unaccounted for pupae (= pupae that could not be found following treatments; $\pm$ 95% confidence intervals) (A) among host-associated populations when treatments were pooled and (B) among treatments when populations were pooled. Considered separately, treatments in panel (A) not sharing a letter and populations in panel (B) not sharing a letter had statistically different percentages of unaccounted for pupae at $\alpha = 0.05$ (n = numbers of eclosion containers).

Fig. 3.8 Percentages of flies that eclosed from each population in the diapause depth experiment ($\pm 95\%$ confidence intervals), where pupae were not placed in a 5°C winter, but rather exposed to $22\text{--}23^{\circ}\text{C}$ continuously. Populations not sharing a letter had statistically different percentages of non-diapausing flies at $\alpha = 0.05$ (n = numbers of eclosion containers).

Fig. 3.9 Days to eclosion following pupation for each of the four host-associated populations reared under constant 22–23°C and 15L:9D in the diapause depth experiment.

Fig. 3.10 (A) Field-documented eclosion curves (solid lines) from the 2013 season closely matched laboratory eclosion curves (dashed lines) for each population (black = black hawthorn; red = early apple; green = late apple; blue = ornamental hawthorn) when regressed against degree days using base temperature 6.4°C. Beginning dates of degree day accumulation was 01 March 2013 for the field eclosion curves (using temperature data recorded at Pearson Field Airport [airport code KVUO], Vancouver, WA, located ~5 km from the study site); and beginning date for the laboratory eclosion curve was the first day that the pupae were removed from the 5°C winter into 22–23°C conditions. (B) Specific degree days corresponding to median relative cumulative eclosion both in the field and in the laboratory; color-coding identical to (A).

Chapter 4:

Life History Adaptations Traverse Trophic Levels

Abstract

Hymenopteran parasitoid wasps are a major cause of mortality for the phytophagous fruit flies of the genus *Rhagoletis* (Diptera: Tephritidae). These parasitic wasps have life cycles very similar to that of their fly hosts. For example, each has one generation per year with a limited adult life span (< 30 days). Wasp life history must therefore be precisely timed so diapause termination and subsequent eclosion occurs when prey are abundant. *Rhagoletis pomonella* has been shown to shift diapause life history timing to adapt to new hosts' phenologies, resulting in races specializing on separate hosts in sympatry. Wasps that parasitize this species might play a role in the host formation process by enabling newly derived fly populations to grow rapidly due to a temporary release from parasitoid attack following a host shift. However, the wasps also appear to 'follow' the flies within a few generations, phenologically adapting to the novel host environments in a manner that can be described as 'cascading' host shifts. However, the exact manner in which selection interacts with the environment to result in coincident scheduling of life histories remains unknown. In the present series of experiments, I subjected pupae of *Rhagoletis pomonella* harboring parasitoid wasps to four different thermal regimes prior to the onset of a cold winter treatment to test the hypothesis that the length of pre-winter intervals of exposure to elevated ambient temperatures modulates the length of time wasps wait before eclosing after an episode of overwinter diapause. My

results show that pre-winter thermal manipulations do not affect post-winter eclosion of wasps, suggesting a genetic underpinning in the adaptation of wasp eclosion to coincide with very specific points in the larval life cycles of dipteran hosts.

Introduction

Parasitism is the most widespread strategy of life (Price 1977; Windsor 1998). Numerically, this can only make sense when parasites themselves host other parasites, which they do (Poulin & Morand 2000). A related system is one in which plant-eating (phytophagous) insects are parasitized by insect-eating (entomophagous) insects that eventually kill their host (= parasitoids). Given that many parasites and parasitoids have strong host preferences and specializations (Bush 1975; Poulin & Morand 2000), this framework constitutes a scenario wherein if and when ecologically divergent selection initiates speciation in the primary parasite (e.g., the phytophage), the trophically dependent, higher order parasite (e.g., the entomophage) should potentially also experience ecologically divergent selection from its ancestral lineage. Hence, the process of biodiversification in and of itself generates biodiversity (Hudson et al. 2006; Stireman et al. 2006; Forbes et al. 2009; Feder & Forbes 2010).

Parasites must coordinate their metamorphic stages with host phenologies to ensure reproduction and survival (Ehrlich & Raven 1964). In the case of the phytophagous apple maggot fly, *Rhagoletis pomonella* Walsh (Diptera: Tephritidae), adults oviposit into the ripe fruits of host plants belonging to the family Rosaceae, where they hatch and larvae feed on fruit flesh for 2 – 5 weeks before emerging from the fruit to burrow ~1 cm deep into the soil and form puparia in which they overwinter in a facultative pupal diapause. A single generation ecloses the following summer (i.e., they are univoltine) just prior to when natal host fruits ripen. Then, within the relatively brief 30-day life span of the adult, they feed on yeast, bacteria, and insect honeydew, until

reaching reach sexual maturity within 7 days, and visually and chemotactically locate host fruits upon which they mate and oviposit (Dean & Chapman 1973; Boller & Prokopy 1976).

Rhagoletis pomonella are in turn attacked by several species of hymenopteran ecto- and endoparasitoid wasps (Table 4.1) whose life cycles closely resemble that of *R. pomonella*. They too are univoltine, have a facultative diapause regulated to closely match reproductive phenologies of their fly hosts, and use tactile and chemical plant cues to locate hosts within fruits (see Gut & Brunner 1994 and Forbes et al. 2009, 2010 for detailed discussions of the biology, ecology, and geographic distributions of the various parasitoid wasps). Each species of wasp is specially adapted to oviposit either into or onto *R. pomonella* eggs, larvae, or pupae (Table 4.1). The female wasp deposits her egg beneath the dermis of the insect host, where the egg remains dormant. When the fly larva emerges from its host fruit and pupates in the soil, the wasp larva hatches and consumes its host, at which time either (1) the wasp diapauses through winter as a fourth instar larva and briefly pupates the following summer within the fly's puparium before eclosing as an adult, or (2) foregoes diapause and ecloses as a second annual generation if exposed to consistently high temperatures and photoperiods. Parasitoid attack is lethal for host flies and can kill up to 90% of the fly larvae (Gut & Brunner 1994), but more typically accounts for 1% and 50% of mortalities (Feder 1995; Forbes et al. 2009; Hood et al. 2012), depending on the wasp species.

Rhagoletis pomonella is a model system for studying ecologically-driven adaptive radiation in sympatry (Feder 1998). Now considered a classic example of incipient

sympatric speciation in action, *R. pomonella* underwent a host shift in the Hudson Valley of New York, United States (US) sometime in the mid-1800s from its ancestral host hawthorn (*Crataegus* spp.) to introduced, domesticated apple (*Malus domestica* Borkh. [Bush 1994]). This was accomplished when a local population of flies shifted its seasonal eclosion timing ~10 days earlier, thereby facilitating the exploitation of the newly available host, apple, which ripens 2 – 4 weeks prior to hawthorn fruits (Feder et al. 1993). This phenological shift, coupled with the reinforcing effects of strong natal host fidelity (flies mate directly on, or very near host fruit), generated an apple host race that is partially reproductively isolated from the ancestral hawthorn race across the eastern United States (Feder 1998). Flies that experienced the original shift from hawthorn to apple likely found an environment of reduced wasp predation because (1) diapause phenologies of wasps were initially asynchronous with the novel apple race flies; (2) the wasps lacked visual and chemical cues enabling location of *R. pomonella* within apple fruit; and (3) apples are larger than hawthorn fruits and thereby allowed fly larvae to burrow deeper into the fruit, thereby escaping the wasps' ovipositors (Feder 1995). The temporary escape from parasitoid wasps therefore may have helped facilitate the establishment of the apple host race (Feder 1995), although eventually the wasps appear to have 'found' those flies infesting apples (Forbes et al. 2010), potentially initiating a process of divergence among parasitoid wasps attacking the two distinct fly host races, a process termed 'sequential speciation' (Feder & Forbes 2010).

The very recent (40 – 65 ya) introduction of *R. pomonella* to the Pacific Northwestern (PNW) United States (AliNiazee & Penrose 1981; Dowell 1988; Hood et

al. 2013; Sim 2013) provides a unique opportunity to examine the propensity for host race formation not only in this fly species but also in its parasitoid host community, and on an even more proximate time scale than the Northeastern U.S. hawthorn-to-apple host shift. Since being introduced in the mid-1900's to the Portland, Oregon, US, region via larval-infested apples (Sim et al. 2012; Linn et al. 2012; Hood et al. 2013; Sim 2013), *R. pomonella* now exploit in addition two hawthorn species: the native black hawthorn (*Crataegus douglasii* Lindl.), which fruit before local apple varieties, and the introduced English ornamental hawthorn (*C. monogyna* Jacq.), which ripen after apples. Eclosion times of each host-associated population have demonstrated consistent phenological shifts both earlier and later to match the fruit availability times of their newly colonized hawthorn hosts (Tracewski et al. 1987; Mattsson 2015: Chapters 1 & 2).

Parasitoid wasps also attack PNW populations of *R. pomonella* associated with the native and introduced ornamental hawthorn hosts (Gut & Brunner 1994; Forbes et al. 2010). Increasingly, data suggest that populations of *R. pomonella* are specializing to the new hawthorn hosts in the PNW through shifts in their diapause phenology (Mattsson 2015, Chapters 1 & 2) as well as using odor discrimination among host fruit volatiles (Sim et al. 2012; Linn et al. 2012), mechanisms that together enhance host fidelity. The question with respect to the higher level host–parasitoid system therefore is: are their parasitoid wasps also in the nascent stages of speciation? The parasitoid species that attack *R. pomonella* have an even shorter adult life span than their hosts (2 – 3 weeks: Lathrop & Newton 1933), requiring their eclosion and reproduction times to be highly precise in order to enable parasitizing the flies.

In this report, I present data on wasp predation rates, as well as eclosion phenologies of wasps emerging from pupae associated with four primary host-associated populations of *R. pomonella* (black hawthorn, early-fruiting apple, late-fruiting apple, and ornamental hawthorn) at a site in southwest Washington.

Methods

Background

The findings here are derived from a laboratory experiment I performed to investigate how *Rhagoletis. pomonella* populations in the PNW synchronize their diapause schedules to match host fruit ripening. Under standardized laboratory conditions, I manipulated pre-winter intervals experienced by larvae and pupae reared from infested fruit of black hawthorn, early- and late-ripening apples, and ornamental hawthorn (see ‘*Sample Collection*’ below for collection and laboratory rearing procedures) to test a ‘thermal accumulation hypothesis’. This hypothesis predicts that when exposure by the flies to ambient temperatures before the onset of winter is prolonged (more typical of the exposure regimen of early season flies), it may offer these individuals the opportunity to transduce thermal energy to advance their development, allowing them to then rapidly eclose the following year in order to synchronize with their early ripening natal host fruits (and vice versa for late season populations, which might require longer exposures the summer following pupal diapause in order to develop into adults). Alternatively, excessively long or short pre-winter intervals might stress the organisms and therefore be a trait upon which natural selection acts to sculpt the

developmental rates and the diapause regimen to fine-tune eclosion timing of the population.

The experimental pre-winter intervals (temperature = 22-23°C; photoperiod = 15 Light : 9 Dark) were designed to simulate those typically experienced in nature by each of the four host-associated populations: black hawthorn = 60 days; early-apple varieties = 45 days; late-apple varieties = 30 days; ornamental hawthorn = 15 days (Fig. 4.1). Fly pupae reared from each host species were subdivided to experience each pre-winter treatment interval (reciprocal transplantation), followed by a prolonged (25 wk) period in a 5°C chamber to simulate winter and enable flies to break diapause.

The results from that experiment contradicted my predictions: shortened and prolonged pre-winter intervals did not affect post-winter eclosion times, thus indicating there exist genetic underpinnings to the seasonally adapted diapause schedules of the fly populations. Parasitoid wasps also eclosed during that experiment and form the basis of the analysis presented herein, enabling me to begin to examine the cascading effects of life history shifts of fly hosts to their trophically dependent parasitoids.

Sample Collection

In the summer and fall of 2013, I collected fruit infested with *Rhagoletis pomonella* larvae, many of which harbored parasitoid wasps (Table 4.2) from black hawthorn, apple, and ornamental hawthorn trees growing within 100 m of Burnt Bridge Creek Greenway (BBCG), Vancouver, Washington, USA (45° 37.9' N; 122° 37.1' W; Fig. 4.2). I also collected infested fruit from an additional ornamental hawthorn tree

located near Mount Tabor Park in Portland, Oregon, USA (45°30.63'N, 122°35.23'W). [See Mattsson (2015, Chapter 2) for a more complete description of sampling protocol.] Fruits were promptly taken to the laboratory and spread atop wire mesh baskets placed in plastic containers into which larvae emerging from fruit dropped and pupated. I collected those pupae every other day and distributed them among 4 Petri dishes containing moist vermiculite, each assigned to one of the specific pre-winter treatments mentioned above.

Following pre-winter treatments, Petri dishes were placed into a refrigerator (photoperiod = 0L:24D; temperature = 4 – 6°C) for 25 weeks in order to simulate natural PNW overwintering conditions (Bates 1975; Zheng et al. 1993). After the 25 wk overwintering period, I transferred the contents of the Petri dishes into 480 ml beverage containers (SOLO® Cup Company, Lake Forest, IL) covered with paper towels that were kept moist and secured with rubber bands for the remainder of the study. Eclosion containers were haphazardly distributed and rotated weekly in a room with 15L:9D photoperiod and 22 – 23°C. I aspirated eclosing adult wasps from containers every other day, and transferred them into 1.5 ml micro centrifuge tubes which were then placed into a –80°C freezer for genetic preservation. I monitored the eclosion containers for 180 ≤ 300 days.

Data analysis:

To calculate the total predation rate of each *Rhagoletis pomonella* population, I summed the numbers of wasps eclosed with the numbers of wasps found (by dissection)

within *R. pomonella* pupae remaining at the end of the study. I then divided this sum by the total numbers of *R. pomonella* pupae that entered the experiment.

To compare mean number of days to eclosion (DTE) following removal from overwintering conditions, I used one-way ANOVA (if the data were homoscedastic according to Brown-Forsythe tests, $\alpha = 0.05$) or Welch's ANOVA (when data were heteroscedastic) among treatments within populations, using each individual eclosing wasp as a replicate. When the mean DTE were significantly different ($P < 0.05$), Tukey's Honestly Significant Difference (HSD) post-hoc analyses were conducted to determine the degree of difference between pre-winter treatment pairs either within or between populations. No wasps emerged from apple-infesting flies, so DTE between wasps from the two hawthorn populations were compared using either Student's t-tests (when data were homoscedastic) or Welch's t-tests (when data were heteroscedastic).

Results

Wasps parasitized black- and ornamental hawthorn-associated populations of *Rhagoletis pomonella* (hereafter 'black hawthorn wasps' and 'ornamental hawthorn wasps'), but neither apple population. Parasitism rates depended on time of sampling (Table 4.2), and were consistently higher for collections occurring later in the season, at times when the hawthorn berries were beginning to shrivel up and fall from the tree. These observed rates are likely approximations of the true rate of parasitism. To obtain more accurate rates of parasitism, puparia of *R. pomonella* must be excavated from beneath the host tree after the majority of fruit have abscised (Lathrop & Newton 1933).

Pre-winter treatments did not affect mean DTE for black hawthorn wasps (one-way ANOVA, $F_{3,86} = 0.189$, $P = 0.904$; Fig. 4.3), where the mean ($\pm$ standard error in days) DTE was 76.2 (± 0.80), but did affect ornamental hawthorn wasps (one-way ANOVA, $F_{3,181} = 4.751$, $P = 0.003$). In direct opposition to predictions of the thermal accumulation hypothesis, the 60-day group of ornamental hawthorn wasps eclosed later, not earlier, than the 30-day group by ~ 11 d (Tukey HSD, $P = 0.002$) or the 45-day group by 8 d ($P = 0.038$; Fig. 4.3).

I tested for pairwise differences between the two populations for each treatment group. These revealed that, for every treatment group, ornamental hawthorn wasps eclosed later than black hawthorn wasps (Welch's two-sample t-test, 15-day treatment: $t_{74.6} = -19.7$, $P < 0.0001$; 30-day treatment: $t_{63.1} = -20.2$, $P < 0.0001$; two-sample t-test, 45-day treatment: $t_{49} = -15.7$, $P < 0.0001$; Welch's two-sample t-test, 60-day treatment: $t_{62.7} = -20.4$, $P < 0.0001$). Black hawthorn wasps eclosed 53.4, 48.1, 52.7, and 60.4 days before ornamental hawthorn wasps within the 15-, 30-, 45-, and 60-day treatments, respectively.

It is particularly relevant from a community ecology perspective to compare host fly and parasitoid wasp eclosion phenologies (Fig. 4.4). Mean DTE for black hawthorn wasps was 31 days after the mean DTE of its fly host population (76.2 [± 0.80] versus 45.3 [± 0.51], respectively). The primary parasitoids of *R. pomonella* that infest black hawthorn are *Diachasmimorpha mellea* Gahan, a larval parasitoid. Thus, in nature, the time lapse between eclosion times of host and parasitoid makes biological sense: there is a 3 – 4 week lag between fly eclosion to peak larval abundance, allowing 7 – 10 d for

flies to reach sexual maturity, and a 2 – 5 week window of larval feeding within fruit (Dean & Chapman 1973; Mattsson 2015, Chapter 1).

The parasitoid known to attack ornamental hawthorn-associated *R. pomonella* is *Opius downesi* Gahan, also a larval specialist. Therefore the reason for the relatively long time lapse, ~65 days, between eclosion of this population of flies (mean DTE = 64.8 [± 0.53]) to eclosion of its associated wasps (129.6 [± 1.07] DTE) is unclear, given what is known about developmental rates and life expectancy of *R. pomonella*.

Discussion

I demonstrated that parasitoid wasps continue to parasitize populations of the fruit fly, *R. pomonella*, that in turn infest two hawthorn species whose fruiting times are temporally offset in western Washington. *Rhagoletis pomonella* also is an abundant parasite of domesticated apple in the region, yet no parasitoid wasps were reared from apple-associated pupae in this study.

Wasp species identifications are forthcoming, and it is anticipated that at least two separate wasp species are specialized on the two fly populations. According to previous investigations, *D. mellea* exclusively attacks the larvae of the (early-season) black hawthorn-infesting population, while *O. downesi* exclusively attacks larvae of the (late-season) ornamental hawthorn-infesting populations of *R. pomonella* (AliNiazee 1985; Gut & Brunner 1994). These wasps may have shifted from populations that attack endemic *Rhagoletis* spp. in the region, to begin attacking *R. pomonella* following its recent introduction via larval-infested apples.

One way to test this host-shifting hypothesis would be to collect fruits of host plants infested by congeners of *R. pomonella* whose fruiting phenologies are similar to those of black and ornamental hawthorn, and then rear parasitoids from fly pupae to see if the same species attack both endemic and introduced *Rhagoletis* spp. For example, two species of Oregon grape (*Mahonia* spp.) grow extensively in the area and are parasitized by *R. berberis* Curran; like black hawthorn, its fruits ripen in July. *Rhagoletis zephyria* Snow, the snowberry maggot, infests snowberry (*Symphoricarpos albus* [L.] S.F. Blake), which has a prolonged ripening arc beginning in August and extending through October, straddling ripening times of ornamental hawthorn. In fact, *R. zephyria* is a known host of *O. downesi* (the parasitoid also commonly reared from ornamental hawthorn-infesting *R. pomonella*) and AliNiazee (1985) posited that, indeed, a shift from *R. zephyria* to *R. pomonella* is more likely than a combined introduction of the fly and the wasp from the eastern US, since the introduced apple flies are not even attacked by this species (as shown by AliNiazee 1985; Gutt & Brunner 1994; and this study). Genetic surveys would provide a means to generate co-phylogenies of parasitoid wasps and their *Rhagoletis* hosts to begin to resolve the evolutionary and ecological relationships within this host–parasite network of species.

Even without definitively knowing the wasp species' identities, it is clear that black and potentially ornamental hawthorn wasps are phenologically adapted to their fly hosts. Their eclosion schedules were virtually unaffected by the manipulation of pre-winter eclosion times, suggesting that eclosion is timed by other abiotic or endogenous cues not measured in this study. The extended time interval between the mean eclosion

times of ornamental hawthorn flies and their wasps echoes puzzling results from a previous field study (Mattsson 2015, Chapter 1). There, I detected an unusually long interval of ~70 days between ornamental fly eclosion (mean = 17 July) and peak larval infestation times (26 September) of ornamental hawthorn fruits. Also, ornamental hawthorn fruit ripened much later than would be expected if flies are phenologically adapted to their host fruit (Fig. 4.5). Given these current lines of evidence, I suspect that the ornamental hawthorn-associated population of *R. pomonella* either (1) has an adult life span longer than 30 days or (2) larvae feed in fruits for a longer time period and develop more slowly than black hawthorn larvae, perhaps related to the different rates at which their host fruits ripen and abscise (by examining the slopes of fruit growth and softening in Fig 4.5, one can see that black hawthorn fruits rapidly ripen, while ornamental hawthorn fruits have a protracted ripening period). One hypothesis that could be derived from this is that ornamental hawthorn wasps oviposit onto a specific larval instar not available until later in the season, thereby shifting their eclosion time to more closely match when those larval instars of *R. pomonella* are most abundant.

We are only beginning to unravel the connections among environments and primary producers, parasites, and parasitoids. The data presented here support a hypothesized co-evolutionary speciation accelerator linking three trophic levels wherein subtle shifts in the phenology of the producer lead to a cascade of downstream adaptations.

Table 4.1 Parasitoid wasps known to infest species within the *Rhagoletis pomonella* species complex in North America. Occurrence data were compiled from Gut & Brunner (1994) and Forbes et al. (2010). ‘Life Stage’ refers to the *Rhagoletis* spp. metamorphic life stage attacked by the wasp, and ‘Region Found’ indicates whether the wasp has been found east or west of the Continental Divide.

Wasp Family	Current Name of Wasp	Host (fruit infested by host, binomial name of host's host plant)	Life Stage	Region Found
Braconidae	<i>Utetes canaliculatus</i>	<i>Rhagoletis pomonella</i> (apple, <i>Malus domestica</i>) <i>Rhagoletis pomonella</i> (downy hawthorn, <i>Crataegus mollis</i>) <i>Rhagoletis pomonella</i> (hawthorn, <i>Crataegus rosei</i>) <i>Rhagoletis pomonella</i> (Mexican hawthorn, <i>Crataegus mexicana</i>) <i>Rhagoletis pomonella</i> (hawthorn, <i>Crataegus gracilior</i>) <i>Rhagoletis</i> nr. sp. <i>mendax</i> (flowering dogwood, <i>Cornus florida</i>) <i>Rhagoletis zephyria</i> (snowberry, <i>Symphoricarpos albus</i>)	Egg	East
Braconidae	<i>Utetes lectoides</i>	<i>Rhagoletis pomonella</i> (apple, <i>Malus domestica</i>) <i>Rhagoletis pomonella</i> (ornamental hawthorn, <i>Crataegus monogyna</i>) <i>Rhagoletis zephyria</i> (snowberry, <i>Symphoricarpos albus</i>)	Egg	East & West
Braconidae	<i>Utetes richmondii</i>	<i>Rhagoletis mendax</i> (blueberry, <i>Vaccinium corymbosum</i>) <i>Rhagoletis</i> nr. sp. <i>mendax</i> (sparkleberry, <i>Vaccinium arboreum</i>) <i>Rhagoletis cornivora</i> (shrubby dogwood, <i>Cornus amomum</i>)	Egg	East

Braconidae	<i>Diachasma alloeum</i>	<p><i>Rhagoletis pomonella</i> (apple, <i>Malus domestica</i>)</p> <p><i>Rhagoletis pomonella</i> (downy hawthorn, <i>Crataegus mollis</i>)</p> <p><i>Rhagoletis pomonella</i> (eastern mayhaw, <i>Crataegus opaca</i>)</p> <p><i>Rhagoletis pomonella</i> (fanleaf hawthorn, <i>Crataegus flabellata</i>)</p> <p><i>Rhagoletis pomonella</i> (green hawthorn, <i>Crataegus viridis</i>)</p> <p><i>Rhagoletis pomonella</i> (blueberry hawthorn, <i>Crataegus brachycantha</i>)</p> <p><i>Rhagoletis pomonella</i> (plum, <i>Prunus domestica</i>)</p> <p><i>Rhagoletis pomonella</i> (Asian crab apple, <i>Malus floribunda</i>)</p> <p><i>Rhagoletis mendax</i> (blueberry, <i>Vaccinium corymbosum</i>)</p> <p><i>Rhagoletis mendax</i> (deerberry, <i>Vaccinium stamineum</i>)</p> <p><i>Rhagoletis mendax</i> (dwarf huckleberry, <i>Vaccinium corymbosum</i>)</p> <p><i>Rhagoletis</i> nr. sp. <i>mendax</i> (sparkleberry, <i>Vaccinium arboreum</i>)</p> <p><i>Rhagoletis</i> nr. sp. <i>mendax</i> (flowering dogwood, <i>Cornus florida</i>)</p> <p><i>Rhagoletis zephyria</i> (snowberry, <i>Symphoricarpos albus</i>)</p> <p><i>Rhagoletis zephyria</i> (snowberry, <i>Symphoricarpos occidentalis</i>)</p>	Larva	East
Braconidae	<i>Diachasmimorpha mellea</i>	<p><i>Rhagoletis pomonella</i> (apple, <i>Malus domestica</i>)</p> <p><i>Rhagoletis pomonella</i> (downy hawthorn, <i>Crataegus mollis</i>)</p> <p><i>Rhagoletis pomonella</i> (hawthorn, <i>Crataegus rosei</i>)</p> <p><i>Rhagoletis pomonella</i> (Mexican hawthorn, <i>Crataegus mexicana</i>)</p> <p><i>Rhagoletis pomonella</i> (black hawthorn, <i>Crataegus douglasii</i>)</p> <p><i>Rhagoletis mendax</i> (blueberry, <i>Vaccinium corymbosum</i>)</p> <p><i>Rhagoletis zephyria</i> (snowberry, <i>Symphoricarpos albus</i>)</p>	Larva	East & West
Braconidae	<i>Opius downesi</i>	<p><i>Rhagoletis pomonella</i> (ornamental hawthorn, <i>Crataegus monogyna</i>)</p> <p><i>Rhagoletis zephyria</i> (snowberry, <i>Symphoricarpos albus</i>)</p> <p><i>Rhagoletis tabellaria</i> (hawthorn, <i>Crataegus stolonifera</i>)</p>	Larva	East & West
Eupholidae	<i>Tertrastichus</i> spp.	<p><i>Rhagoletis pomonella</i> (ornamental hawthorn, <i>Crataegus monogyna</i>)</p> <p><i>Rhagoletis pomonella</i> (black hawthorn, <i>Crataegus douglasii</i>)</p>	Larva	West

Pteromalidae	<i>Pteromalus</i> sp.	<i>Rhagoletis pomonella</i> (black hawthorn, <i>Crataegus douglasii</i>) <i>Rhagoletis pomonella</i> (ornamental hawthorn, <i>Crataegus monogyna</i>)	Larva	West
Diapriidae	<i>Coptera pomonellae</i>	<i>Rhagoletis pomonella</i> (apple, <i>Malus domestica</i>) <i>Rhagoletis pomonella</i> (downy hawthorn, <i>Crataegus mollis</i>) <i>Rhagoletis suavis</i> (walnut, <i>Juglans</i> sp.)	Pupa	East

Table 4.2 Fruits of black hawthorn (*Crataegus douglasii*) and ornamental hawthorn (*C. monogyna*) were infested with both *R. pomonella* and parasitoid wasps. Wasp parasitism (Parasitism Rate) increased for collections made later in the season. No parasitoid wasps were reared from *R. pomonella* infesting apples.

Host Population	Fruit Collection Date	# <i>R. pomonella</i> Pupae	# Wasps	Parasitism Rate
Black Hawthorn	12+18 July	1011	68	0.067
Black Hawthorn	12-Aug	56	29	0.518
Ornamental Hawthorn	6-Sep	251	10	0.040
Ornamental Hawthorn	26-Sep	1290	279	0.216

Fig. 4.1 Populations of *R. pomonella* that infest alternative hosts have disjunct pupation times at a site in southwest Washington (see text for details): bold vertical arrows descending from host names are pointing at their unique, natural pupation times on the horizontal bold arrow, which represents time moving left to right. The four other faint arrows radiating from each host represent experimental reciprocal transplants, where pupae reared from each host were subjected to pre-winter intervals experienced by conspecifics. Horizontal bars below the time arrow indicate the exact pre-winter intervals used in the experiment, followed by 25 weeks at 5°C, followed by eclosion of flies and wasps.

Fig. 4.2 (A) *Rhagoletis pomonella* was introduced to Portland, OR, USA sometime in the mid-1900s and spread north and south into Washington and Oregon (gray arrows), as well as eastward into the Columbia River Gorge toward the fruit growing regions of central Washington (shaded area). (B) Burnt Bridge Creek Greenway, Vancouver, WA, USA where the majority of the fruits from the four different hosts infested with *R. pomonella* larvae were sampled for this study. One additional ornamental hawthorn tree was sampled at an off-map site (see text for details), as indicated in the lower right. General locations of host species are represented by symbols, but due to scaling and resolution constraints there are not individual symbols for each and every tree sampled (see Mattsson 2015, Chapter 2 for exact numbers of trees sampled).

Fig. 4.3 Pre-winter treatment intervals (x-axes) did not affect the length of time to eclosion of adult wasps associated with black hawthorn-infesting *R. pomonella* following removal from over-wintering conditions (25 weeks at 5°C). Ornamental hawthorn wasps in the 60-day pre-winter treatment eclosed slightly later than wasps in the 30-day and 45-day treatments. Treatments sharing a letter have statistically identical values. Sample sizes are individual wasps.

Fig. 4.4 Cumulative relative eclosion curves for (A) four populations of *R. pomonella* fruit flies associated with different hosts in the Pacific Northwestern United States and (B) the flies' associated parasitoid wasps ('black hawthorn wasps' and 'ornamental hawthorn wasps'). Pre-winter treatments had virtually no effect on post-winter days to eclosion for any of the populations of flies or wasps, but are shown here to demonstrate the slight variation associated within each population (for unexplained reasons, wasps in the 60-day pre-winter treatment had an extra lag-time toward the latter end of the eclosion curve). Numbers in parentheses are mean numbers of days to eclosion ($\pm$ standard error in days) following removal from overwintering conditions, and sample sizes are numbers of individuals.

Fig. 4.5 Cumulative eclosion curves (solid black line) of (A) black hawthorn- and (B) ornamental hawthorn-associated populations of *R. pomonella*, based on 2013 field measurements (see Mattsson 2015, Chapter 1 for details). Fruit softness (filled triangles) and fruit diameter (open circles) of the host fruits are shown to demonstrate the pronounced time-lag between eclosion and fruit ripening in the ornamental hawthorn-associated population of *R. pomonella*, echoing the disparity between ornamental fly eclosion and ornamental wasp eclosion found in this study.

Chapter 5:

Summary, Conclusions, and Future Directions

These investigations built upon one another to test a broad hypothesis: host races have evolved rapidly from a single population recently introduced to the Pacific Northwestern United States. First, I needed to establish whether fruits of the alternative host species actually do ripen at different times. In large part, they do (Table 1.2). This is a critical finding because allochronic isolation due to temporally isolated resource islands is the central focus of this thesis, as it is likely a primary barrier impeding gene flow among geographically overlapping populations (Coyne & Orr 2004:166–167; notwithstanding Mayr 1963:475–477). I next established that adult eclosion times in the field have shifted to accommodate the distinct ripening times of each plant host species, except in the case of black hawthorn-associated and early apple-associated flies, where I found no temporal separation in the field (Fig 1.3). I then devised a laboratory analysis to tease apart whether environmental or genetic influences were responsible for the differing phenologies among distinct host-associated fly populations. Manipulation of pre-winter temperature did not affect the time interval to eclosion following post-winter heating. When laboratory and field eclosion data were regressed against degree days, they were, somewhat surprisingly, nearly identical (Fig 3.10). I therefore conclude that either (1) each plant host-associated fly population has undergone selection for transducing thermal

energy into adult development at different rates during post-winter heating, as determined by previous generations that successfully reproduced based on their individual eclosion times; or (2) an as yet unknown abiotic stimulus is acting as a cue prior to when larvae emerge from fruit, a stimulus that would not have been detected in this study because I did not manipulate environmental variables before this point in their lives.

Taken together, my results suggest that differential diapause phenologies among these newly established plant host-associated fly populations are genetically determined rather than the result of plasticity of diapause or eclosion phenotypes. These are important lines of evidence suggesting that allochronic isolation may also have contributed to speciation among recently diverged congeners of *R. pomonella*. For instance, *R. mendax*, which attacks the very early ripening blueberry bush (*Vaccinium* spp.) in the east, and *R. zephyria*, who attacks the late ripening snowberry bush (*Symphoricarpos* spp.) across North America, might both have radiated from a common ancestor with *R. pomonella* into their respective host race niches following host shifts similar to those observed in this work (Fig 1.1; Dambroski & Feder 2007; Gavrilovic et al. 2007). Via (2009) discussed the importance of combining analyses of current populations exhibiting inchoate population divergence—which she termed ‘the magnifying glass’ approach—with observations of more anciently diverged sibling species—which she called ‘the spyglass’ approach. The various *Rhagoletis* species complexes fulfill these criteria to perfection. The *Rhagoletis pomonella* species group in particular is a group exhibiting potentially very recent (40 – 65 ya) population divergence (Sim et al. 2012; this work), slightly less recent (~160 ya) population divergence in the

eastern United States (Bush 1994; Feder 1998; Michel et al. 2010), as well as macroevolutionary divergence in recent times, specifically in the Nearctic assemblage of the genus *Rhagoletis* (Fig 1.1; Feder et al. 2003a, b; Smith et al. 2005).

Eclosion time is not the only trait under divergent ecological selection. *Rhagoletis* use the volatile compounds emitted from ripe host fruit as key olfactory cues to find and distinguish among distinct host plants (Prokopy & Bush 1973; Roitberg et al. 1982; Linn et al. 2003; Nojima et al. 2003; Forbes & Feder 2006). The behaviorally active compounds of apple and downy hawthorn fruit from eastern North America have been identified, and synthetic blends developed that result in flies orienting to the blends at the same level as whole fruit extracts in flight tunnel assays (Linn et al. 2003). Subsequent laboratory and field collection studies have shown that apple-origin flies from the eastern US preferentially orient to their natal apple fruit blend and are antagonized (tend to avoid) by the alternative non-natal hawthorn. In contrast, the reverse is true for downy hawthorn flies. Because *R. pomonella* mate only on or near the fruit of their respective host plants, the differences in fruit odor discrimination translate into positive assortative mating, contributing to premating reproductive isolation between eastern apple and downy hawthorn flies.

Similarly, in the Pacific Northwest, volatile fruit blends have been developed for black hawthorn, apple, and ornamental hawthorn (Linn et al. 2012). As in the East, black hawthorn, apple, and ornamental hawthorn flies prefer natal host blends while avoiding non-natal volatiles. Sim et al. (2012) and Linn et al. (2012) reported responses of western black hawthorn-, apple-, and ornamental hawthorn-associated flies to non-natal host fruit

volatiles in the field and laboratory, respectively, providing a metric for the propensity of a given population to accept non-natal host fruit (Powell et al. 2012). Total reproductive isolation likely results from the additive effects of eclosion time differences and host fidelity, in sequence, during the adult life of the fly. I therefore combined the estimates of behavioral host fidelity with my current estimates of allochronic isolation (AI, equation 1 from Chapter 1 of this work) to calculate percent total reproductive isolation (TRI) between host-associated populations using the equation of Ramsey (2003):

$$TRI = (BI * (1 - AI) + AI) * 100 \quad , \quad (\text{equation 2})$$

where BI is ‘behavioral isolation’:

$$BI_{xy} = 1 - \left(\frac{p_x + p_y}{2} \right) * 100 \quad , \quad (\text{equation 3})$$

where p_x is the proportion of flies associated with host x that positively orientated toward non-natal host fruit y , and p_y is the proportion of flies associated with host y that positively oriented toward non-natal host fruit x . The combined effects of these two mechanisms may greatly reduce overlap between host populations at my research site, for instance, to just 1.3% in the case of black hawthorn versus ornamental hawthorn (Table 5.1). Estimates of TRI due to allochronic and behavioral isolation between the apple and downy hawthorn population at the Grant, Michigan, site is 12.4%, based on my calculations using allochronic isolation data from Feder et al. (1993) and behavioral response data reported by Linn et al. (2003). Given that the apple and downy hawthorn

populations in the east, including at the Grant, MI, site, are genetically differentiated host races as measured in multiple studies, it is quite likely that the populations at my research site in Vancouver, WA, are likewise genetically differentiated.

Future studies are needed to further test hypotheses associated with rapidly evolving host races of *R. pomonella* in the PNW. Most importantly, the spatial extent of host-associated phenology shifts across time (generations) among populations of the PNW must be established. Equally important is identifying whether there is a genetic basis to the different eclosion phenotypes and, if so, which genetic loci are affecting the various phenotypes. Also unknown is the degree of genetic exchange among populations specializing on different hosts plants at the local (site) level versus gene flow between populations specialized on the same host at spatially disjunct sites. In addition to the critical aforementioned problems, I list the following avenues of research for further describing the evolutionary status of PNW populations:

- A rigorous assessment of cryptic morphological characteristics among host-associated populations should be conducted, and could reveal subtle but interesting morphological differences conducive to reproduction and ecological specialization on alternative plant species, in addition to providing a baseline for future comparisons in the event of increased specialization of fly races infesting different hosts. An analysis based on the presence or absence of morphological features is one approach, as undertaken by Smith et al. (2005).
- The genetic and hormonal regulation of diapause, and its interaction with

the environment across life stages, remains to be described in this and other insect species. However, careful preliminary work already has begun to establish key genetic expression events that occur during the diapause syndrome in *Rhagoletis pomonella* (Ragland et al. 2011), and results indicate diapause is clearly a complex phenomenon integrating multiple genetic and chemical products.

- Post-zygotic selection against immigrants reinforces reproductive isolation because intermediate phenotypes are at a disadvantage and cannot compete with individuals possessing alleles conferring the specialized phenotype (Via 2001). This could be the case for phenologically or chemotactically intermediate hybrids among these populations of *R. pomonella*.
- *Crataegus suksdorfii* [(Sarg.) Kruschke] is second species of black hawthorn in the PNW, in addition to *C. douglasii*. Preliminary evidence that I gathered (not included in this work) suggests that *R. pomonella* have not successfully colonized this black hawthorn species in the Portland, OR / Vancouver, WA area. Ripening times of *C. suksdorfii* are variable across the region, but in general appear to slightly precede those of locally sympatric *C. douglasii*. Habitat choice and viability experiments in the laboratory between the two local black hawthorn species are needed to provide insights into the determinants of host acceptance behavior. Host shifting could facilitate dispersal using these alternative black hawthorn

species from densely populated regions of western Washington and Oregon to protected areas in the central regions of these states.

Finally, while executing these experiments and preparing the present work, I was struck by a feeling of concern that my results, and the phenomenon they attempt to explain, are obvious, not surprising, and potentially uninteresting. Yet, when one scans the literature of the past century, they will find a steady flow of opinions and proclamations challenging and championing the plausibility of speciation caused by divergent ecological selection in the absence of geographic isolation (e.g., references in Mayr 1963, and Via 2001). The preliminary evidence I provide here is yet another example of how, when a population harbors a variable phenotype (in this case eclosion/diapause phenology), a small proportion of individuals possessing a phenotype more adaptively suited to a newly available habitat or ecological niche (earlier or later fruiting hawthorns) can splinter off to become a partially isolated deme. The current scenario reflects insights by Simpson (1984), who envisioned ‘adaptive zones’ within environments consisting of finite divisions of ecological niches that can be colonized by organisms, resulting in uniquely adapted forms. The field of evolutionary biology almost unanimously agrees that partial reproductive isolation can evolve in sympatry, and it has been demonstrated in several species. The problem that remains is demonstrating whether incipient ‘host races’ will eventually sever genetic exchange and become independent species. It is stimulating to know that fundamental questions in evolutionary biology remain unanswered.

Table 5.1 Estimates of percent reproductive isolation among host populations as a result of (a) adult eclosion times in the field (Chapter 1, this work), (b) flight activity times in the field (Chapter 1, this work), (c) behavioral responses to fruit volatiles recorded in laboratory wind tunnels (Linn et al. 2012), (d) behavioral responses to olfactory stimuli recorded in the field (Sim et al. 2012), (e) total reproductive isolation (TRI) estimates using field eclosion and laboratory behavioral isolation (BI; and (f) TRI estimates using field eclosion and field behavioral isolation (see text for formulae). Data collected from co-occurring populations of *R. pomonella* in southwestern Washington State.

(a) Eclosion	Black hawthorn	Early apple	Late apple
Early apple	24.4%	—	—
Late apple	55.7%	43.9%	—
Orn haw	92.6%	87.1%	59.3%
(b) Activity			
Early apple	14.7%	—	—
Late apple	31.3%	11.5%	—
Orn haw	48.3%	36.6%	18.9%
(c) Behavioral (Lab)			
Early apple	69.7%	—	—
Late apple	69.7%	0.0%	—
Orn haw	82.0%	90.1%	90.1%
(d) Behavioral (Field)			
Early apple	64.7%	—	—
Late apple	64.7%	0.0%	—
Orn haw	69.1%	77.8%	77.8%
(e) TRI (BI = lab)			
Early apple	77.1%	—	—
Late apple	86.6%	43.9%	—
Orn haw	98.7%	98.7%	96.0%
(f) TRI (BI = field)			
Early apple	73.3%	—	—
Late apple	84.3%	43.9%	—
Orn haw	97.7%	97.1%	91.0%

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Appendix: Supplementary data for Chapter 2

Table A.1 Dates of fruit collection for measuring firmness (F), size (S), and larval abundance (L).

<u>Date</u>	<u>Black haw</u>	<u>Early apple</u>	<u>Late apple</u>	<u>Orn. haw</u>
12-Jun-13	F, S			
15-Jun-13	F, S			
17-Jun-13	F, S			
19-Jun-13	F, S			
22-Jun-13	F, S			
24-Jun-13	F, S			
26-Jun-13	F, S	F, S	F, S	
29-Jun-13	F, S			
1-Jul-13	F, S			
3-Jul-13	F, S	F, S	F, S	F, S
6-Jul-13	F, S			
8-Jul-13	F, S			F, S
10-Jul-13	F, S	F, S	F, S	
12-Jul-13	F, S, L	F, S	F, S	
13-Jul-13	F, S			
15-Jul-13	F, S			F, S
17-Jul-13	F, S	F, S	F, S	
18-Jul-13	L	F, S, L	F, S, L	
22-Jul-13		F, S	F, S	F, S
29-Jul-13				F, S
31-Jul-13		F, S, L	F, S, L	
3-Aug-13				F, S
6-Aug-13		F, S	F, S	F, S
10-Aug-13				F, S
12-Aug-13	L			F, S
14-Aug-13		F, S, L	F, S, L	F, S
16-Aug-13				F, S
19-Aug-13				F, S
24-Aug-13			F, S	F, S
26-Aug-13				F, S
1-Sep-13			F, S	F, S
6-Sep-13				F, S, L
10-Sep-13			F, S	F, S
19-Sep-13			F, S	F, S
26-Sep-13				L

Table A.2 Mean dates of eclosion ($\pm$ standard error in days) for individual eclosion tents as well as host population (BH = black hawthorn; EA = early apple; LA = late apple; OH = ornamental hawthorn), and the mean of the individual tent means (Population Ordinal Mean) for each population. Ordinal date 165 = 14–Jun.

<u>Host</u>	<u>Tent ID</u>	<u># Flies</u>	<u>Mean Ordinal Date</u>	<u>Tent s.e.</u>	<u>Median Ordinal Date</u>	<u>Pop. Ordinal Mean</u>	<u>Pop. s.e.</u>
BH	DGBH 02	22	180.68	3.07	181.50	174.12	3.14
BH	DGBH 03	17	177.24	2.06	176.50	–	–
BH	DGBH 05	4	168.50	0.66	168.50	–	–
BH	DGBH 06	3	167.00	1.60	167.50	–	–
BH	DGBH 07	2	184.50	3.10	184.00	–	–
BH	DGBH 09	5	166.80	2.24	180.00	–	–
EA	BOB AP	3	180.67	8.36	172.50	177.41	2.95
EA	WILKAP 01	49	171.53	1.10	169.50	–	–
EA	WILKAP 04	63	180.05	1.25	176.50	–	–
LA	WILKAP 02	22	182.73	1.88	179.50	186.47	1.81
LA	WILKAP 03	7	187.14	1.83	188.50	–	–
LA	WILKAP 06	4	186.25	4.31	188.50	–	–
LA	WILKAP 07	3	183.33	3.29	181.50	–	–
LA	WILKAP 08	16	192.88	3.05	191.00	–	–
OH	DISCOH 04	1	194.00		193.50	198.36	1.79
OH	ALKIOH 01	44	198.45	1.41	197.50	–	–
OH	ALKIOH 02	4	198.25	2.01	196.50	–	–
OH	BBCOH	4	202.75	2.86	204.00	–	–

Table A.3 Eclosion tents dampened fluctuations in soil temperatures within the enclosure. Presented are only instances where significant temperature differences (inside vs. outside tent) were detected (paired t-tests). Significant *P*-values ($\alpha = 0.05$) are in bold. “Area” refers to the three boxed regions indicated in Fig. 2B.

Temperature (°C) Difference					
(Inside – Outside)					
<u>Area</u>	<u>Mean min</u>	<u>Mean Max</u>	<u>t value</u>	<u>df</u>	<u><i>P</i></u>
1	0.4 (se±0.04)	—	-9.66	118	< 0.0001
2	0.27 (se±0.12)	—	-2.99	112	< 0.0200
2	—	-2.18 (se±0.16)	13.94	112	< 0.0001
3	—	-0.56 (se±0.2)	2.89	95	< 0.0070

Fig. A.1 Mean diameter ($\pm$ se) and firmness ($\pm$ interquartile range) for hawthorn and apple fruit at the Vancouver, WA sites through time.