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NATURAL HYBRIDIZATION AND SPECIATION IN *RHAGOLETIS* (DIPTERA: TEPHRITIDAE)

A Thesis in

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ABSTRACT

Hybridization has long been regarded as an important source of evolutionary novelty in plants. Zoologists, in contrast, have regarded hybridization merely as an artifact of incomplete reproductive isolation. Especially the formation of new animal species via hybridization has been deemed highly unlikely (unless it results in parthenogenetic, polyploid taxa). Here I present the first case of hybridization in an insect that has resulted in an isolated, diploid and bisexually reproducing population.

I discovered the infestation of invasive honeysuckle, *Lonicera* spp., by flies belonging to the *R. pomonella* species complex (Diptera: Tephritidae). Because all members of this species complex are native to North America, the infestation of *Lonicera* has to be the result of a recent host shift. Multilocus nuclear genotypes and mitochondrial DNA sequences showed that the *Lonicera* Fly originated via hybridization between the blueberry maggot *R. mendax* and the snowberry maggot *R. zephyria*. The same data also provide indirect evidence for the reproductive isolation of the *Lonicera* Fly from its parent taxa.

I tested the acceptance of different host fruits by the *Lonicera* Fly, *R. mendax* and *R. zephyria* in behavioral experiments. These experiments suggest that, compared to host races and described sibling parent species, the *Lonicera* Fly shows an intermediate level of behavioral isolation from its parent taxa. At the same time, the *Lonicera* Fly's host selection behavior indicated a higher phenotypic variability when compared to *R. mendax* and *R. zephyria*.

This unique combination of host shift and hybridization suggests sympatric speciation via host shift as the mechanism by which the hybrid *Lonicera* Fly formed. I discuss how the increased phenotypic variability of hybrid origin populations could facilitate adaptive speciation. I further argue that hybrid speciation in parasitic animals could be common because the ecological conditions of the *Lonicera* Fly system are frequently encountered in nature.

Finally I integrate the findings of this study with previous results on speciation and hybridization in the genus *Rhagoletis* and other animal taxa. I propose the term "biological metaspecies" to describe the evolution of the *Rhagoletis pomonella* species group. A biological metaspecies is a group of ecologically differentiated sibling taxa that are linked by occasional hybridization. Each single taxon might lack the necessary variation to colonize a new niche, but such variation could be maintained on the metaspecies level and become mobilized by hybridization.

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Chapter 1

INTRODUCTION

The enormous diversity of living organisms is one of the most fascinating properties of life on earth. Humans have always attempted to order and classify organismal diversity, and it is therefore not surprising that systematics and taxonomy established themselves among the earliest fields of biology. At the core of our modern taxonomic system is the species is the most fundamental unit of classification (Mayr 1982). According to Mayr (1982), species "represent the lowest level of genuine discontinuity above the level of the individual." For a long time, species were (and sometimes still are) regarded static, immutable entities.

Explanations for the origin of organismal diversity have been developed throughout history. But speciation, the mechanism(s) by which new species are formed, did not become established as a scientific problem until Charles Darwin published "The Origin of Species" in 1859 (Darwin 1859). Darwin refuted the idea that species are static and immutable entities, but hypothesized instead that all recent species evolved from a single origin of life by a series of speciation events. Almost 150 years after "The Origin of Species" was published, understanding speciation is still one of the great challenges of biology (Barton 2001a).

Darwin noted also that the values of biological traits are not uniformly distributed within a species, but that organisms show individual, heritable differences. This variation serves as the raw material for natural selection, which Darwin described as one of the major mechanisms of evolution. The term "species" suddenly took on two different

meanings. On the one hand, it is still used in its pre-Darwinian meaning as a taxonomic unit of classification. On the other hand, it has also become a description for an evolutionary unit of organisms (Hey 2001). It is commonly acknowledged today that the evolutionary mechanisms of "microevolution" (= the changes within a population) are not different from the mechanisms of macroevolution (= the formation of new "taxa"). Speciation is one outcome of population-level processes. We therefore expect a gradient of discontinuities among groups of organisms. It is difficult to determine at what level these discontinuities are "genuine," and correspond to a "species" according to Mayr's definition (Mayr 1982). This is the reason why species definitions are bound to be arbitrary, yet they remain a necessary conceptual tool for the study of speciation (Barton 2001a). For this study, I will adopt Templeton's cohesion species concept (Templeton 1989). According to this concept, species are distinguished by either unique or unique combinations of genetic characters and ecological differences.

Stepping back from the population level and taking a phylogenetic perspective, there are, in principle, two different possibilities for the formation of a new species. The first is the splitting of a single ancestral species into two derived species. This results in a bifurcating species tree. In the second possibility, two (or more) ancestral species hybridize, and the resulting hybrid offspring forms a new lineage. Such speciation by hybridization produces reticulating species trees.

Some authors, (e.g., Dowling and Secor 1997) distinguish between "hybrid speciation" and "speciation by introgressive hybridization." They define "hybrid speciation" as the "instantaneous" evolution of reproductive isolation following allo-

polyploidization. Allo-polyploidization has been described in many plant species and could account for more than 50% of plant diversity (Masterson 1994). Polyploidization appears to play a much smaller role in animal speciation, but there is still a number of examples of hybrid speciation in animals (Bullini 1994).

During speciation by introgressive hybridization, hybrid offspring do not become immediately reproductively isolated, but can still interbreed with the parental taxa to some extent. This results in the formation of a "group of recombinant individuals" (Dowling and Secor 1997), which may evolve reproductive isolation from its parents. Well-documented examples for introgressive hybridization are rare both in animals and in plants (Dowling and DeMarais 1993; Rieseberg 1997). It is unclear whether speciation by introgressive hybridization is a mode of speciation that occurs only rarely in nature or whether it is just difficult to document (Dowling and Secor 1997). Unlike polyploidization, it does not leave a characteristic signature in the hybrid species' genome.

The mechanisms by which animal hybrid swarms become reproductively and ecologically isolated are poorly understood. The literature contains only three examples of speciation by introgressive hybridization in animals. They comprise three fish (DeMarais et al. 1992; Salzburger et al. 2002; Smith et al. 2003) and one mammalian example (Wayne and Jenks 1991). These studies provide morphological and genetic evidence that make a hybrid origin the most likely explanation for the origin of an extant species, but do not report empirical data on potential mechanisms of speciation. In all four cases the authors explicitly or implicitly state that allopatric speciation is the most likely mechanism by which these animal hybrid taxa became isolated.

In plants, the work by Rieseberg and coworkers provides the best-documented example for speciation by introgressive hybridization (Rieseberg et al. 1995; Rieseberg et al. 1996). These authors showed that certain species of sunflowers formed by hybridization between two parent taxa with the same chromosome number. By experimental studies Rieseberg et al. (1995 and 1996) showed that, although the hybrid taxa had the same chromosome number as their parents, they were distinguished by chromosomal reorganizations from the parent taxa that serve as a reproductive barrier. Rieseberg (1997) uses the term "homoploid" to distinguish examples like the hybrid sunflowers from "polyploid" hybrid species. Dowling and Secor (1997) propose a parallel term for animal hybrids that originated by introgressive hybridization. They term such taxa "diploid, bisexual hybrid species."

Speciation via hybridization has to proceed, at least initially, in sympatry (Rieseberg 1997). Mayr (1963) also classifies speciation via introgressive hybridization as a form of sympatric speciation. According to Mayr, speciation via introgressive hybridization and other forms of sympatric speciation in animals are unlikely, because they fail to provide a mechanism to overcome the two "ever-present problems of speciation" (Mayr 1963). The two problems he references are the evolution of reproductive isolation from the parent taxa and the evolution of ecological compatibility with the parent species.

Mayr's judgement on the probability of sympatric speciation was challenged by authors that worked on host-specialist insect species (Bush 1966; Tauber and Tauber 1977; Wood and Guttman 1983). Bush (1966) proposed the genus *Rhagoletis* as an example for sympatric speciation and as a result of subsequent research *Rhagoletis* has become one of the most compelling cases for this mode of animal speciation (Berlocher

and Feder 2002). Bush had observed that almost all species of the tephritid genus *Rhagoletis* in eastern North America are specialist fruit parasites, each of which occupies separate host plants. He further noted that the distributions of these taxa show a high degree of overlap. Bush therefore concluded that it was more likely that *Rhagoletis* radiated by sympatric speciation following shifts than by allopatric speciation (Bush 1966). This idea was informed by the example of the apple maggot, *R. pomonella*. *R. pomonella* is native to eastern North America and uses hawthorn, *Crataegus* spp., as its native host plant. After the introduction of domestic apple to North America by European colonists, *R. pomonella* also adopted this newly introduced plant as a host (Bush 1966). Bush hypothesized that a shift by a specialized fruit parasite can present a solution to Mayr's two problems (Mayr 1963) and result in sympatric speciation (Bush 1992).

1. *Reproductive isolation*. The major driving force behind all models of sympatric speciation is disruptive selection on fitness loci between subpopulations that use different resources. The greater the selection differential, the greater the likelihood of sympatric speciation (Udovic 1980). Subpopulations of host-specific insects, like *R. pomonella*, that occur on two different hosts are therefore likely candidates for sympatric speciation. However, disruptive selection on fitness loci alone will not result in reproductive isolation, unless there is assortative mating between differentially adapted genotypes. Udovic (1980) developed a simple model of sympatric speciation using one locus for habitat-specific fitness and one locus for assortative mating. If the two loci are unlinked, the combined effect of selection and assortative mating has to be very strong to cause sympatric speciation. But with increasing linkage disequilibrium between the two loci, the conditions for sympatric speciation are relaxed (Udovic 1980). In *R. pomonella*,

mating takes place on the host plant (Prokopy et al. 1971), and host choice is assumed to equal mate choice (Prokopy and Papaj 2000). Assortative mating and host specific fitness are thereby epistatically linked. This leads, like physical linkage, to relaxed conditions for the development of reproductive isolation in sympatry (Diehl and Bush 1989).

2. *Ecological compatibility*. If the ecological differences between the old and the new host are great enough, a host shift by specialist parasites is likely to result in ecological isolation. The *R. pomonella* host races on hawthorn and apple differ in their phenology (Filchak et al. 2000), host fruit acceptance (Prokopy et al. 1988), and response to host-specific fruit volatiles (Linn et al. 2003). Host fidelity of *R. pomonella* host races has also been demonstrated by a mark – release – recapture experiment in the field (Feder et al. 1994).

That this scenario did indeed result in partial reproductive isolation was demonstrated by McPheron et al. (1988) and Feder et al. (1988). They observed that host-associated populations on apple and hawthorn showed differences in the frequency of allozyme alleles at a number of "diagnostic" loci. Genetically differentiated host races have been also described for other taxa (Dres and Mallet 2002). Examples include two other tephritid flies, *Eurosta solidaginis* (Abrahamson and Weis 1997; Craig et al. 1993; Waring et al. 1990) and *R. cerasi* (Boller et al. 1998; Schwarz et al. 2003), an aphid species (Via 1999), a beetle (Abrahamson et al. 2003), and moths (Emilianow et al. 2004; Nason et al. 2002). But examples of sympatric speciation are not limited to host-specific insects. Sympatric speciation has also been implicated as a mode of speciation in fish (e.g., crater lake cichlids, Schliewen et al. 1994), and has become accepted as one possible mode of animal speciation (Via 2001).

Much evidence indicates that Mayr's two "ever-present problems of speciation" (Mayr 1963) can be overcome in sympatry. Does this open the possibility for speciation via introgressive hybridization in animals? It is conceivable that a population of hybrid origin could become reproductively isolated following, for example, a host shift. In that case, hybridization could be merely coincidental, and not play any functional role in the process of speciation. Strictly speaking, this would not constitute speciation *via* hybridization, but the speciation of a hybrid population via host-race formation.

Even if introgressive hybridization does not result in chromosomal rearrangements (as in sunflowers, Rieseberg et al. 1995; Rieseberg et al. 1996), introgressive hybridization could play a functional role in sympatric speciation. One factor that is often ignored in models of adaptation and sympatric speciation is the origin and maintenance of genetic variation (Gavrilets in press). Models of sympatric speciation depend on the adaptation to new resources. But where does the phenotypic variation originate that is necessary for the colonization of new niches?

Authors have proposed two principal sources, novel mutations and existing preadaptations (Floate and Whitham 1993). Novel variation can also be introduced to populations by migration (Hartl and Clark 1997) from another subpopulation of the same species. If migration crosses the borders of taxonomically described species, it is referred to as hybridization, but there is no qualitative difference between the two processes (Ellstrand and Schierenbeck 2000). Hybridization is a fast mechanism for generating novel phenotypic variation, especially under quantitative genetic models (Barton 2001b), that could generate the necessary variation for fast ecological adaptation. Hybridization

could thereby play a functional role in speciation.

The role of hybridization as an important source of evolutionary and ecological novelty was first recognized by plant biologists (e.g., Anderson 1948). Anderson (1948) noticed that hybrid origin individuals often colonized disturbed habitats that were not used by either parental taxon. This has also been observed in hybrid sunflower species that occupy extreme habitats, such as salt marches (Rieseberg et al. 2003). Arnold (1997) showed that hybrid zones between species of *Iris* coincide with ecological transitions and that some hybrid genotypes are better adapted to the intermediate habitat.

In animals, hybridization has been traditionally regarded as an evolutionary dead end (Mayr 1963). Hybridization and hybrid zones have been primarily studied as a laboratory for the reproductive isolation between species (Barton and Hewitt 1985). There is also evidence, however, that hybridization can have adaptive advantages in animals. Lewontin and Birch (1966) demonstrated that introgressive hybridization between two tephritid species in the genus *Bactrocera* could have resulted in the greater temperature tolerance and range expansion of one of the flies. Grant and Grant (1992) showed that, following a change in weather patterns, hybrid Darwin finches had a higher fitness than the parentals. A recent study on hybridization between mosquitoes in the eastern United States suggests that hybrid genotypes could be more successful in using birds as a host (Fonseca et al. 2004). This latter study corresponds to Ellstrand and Schierenbeck's hypothesis that hybridization triggered the evolution of invasiveness in introduced plants (Ellstrand and Schierenbeck 2000). Arnold (2004) cites evidence that introgressive hybridization played an important role in the domestication of animals.

In 1997, I discovered a new population within the *Rhagoletis pomonella* species complex, the *Lonicera* Fly. A preliminary population-genetic analysis suggested that the *Lonicera* Fly could have formed by hybridization between two native *Rhagoletis* species – the blueberry maggot, *R. mendax*, and the snowberry maggot, *R. zephyria*. The *Lonicera* Fly is only found on brushy honeysuckle, *Lonicera* spp., an invasive weed, that has been introduced to North America within the last 250 years (Rehder 1947). The infestation of *Lonicera* is therefore the result of a recent host shift by *Rhagoletis*.

The *Lonicera* Fly could represent a unique combination between hybridization and host shift. It belongs to a genus of tephritid fruit flies in which host shifts are known to have resulted in reproductive isolation (Berlocher and Feder 2002). It could therefore represent the first example for the speciation of an insect hybrid population.

This thesis consists of two major parts. A population genetic study tests the hypothesis that the *Lonicera* Fly originated by hybridization. It also examines genetic evidence for the reproductive isolation of the *Lonicera* Fly (Chapter 2). The reproductive isolation of the *Lonicera* Fly is also the topic of a behavioral study on the host fruit acceptance and preference of the *Lonicera* Fly and its parents. I will further discuss the behavioral results under the question of whether hybridization plays a functional role in the speciation of the *Lonicera* Fly (Chapter 3). I also report a study on the host-independent sexual isolation of *R. mendax* and *R. zephyria*. I conclude with a synthesis of both studies and integration of the results into a model for the evolution of the *Rhagoletis pomonella* species complex.

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Chapter 2

HOST SHIFT TO AN INVASIVE PLANT TRIGGERS RAPID ANIMAL HYBRID SPECIATION

Dietmar Schwarz, Benjamin M. Matta, Nicole S. Shakir-Botteri and Bruce A. McPheron

Hybridisation as a source of evolutionary novelty, in particular its role in speciation, is well described in plants (Arnold 1997; Rieseberg 1997). In animals, however, it has been regarded as a process of little evolutionary consequence (Mayr 1963). We report the first case of diploid, bisexual hybrid speciation in an insect. The two parental species are host-specific true fruit flies native to North America (Bush 1966), but the hybrid "*Lonicera* Fly" completes its development on introduced, invasive honeysuckle (Rehder 1947), suggesting rapid speciation within the last 250 years. We use multiple nuclear and mitochondrial markers to demonstrate that the *Lonicera* Fly formed via hybridisation and that it now represents an independently evolving population. The host shift leading to the *Lonicera* Fly presents an ecologically robust scenario for animal hybrid speciation – in the face of the enormous obstacles for such (Mayr 1963) – because it simultaneously offers a novel ecological niche and a mechanism for reproductive isolation (Berlocher and Feder 2002). We speculate that the novel genetic and phenotypic variation that resulted from hybridisation are likely to have facilitated the acquisition of a new host and the subsequent sympatric speciation via host-race formation (Kondrashov and Mina 1986). The necessary conditions for our proposed mechanism are commonly found in

parasitic animals (Price 1980), and the frequency of hybrid speciation in animals may increase because of anthropogenic blending of previously isolated communities (Mack et al. 2000).

In 1997, we discovered the infestation of non-native, brushy honeysuckle forms, *Lonicera* spp., by tephritid fruit flies within the *Rhagoletis pomonella* species complex in the mid-Atlantic United States (see supplementary material). All taxa within the *R. pomonella* species complex are specialized fruit parasites, each occupies only a very limited range of host plants (Bush 1966). The infested *Lonicera* plants represent a mixture of parentals (*L. morrowii*), described hybrids (*L.* x *bella* and *L.* x *amoena*) and introgressed forms that originated from Asian introductions to North America during the past 250 years (Rehder 1947). These forms are widely distributed (http://plants.usda.gov) and abundant invasive weeds throughout the northeastern United States (Hunter and Mattice 2002). While the introduced honeysuckle taxa serve as hosts for *Rhagoletis* in Asia and Europe (Smith and Bush 2000) no infestation of introduced or native Lonicera by *Rhagoletis* has been described in North America (Wasbauer 1972). The new insect colonists, however, belong to a monophyletic group of *Rhagoletis* that consists entirely of native North American taxa, most of which overlap in distribution with our newly discovered infestation of *Lonicera* (Berlocher 2000). This indicates that the infestation of invasive honeysuckle forms is the result of a recent host shift. Previous studies that sampled hundreds (sometimes thousands) of individuals of *Rhagoletis* species from multiple locations within their range (table 2-1) showed that these taxa are characterized by unique high frequency (="private") alleles (Berlocher 2000). Given this previous information, we expected to unequivocally assign this as yet undescribed population on

Lonicera to a known *Rhagoletis* taxon. Instead we found that the flies from *Lonicera*, subsequently referred to as "*Lonicera* Fly," showed a unique mixture of species specific allozyme alleles that indicate that the *Lonicera* Fly formed via hybridisation between the blueberry maggot, *R. mendax*, and the snowberry fly, *R. zephyria* (Table 2-1). The *Lonicera* Fly samples exhibit mixtures of private alleles for both *R. zephyria* (*Had*^{111}) and *R. mendax* (*Fum*^{158}) and lack two common *R. pomonella* alleles (*Dia-2*^{100} and *Aat-2*^{100}). The hybrid origin of the *Lonicera* Fly is further supported by additional allozyme and sequence-based markers from five of the six nuclear linkage groups in the *R. pomonella* species complex (Roethele et al. 2001) and the mitochondrial genome (Figure 2-1). In addition to *Had* and *Fum* in linkage group III, the *Lonicera* Fly possesses alleles that are private in each parent species at both P1700 (Roethele et al. 2001), linkage group V) and the mitochondrial cytochrome oxidase II (COII) gene. At *Aat-2*, *Idh*, and *Pgm*, all located in linkage group I, the *Lonicera* Fly shows intermediate allele frequencies. The same pattern is observed at *Mpi* (linkage group II) and P2963 (linkage group IV), although frequency differences between the *Lonicera* Fly and *R. zephyria* are small (Table 2-2). Our analysis of multilocus genotypes using the model-based clustering approach implemented by STRUCTURE (Pritchard et al. 2000), assuming two populations) shows that all *Lonicera* Fly individuals are consistently classified as intermediates of the two extremes of the ancestry gradient between *R. mendax* and *R. zephyria* (Figure 2-2). The *Lonicera* Fly individuals show a high variance in their ancestry coefficient and span almost the entire spectrum between these putative ancestors, with more individuals being similar to *R. zephyria* than to *R. mendax*. These results – consistent across independent linkage groups – offer strong evidence for the hybrid origin of the *Lonicera* Fly against

the competing hypothesis of incomplete lineage sorting (see supplementary material). The hypothesis that the *Lonicera* Fly is an old, undescribed non-hybrid species is also at odds with the known history of non-native *Lonicera* introduction to North-America, which suggests a recent *Lonicera* Fly origin following a host shift. We considered the alternative hypothesis of host shift followed by non-hybrid speciation by either one of the two putative parental species. Given the sampling of *Rhagoletis* (Table 2-1), a *R. mendax* / *R. zephyria* hybrid origin is a much more parsimonious explanation than the drift- or selection-induced increase of extremely rare or previously undetected alleles on honeysuckle (see supplementary material). Furthermore it is highly unlikely that such a process would have led to a consistent intermediate pattern over all examined nuclear linkage groups and the mitochondrial genome (see supplementary material).

What is the current status of the *Lonicera* Fly? At one extreme of a gradient of isolation from the parent taxa, the flies on *Lonicera* could represent a "hybrid zone" (Barton and Hewitt 1985) that is maintained by constant immigration from both parental species. In this case we would expect a high incidence of F1 hybrids (and backcrosses) and strong deviations from Hardy-Weinberg and linkage equilibrium (HWLE) (Barton and Hewitt 1985). At the other extreme, the *Lonicera* Fly may represent a reproductively isolated hybrid species that has evolved from its original hybrid origin and now represents an independent evolutionary entity (Dowling and Secor 1997); Rieseberg 1997). These two alternatives may be taken as the traditional "zoological" and "botanical" views of the evolutionary outcomes of hybridisation (Arnold 1997). To contrast this apparent dichotomy we used a recent statistical method (NewHybrid, (Anderson and Thompson 2002) to assign individual multilocus genotypes to six different classes of offspring that

would result from two generations of hybridisation (pure parentals, F1 and F2 hybrids and first generation backcrosses). From this analysis we found no evidence that any of 50 sampled *Lonicera* Fly individuals represents an F1 genotype, but rather that their assignment closely resembled a simulated population in HWLE (Figure 2-3). In contrast, when we simulated an F1 hybrid population on the basis of the parental genotypes the analysis clearly identified an F1 origin of the individuals (see Figure 2-4A-C for the assignment to other hybrid classes). In concordance with this, no significant deviations from Hardy-Weinberg equilibrium in the observed *Lonicera* Fly populations were revealed by a permutation test (Table 2-3). The State College population of the *Lonicera* Fly was the only sample sizable enough (Table 2-2) to allow for a meaningful test of linkage disequilibrium among allozyme markers. We observed no significant deviations from linkage equilibrium in this sample except between *Had* and *Fum,* two genes that are known to be in the same linkage group (McPheron and Berlocher 1985) and could be part of a chromosomal inversion (Feder et al. 2003) (Table 2-4). Taken together, our results reject a hybrid-zone model for the *Lonicera* Fly and support the model of the *Lonicera* Fly as a randomly mating, self-sustaining taxon of hybrid origin. Due to limitations on the resolution of our genetic markers we cannot quantify the exact degree of isolation from the parent taxa. To date, however, we have no evidence that the *Lonicera* Fly acts as a bridge for the flow of parent private alleles between *R. mendax* and *R. zephyria* in the eastern United States (see table 2-1). In sum, three findings indicate the reproductive and ecological independence of the *Lonicera* Fly from its parents: (1) the *Lonicera* Fly has a unique combination of parental private alleles, found in no other *Rhagoletis* taxon, (2) the *Lonicera* Fly is in population genetic equilibrium, and (3) the *Lonicera* Fly occupies a

separate ecological niche (Templeton 1989). We therefore conclude that the *Lonicera* Fly is either a fully formed or incipient diploid, bisexual insect hybrid species that evolved following an initial host shift within the past 250 years.

Speciation via hybridisation must involve sympatry, at least initially. This mode of speciation faces the general difficulties of sympatric speciation: the lack of a private ecological niche and the absence of reproductive isolation (Mayr 1963). Sympatric speciation via host-race formation is a theoretical solution to these problems (Diehl and Bush 1989), and there is growing empirical evidence, e.g. in the host races of *R. pomonella*, for its action in nature (Berlocher and Feder 2002; Via 2001). There are important theoretical reasons to hypothesize that hybridisation would facilitate sympatric speciation via host shift. Phenotypic variation is a prerequisite for the action of disruptive selection, the key process in all models of sympatric speciation. At the same time strong, host-specific selection is expected to eliminate variation in host specific taxa like *Rhagoletis*. If so, how does the critical variation in traits that are responsible for sympatric speciation originate and how is this variation maintained (Kondrashov and Mina 1986)? Many theoretical models of sympatric speciation do not address this problem, even though some presuppose high levels of initial polymorphism to result in sympatric speciation (Bolnick 2004; Gavrilets). Hybridisation and host shift, as observed in the *Lonicera* Fly, provides one solution and is likely to relax the conditions for sympatric speciation. Given the *Lonicera* Fly's origin by host shift and the conserved life histories of close host association in *Rhagoletis* (Berlocher and Feder 2002) this synergism of hybridization and sympatric speciation provides a plausible mechanism for the origin of the *Lonicera* Fly.

The role of hybridisation in speciation is well known for plants. In animals, hybridisation is classically thought to result in sterile or hybridogenetic taxa (but see (Stöck et al. 2002) and the formation of diploid, bisexual hybrid species was deemed highly unlikely (Mayr 1963). In recent years, however, a handful of studies (three fish: (DeMarais et al. 1992); (Salzburger et al. 2002); (Smith et al. 2003); and one mammal: (Wayne and Jenks 1991) have provided evidence of extant, morphologically differentiated species or populations that appear to originate from introgressive hybridisation. The case of the *Lonicera* Fly, the first diploid, bisexual insect hybrid species, suggests a credible and robust ecological mechanism for hybrid speciation that could occur under commonly observed conditions. Given that hybridisation appears to be frequent in animals (Arnold 1997) and that host-specific lifestyles could represent as much as 50% of animal diversity (Price 1980), the acquisition of hybrid-specific niches via host shift may be a more common phenomenon than previously recognized. Anthropogenic changes offer new opportunities for hybridisation, because previously geographically separated organisms come into contact due human-mediated introductions (Fonseca et al. 2004). At the same time these community alterations provide access to potential new hosts and thereby the opportunity for habitat shifts. Hybridisation between parasites could widen the spectrum of potential new hosts by generating novel phenotypes (Fonseca et al. 2004), a mechanism akin to the idea that plant hybrids serve as a bridge for the acquisition of new hosts by herbivores (Floate and Whitham 1993). Hybridisation and host shifts occur under natural conditions, but human-mediated community changes could increase the frequency of such events. Hybridisation can also be difficult to detect, especially in morphologically cryptic species like *Rhagoletis* that

might account for much of insect diversity. The availability of extensive genetic data for the *R. pomonella* species complex, including reports of local introgression between *R. pomonella* and *R. zephyria* (Feder et al. 1999; McPheron 1990a), is unusual compared to many host-specific organisms. It is likely that specialized parasite hybrid species like the *Lonicera* Fly have gone undetected because of methodological difficulties and the traditional bias against hybridisation as an evolutionary force in zoology (Dowling and Secor 1997). Hybridisation should be considered as a viable hypothesis for the origin of other host-specific animals and we predict that future studies will reveal more cases of parasite hybrid species.

Methods

Sample collection. All samples were collected in central Pennsylvania, U.S.A., in 2000-2002. The *Lonicera* Fly was collected at two locations in State College, Centre County, and one location in Munson, Clearfield County. *R. zephyria* samples were taken from one location in State College and one location in Munson. One *R. mendax* sample was collected near Middleburg, Snyder County (= ”Snyder Cty.”). Two local reference populations for *R. pomonella* were collected from two different hawthorn trees in State College. The five sampling sites in State College were separated by a maximum distance of 4 km from each other. Munson is located 30 km northwest of State College and the *Lonicera* Fly and *R. zephyria* sample were separated by 0.7 km. The *R. mendax* sample location is 60 km east of State College. All samples consist of larvae extracted from

infested fruit, pupae or adults reared from fruit. Larvae, pupae and adult flies were stored at –80°C until further analysis.

Population genetic data collection. Total genomic DNA was extracted from the head or one third of each individual, while the remaining parts were used for allozyme analysis. Seven allozyme loci were examined by standard starch gel techniques (McPheron 1990a; Shaw and Prasad 1970): β-hydroxyacid dehydrogenase (*Had*; E.C. 1.1.1.30), isocitrate dehydrogenase (*Idh*; E.C. 1.1.1.42), NADH-dependent diaphorase-2 (*Dia*-2; E. C.1.6.2.2), aspartate aminotransferase-2 (*Aat*-2; E.C. 2.6.1.1), phosphoglucomutase (*Pgm*; E.C. 5.4.2.2), mannose-6-phosphate isomerase (*Mpi*; E.C. 5.3.1.8), and fumarate hydratase (*Fumh*; E.C. 4.2.1.2). Two additional nuclear loci, developed from a cDNA library (Roethele et al. 1997), were amplified by PCR and scored for restriction length polymorphism. P1700 (T3 = 5'-ACA TAC ATT CTG CAT CTT GCG AAA G-3', T7 = 5'-TTA AGC CGA CTT CTT CTT GAA ACC-3') was polymorphic at one restriction site for *Rsa* 1. To confirm heterozygote genotypes we cloned a limited number of heteroyzgote individuals during the development of our protocol for the restriction digest. We further used restriction enzyme in excess and added previously scored individuals as positive controls to each new batch of digestions. P2963 (T3 = 5'-AGT CAA CGA CCT GCT TAT TT-3', T7 = 5'-TGC ACC TTA ATT CAC GAA AAT C-3') was cut with *Alu* 1, *Ase* 1, and *Tsp*509 1 at four restriction sites and the haplotype inference software HAPLOTYPER was used to determine genotypes (Niu et al. 2002). A 636-bp-long piece of the mitochondrial COII coding region (C2-J-3136 = 5'-CAA AAT AGT GCC TCT CCC-3', TK-N-3772 = 5'-GAG ACC ATT ACT TGC TTT CAG TCA-3', (Simon et al.

1994) was amplified and sequenced. The variation within a 423-bp-long subsection of this sequence was used to characterize individual haplotypes.

Data analysis. Allele and haplotype frequencies were calculated using Arlequin ver. 2000 (Schneider et al. 2000). To compare the allelic composition of the *Lonicera* Fly and its parents in central Pennsylvania, we collected all available published allozyme data for the parents of the *Lonicera* Fly and *R. pomonella* (see references under Figure 1). No population data were available for the two nuclear sequence-based markers and the mitochondrial DNA. Published allele frequencies at each locus were converted into allele counts, and a single allele frequency was recalculated for the North American "superpopulation" of each taxon. All samples gathered as part of this study were combined in a similar fashion. Multilocus genotypes representing *R. mendax* [Snyder Cty. (n = 36)], *Lonicera* Fly [State College (n = 30) and Munson (n = 20)], and *R. zephyria* [State College (n = 30) and Munson (n = 20)] were analyzed using STRUCTURE version 2 (Pritchard et al. 2000). All nuclear markers and mtDNA haplotypes were included except for the monomorphic *Dia*-2 and *Fum*. *Fum* was excluded because it is in strong linkage disequilibrium with *Had* (supplementary material Table 2), and no map distance between these two markers is reported. The known map distances between *Aat-2*, *Pgm* and *Idh* on linkage group I were incorporated into the analysis by using the linkage model in STRUCTURE (Falush et al. 2003) (1,000,000 MCMC replicates and independent allele frequency model). Ancestry coefficients for membership in two or three populations were estimated without any prior knowledge of host-plant origin (i.e., population membership). The same data set, with the exception of the mtDNA haplotypes, was used in the analysis with NewHybrids version 1.1 beta

(Anderson and Thompson 2002). No prior information was used in the assignment of genotypes to hybrid classes that result from two generations of hybridisation (100,000 MCMC replicates). A simulated population of 50 F1 genotypes was generated by randomly drawing with replacement one observed parental genotype from *R. mendax* (Snyder Cty.) and *R. zephyria* (State College and Munson). The simulated F1 genotype then received one allele from each selected parental genotype. Alleles were chosen at random if parents were heterozygous. For the simulation of a population in HWLE, expected genotypes for each locus were calculated based on the observed allele frequencies in the *Lonicera* Fly, and single locus genotypes were randomly drawn without replacement to assemble multilocus genotypes. This process was conducted separately for *Lonicera* Fly State College (n = 30) and *Lonicera* Fly Munson (n = 20). Hardy-Weinberg and linkage equilibrium was tested using 100,000 permutations in Arlequin (Schneider et al. 2000; Slatkin and Excoffier 1996).

Supplementary material

The *R. pomonella* species complex and the origin of the *Lonicera* Fly

The *Rhagoletis pomonella* (Diptera: Tepritidae) species complex consists of several closely related taxa that are all native to North America (for a detailed description see (Berlocher 2000). It consists of four described species (*R. pomonella*, *R. mendax*, *R. zephyria*, and *R. cornivora*), two undescribed species (sparkleberry fly and flowering dogwood fly) and three indeterminate populations (plum fly, mayhaw fly, and Mexican *pomonella*). All taxa are morphologically extremely similar, but occupy different host

plants. All described species and the sparkleberry fly can be distinguished by private allozyme alleles, while the other taxa are characterized by differences in phenology and/or strong allele frequency differences. All taxa occur in eastern North America, with the sparkleberry, plum and mayhaw fly being limited to the southeastern United States. The only exceptions are Mexican *pomonella*, which occurs in geographical isolation in central Mexico, and *R. zephyria*. *R. zephyria* is native to the western United States but overlaps with the natural range of *R. pomonella* in the northern plains states. It has been assumed to be introduced to the north eastern United States by human activity (Feder et al. 1999), but there is evidence that its natural range might have extended further east than previously assumed (Gavrilovic 2001). As described in the main text the *Lonicera* Fly combines private alleles of *R. mendax* and *R. zephyria* but lacks diagnostic alleles for *R. pomonella*. Because these latter *R. pomonella* specific alleles occur only at very low frequencies in the sympatric "flowering dogwood fly" (Berlocher 2000), this taxon cannot be excluded as a contributor to the *Lonicera* Fly genome, but such a three-way hybridisation represents a less parsimonious explanation than a *R. mendax* x *R. zephyria* origin. A similar reasoning applies to other undescribed taxa or populations within the *R. pomonella* species complex that are limited to the Southern United States. The basal *R. cornivora* is excluded because it is fixed for Had^{133}, which is absent from the *Lonicera* Fly.

Preliminary data on the geographic distribution of the *Lonicera* Fly

To date we have detected the infestation of introduced Lonicera spp. at numerous locations ranging from Amherst, Massachusetts, in the east to Wooster, Ohio, in the west and from Geneva, NY, in the north to Baltimore, Maryland, in the south. In a preliminary analysis of samples from Baltimore and from Elmira, New York, we found hybrid origin genotypes at the highly informative *Had* allozyme locus.

Linkage relationships in the *R. pomonella* species complex

All members of the *R. pomonella* species complex have six chromosomes (Bush 1966). Linkage relationships appear to be conserved within tephritid flies despite differences in chromosomal arrangement (Berlocher 1993; Rossler et al. 1994) and synteny extends beyond family limits to *D. melanogaster* (Roethele et al. 2001). For the purpose of this study we therefore assume that markers that are unlinked in *R. pomonella* are also unlinked in the *Lonicera* Fly and its parents.

Power analysis for the detection of *Lonicera* Fly "specific" haplotypes in the parent species

The *Lonicera* Fly most likely formed by hybridisation within the last 250 years. Given such a recent origin it appears unlikely that mitochondrial haplotypes that have been sampled only in the *Lonicera* Fly are the result of a recent mutation in the *Lonicera* Fly.

Instead we expect these haplotypes to represent parental variants that have not been detected by our sampling scheme. To estimate the probability of not sampling a *Lonicera* Fly "specific" haplotype in the parental species – even though it is present – we assumed the following model of hybridisation between *R. mendax* and *R. zephyria*. Both parent taxa contributed equally to the *Lonicera* Fly (this seems reasonable given the observed haplotype frequencies in Figure 1) and no drift or selection occurred during hybridisation. That means that all parental haplotypes were introduced to the *Lonicera* Fly and the relative frequencies of haplotypes derived from each parent remained unchanged. We assume also that neither drift nor selection have altered the haplotype frequencies in the *Lonicera* Fly or its parents since the initial hybridisation. Under these assumptions the expected haplotype frequency in a parent population is twice its frequency in the *Lonicera* Fly. We used Bayesian analysis to calculate the posterior distribution $\pi(p)$ for the haplotype frequencies in the *Lonicera* Fly based binomial likelihoods for the data and uniform ($Beta[1,1]$) priors. The probability of falsely observing a zero-probability of a Lonicera Fly "specific" haplotype in one of the parent taxa given the posterior distribution of the *Lonicera* Fly frequencies is calculated according to the zero-probability of the binomial distribution integrated over the posterior distribution obtained for the haplotype frequencies in the *Lonicera* Fly ($\int Bin(0,n,2p)d\pi$). Note that for calculating the zero-probability in the parent taxa we used $2p$ for defining the binomial distribution to accommodate our assumptions for the hybridization process. There is a posterior probability > 0.05 that either one of the two *Lonicera* Fly "specific" haplotypes was missed in at least one of the two parental species. Our set of assumptions is unrealistic but it should lead to the underestimation of the probability of not sampling a

Lonicera Fly "specific" allele even though it is present. This model does also not take any geographical sampling into consideration. In this case the *Lonicera* Fly could have formed in a different geographical area than the one sampled in our study. If it subsequently became isolated it would mirror the haplotype composition of parental populations that could be different from the samples of parent species in our study.

Incomplete lineage sorting and single-parent host race formation – quantitative evaluation of alternative hypotheses for the formation of the *Lonicera* Fly

If we assume that the *Lonicera* Fly is a previously undescribed, extant species it must have shared a common ancestor with *R. mendax* and *R. zephyria* in the past. After separation allele frequencies at unlinked, neutral nuclear loci will have diverged by random genetic drift. Under this scenario, there will be six equally likely outcomes for the relative ranking of frequencies of a given allele in each taxon (*R. z.* > LF > *R. m.*; *R. z.* > *R. m.* > LF; LF > *R. z.* > *R. m.*; LF > *R. m.* > *R. z.*; *R. m.* > *R. z.* > LF; *R. m.* > LF > *R. z.*). But in the observed data, the estimate for the frequency of the *Lonicera* Fly's most common allele is intermediate between *R. mendax* and *R. zephyria* at all seven unlinked loci (Figure 2-5). Under the assumption that the *Lonicera* Fly is an extant species intermediate frequencies in the *Lonicera* Fly are expected in one out of three loci. The probability that seven unlinked loci show intermediate frequencies equals $(1/3)^7$ and is less than 0.0005.

A similar reasoning can be applied to the alternative hypothesis of single-parent host race formation. In that case either *R. mendax* or *R. zephyria* would have been the sole

population of origin for the *Lonicera* Fly. Assuming again neutral and unlinked markers we expect the allele frequencies of the *Lonicera* Fly to diverge randomly from the parental frequencies. This means that for each locus there is a probability of 1/2 that the allele frequency will shift in the direction of the species that was not involved in the host race formation. But at all seven loci allele frequencies shifted in direction of the respective other parent taxon (Figure 2-5). The probability that this outcome is the result of a random shift in allele frequency following a single-parent host shift is $(1/2)^7$ or less than 0.008.

The intermediate allele frequencies of the *Lonicera* Fly are therefore best explained by an origin via hybridization between *R. mendax* and *R. zephyria*. Even if the assumption of neutrality is violated it is unlikely that selection would shift allele frequencies at unlinked loci in the direction of the respective other sibling taxon.

The hypothesis of a single parent host shift can be examined in an alternative fashion at the diagnostic allozyme locus *Had* (Table 2-1). The *Lonicera* Fly combines Had^{100} (found in *R. mendax* but not in *R. zephyria*) and Had^{111} (found in *R. zephyria* but not in *R. mendax*). If we assume that the *Lonicera* Fly formed by a single parent host shift it must have received both *Had* alleles from either *R. mendax* or *R. zephyria* only. Similar to the question of *Lonicera* Fly "specific" haplotypes (see previous section) we used Bayesian analysis to calculate the posterior distribution $\pi(p)$ for the observed allele frequency of zero for Had^{111} in *R. mendax* and Had^{100} in *R. zephyria*. As in the above example we used binomial likelihoods and uniform ($Beta[1,1]$) priors. Using the sample sizes at *Had* from Table 2-1 we obtained 95% credible intervals for the observed frequency of zero for the

respective *Had* alleles in the two parent taxa. For Had^{111} in *R. mendax* this interval spans the allele frequencies from 0 to 0.0015 and for Had^{100} in *R. zephyria* this interval is 0 to 0.0021. This analysis shows that if diagnostic *Had* alleles were to be present in the respective other parent taxon their frequency would be extremely low. Hybridisation between *R. mendax* and *R. zephyria* is the more parsimonious explanation for the occurrence of both Had^{100} and Had^{111} in the *Lonicera* Fly.

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	R. pomonella (all pop.)	*R. pomonella* (Central PA)	*R. mendax* (all pop.)	*R. mendax* (Central PA)	*Lonicera* Fly (Central PA)	*R. zephyria* (all pop.)	*R. zephyria* (Central PA)
N	*7581*	*105*	*992*	*36*	*243*	*578*	*143*
Had^{100}	0.759	0.772	0.094	0.125	0.157	0.000	0.000
Had^{111}	0.002*&	0.000	0.000	0.000	0.673	0.992	0.986
N	*1982*	*36*	*1089*	*32*	*101*	*207*	*82*
Fumh^{158}	0.001	0.000	0.849	0.923	0.178	0.013**	0.000
N	*6357*	*89*	*959*	*34*	*146*	*503*	*123*
Dia-2^{100}	0.721	0.826	0.001	0.000	0.000	0.024*	0.000
N	*6516*	*98*	*855*	*36*	*240*	*470*	*134*
Aat-2^{100}	0.353	0.470	0.011	0.000	0.000	0.000	0.000

Table 2-1 Pooled allele frequencies of diagnostic alleles for the morphologically described species (except *R. cornivora*) within the *Rhagoletis pomonella* species complex and the *Lonicera* Fly. Central PA = populations surveyed in this study. All pop = previously reported data from populations throughout the ranges of the described taxa (Berlocher 1993; Berlocher 1995; Berlocher 2000; Berlocher and Bush 1982; Feder et al. 1988; Feder et al. 1990a; Feder et al. 1990b; Feder et al. 1999; McPheron 1987; McPheron 1990a; McPheron 1990b; McPheron et al. 1988), except *R. pomonella*, for which only the northern populations are represented. * = allele observed only in zones of described hybridisation between *R. pomonella* and *R. zephyria* in the Pacific Northwest and Minnesota (Feder et al. 1999; McPheron 1990a; McPheron 1990b). ** = allele observed only in the Pacific Northwest (Berlocher and Bush 1982; McPheron 1990b). & = *Had*^{111} has been observed at a frequency of 0.007 in one population (n = 76) of the flowering dogwood fly, an undescribed species in the *R. pomonella* complex (Berlocher 2000).

	R. mendax		LF			*R. zephyria*			
	PS	**Sny. Cty.**	**S.C. Wa.**	**S.C. SCP**	**Mu.**	**S.C. OM**	**Mu.**	**PS East**	**PS West**
Idh									
N		36	174	29	32	63	72	ND	
90		0.000	0.000	0.000	0.016	0.000	0.000		
100		0.556	0.382	0.569	0.469	0.079	0.125		
118	0.409 (0.102; P=26)	0.444	0.612	0.414	0.516	0.921	0.875		0.71 (P=1)
128		0.000	0.006	0.017	0.000	0.000	0.000		
Had									
N		36	179	32	32	70	73		
100		0.125	0.176	0.141	0.063	0.000	0.000		
111	0 (0; P=26)	0.000	0.659	0.625	0.797	0.986	0.986	0.976 (0.024; P=5)	0.995 (0.008; P=5)
122		0.875	0.161	0.234	0.200	0.014	0.014		
Aat-2									
N		36	175	33	32	66	68		
21	0.249 (0.069; P=26)	0.292	0.677	0.561	0.656	0.826	0.934	0.804 (0.119; P=5)	0.865 (0.007; P=2)
50		0.639	0.306	0.409	0.328	0.152	0.059		
75		0.069	0.017	0.030	0.016	0.023	0.007		
Pgm									
N		36	173	34	32	58	66		
80		0.042	0.023	0.000	0.016	0.000	0.000		
92		0.056	0.046	0.029	0.000	0.034	0.008		
100	0.856 (0.063; P=26)	0.903	0.754	0.824	0.625	0.440	0.485	0.501 (0.124; P=5)	0.771 (0.057; P=2)
111		0.000	0.176	0.147	0.313	0.526	0.508		
118		0.000	0.000	0.000	0.047	0.000	0.000		

	R. mendax		LF			*R. zephyria*			
	PS	**Sny. Cty.**	**S.C. Wa.**	**S.C. SCP**	**Mu.**	**S.C. OM**	**Mu.**	**PS East**	**PS West**
Mpi									
N		30	164	33	31	65	71	ND	
33		0.029	0.137	0.091	0.145	0.192	0.225		
66		0.157	0.264	0.318	0.290	0.285	0.261		
100	0.893 (0.063; P=26)	0.814	0.599	0.591	0.565	0.523	0.514		0.23 (P=1)
Fum									
N		32	70	ND	32	16	30	ND	
100	0.122 (0.084; P=26)	0.077	0.771		0.875	1.000	1.000		0.98 (P=1)
158		0.923	0.229		0.125	0.000	0.000		
P1700									
N	ND	36	29	ND	19	17	29	ND	ND
1		0.917	0.104		0.132	0.000	0.000		
2		0.083	0.896		0.868	1.000	1.000		
P2963									
N	ND	36	30	ND	19	20	30	ND	ND
0		0.177	0.200		0.184	0.200	0.117		
1		0.371	0.717		0.763	0.750	0.750		
3		0.016	0.000		0.000	0.000	0.000		
4		0.016	0.000		0.000	0.000	0.000		
5		0.226	0.083		0.053	0.050	0.000		
6		0.000	0.000		0.000	0.000	0.133		
12		0.016	0.000		0.000	0.000	0.000		
13		0.177	0.000		0.000	0.000	0.000		

Table 2-2 Allele frequencies at nuclear loci for the *Lonicera* Fly and its parent taxa. For populations that we examined in this study the frequencies for all observed alleles are shown. N = number of individuals. In addition, we include allele frequency information from previous studies (= PS). We chose the most common allele in the *Lonicera* Fly and calculated the mean frequency of this allele from data on parent species populations in the literature. We report standard deviations and number of populations (= P) in brackets under the mean allele frequency. The parental frequencies of the most common allele in the *Lonicera* Fly were colour-coded to illustrate the intermediacy of the observed *Lonicera* Fly frequencies. Red: < all reported *Lonicera* Fly frequencies; Orange: < *Lonicera* Fly S.C. Wa.; Blue > all reported *Lonicera* Fly frequencies; Light Blue: < *Lonicera* Fly S.C. Wa.. In almost all cases *R. mendax* and *R. zephyria* frequencies diverge consistently from the *Lonicera* Fly frequencies in opposite directions. *R. mendax*: PS = data from (Berlocher 1995), Sny. Cty. = Snyder County. *Lonicera* Fly: S.C. Wa = State College Waupelani, S.C. SCP = State College Slab Cabin Park, Mu. = Munson. R. zephyria: S.C. OM = State College Old Main, Mu. = Munson, PS East = data for populations east of the Rocky Mountains (Berlocher 2000; Feder et al. 1999), PS West = data for populations west of the Rocky Mountains (Berlocher and Bush 1982; Feder et al. 1999; McPheron 1990b). ND = no data.

	LF Waupelani				LF Munson			
locus	N	P-value	Obs H	Exp. H	N	P-value	Obs. H	Exp. H
P1700	29	0.013	0.069	0.189	19	-		
P2963	30	-			19	-		
Idh	174	-			32	-		
Had	179	-			32	-		
Aat-2	175	-			32	0.020	0.281	0.479
Pgm	173	-			32	-		
Mpi	164	-			31	-		
Fumh	70	-			32	-		

Table 2-3 Permutation test for deviations from Hardy-Weinberg equilibrium in *Lonicera* Fly populations (Schneider et al. 2000). Only p-values <0.05 are shown (- = p > 0.05). Neither of the two displayed deviations is significant after a sequential Bonferroni correction. Obs. H = observed heterozygosity, Exp. H = expected heterozygosity.

	Idh	*Had*	*Aat*-2	*Pgm*	*Mpi*
Had	-				
Aat-2	-	0.027			
Pgm	-	-	0.009		
Mpi	-	-	-	-	
Fumh	-	<0.001*	-	-	-

Table 2-4 Permutation test for deviations from pairwise linkage equilibrium between allozymes in *Lonicera* Fly State College (Schneider et al. 2000). Only p-values <0.05 are shown (- = p > 0.05). After sequential Bonferroni correction only *Had* deviates significantly from linkage equilibrium (indicated by *****).

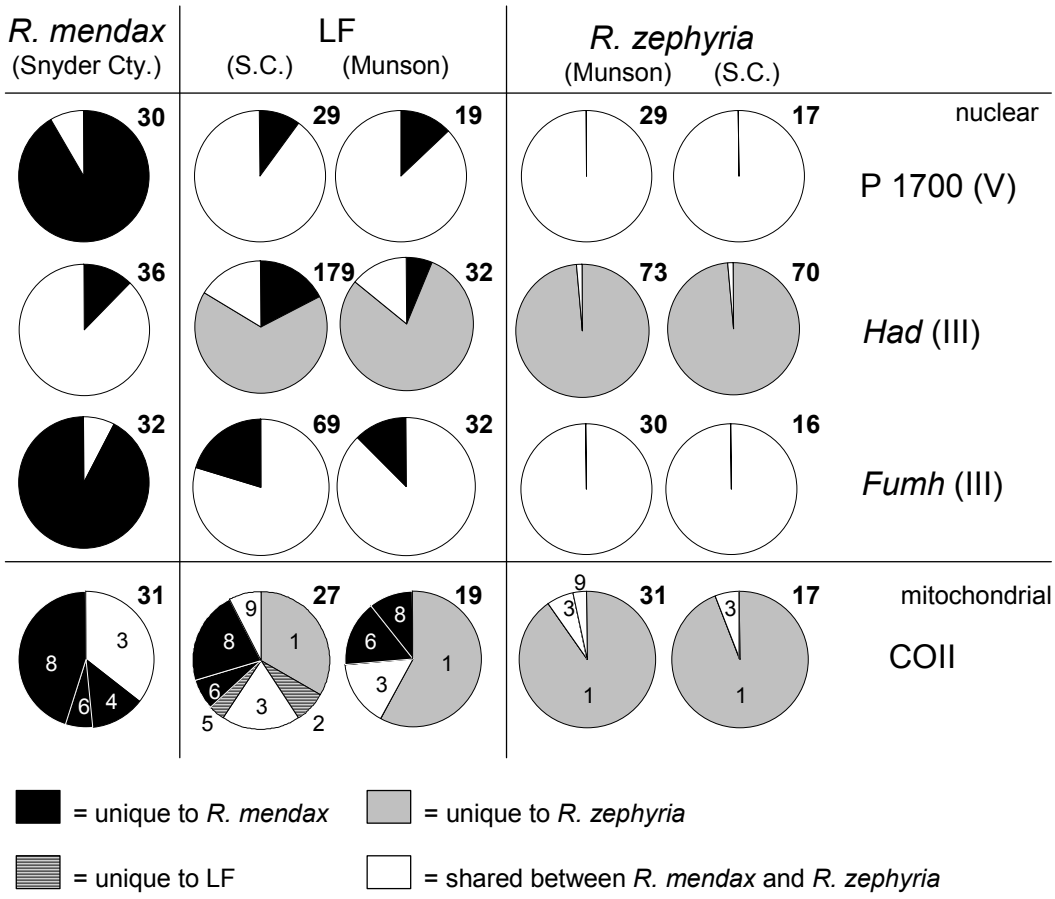

Figure 2-1 Allele frequencies at hybrid diagnostic nuclear and mitochondrial loci in *Lonicera* Fly and parent populations from Central Pennsylvania. Black = *R. mendax* private allele, grey = *R. zephyria* private allele, white = shared allele, hatched = haplotype only found in *Lonicera* Fly. Small integers identify different mitochondrial haplotypes. Large, bold integers indicate the number of sampled individuals. There is a posterior probability > 0.05 that either one of the two *Lonicera* Fly "specific" haplotypes was missed in at least one of the two parental species. This result is based on a simple model that assumes equal contributions from both parents, no genetic drift, and no geographic differences in parental haplotype composition (supplementary material).

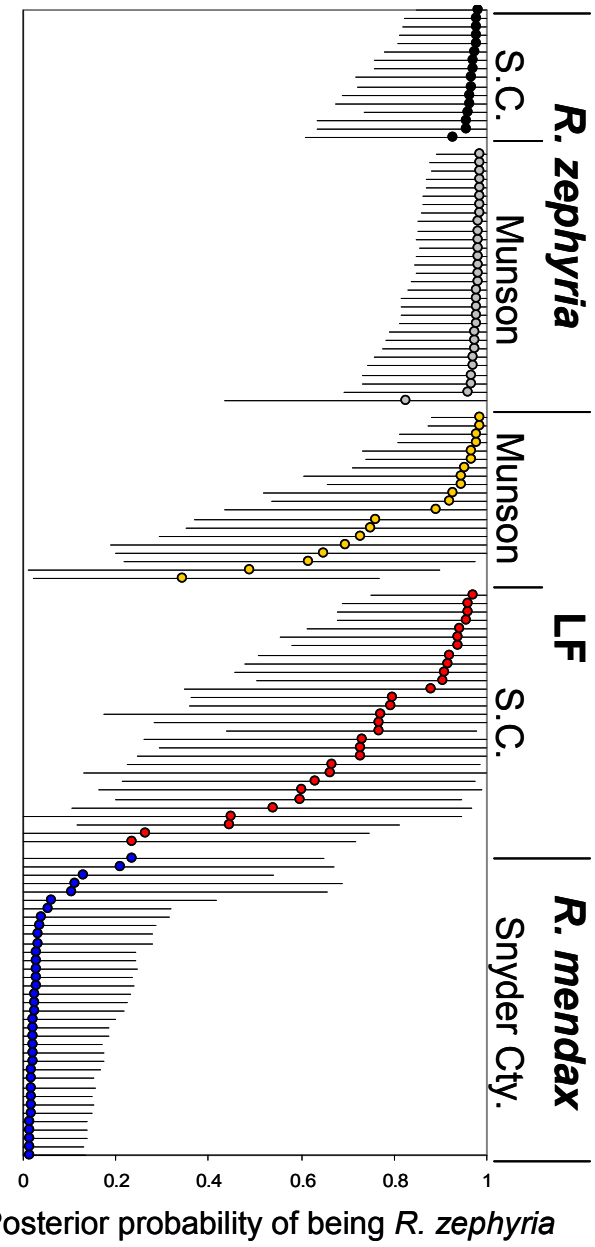

Figure 2-2 Assignment of ancestry from two parent populations to individuals of the *Lonicera* Fly and its parents without any prior information of population membership (as implemented in STRUCTURE). Blue = *R. mendax* Snyder Cty., Red = *Lonicera* Fly State College, Orange = *Lonicera* Fly Munson, Grey = *R. zephyria* Munson, Black = *R. zephyria* State College. Black bars represent 95% credible intervals.

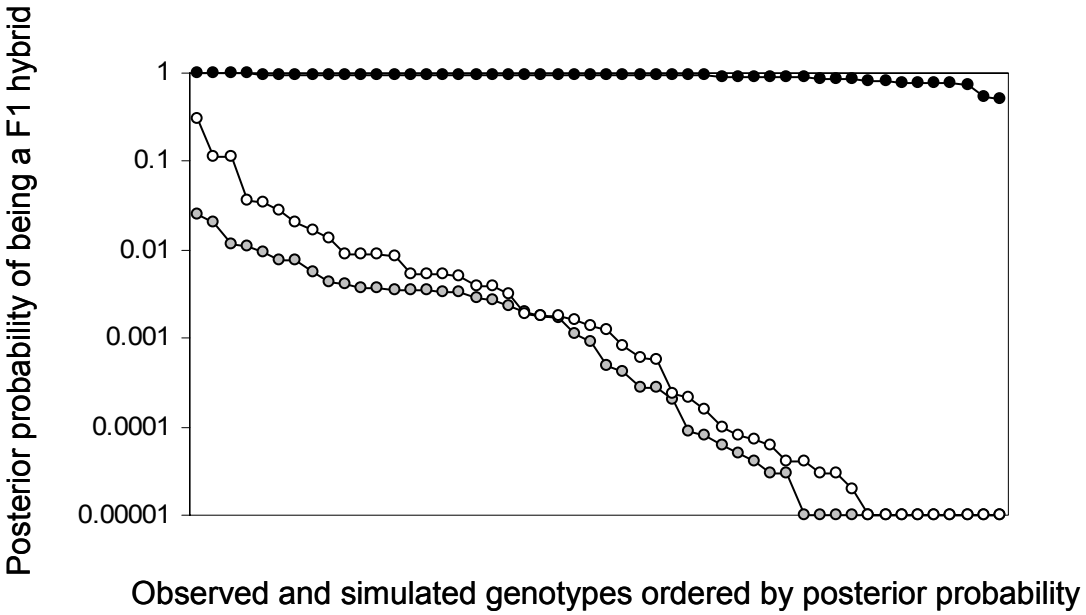

Figure 2-3 Posterior probability for the assignment of observed *Lonicera* Fly individuals as F1 hybrids between *R. mendax* and *R. zephyria*. Grey = observed *Lonicera* Fly individuals, white = simulated population in Hardy-Weinberg and linkage equilibrium based on observed *Lonicera* Fly allele frequencies, black = simulated population of F1 crosses between *R. mendax* and *R. zephyria* using observed parental genotypes. Logarithmic scale.

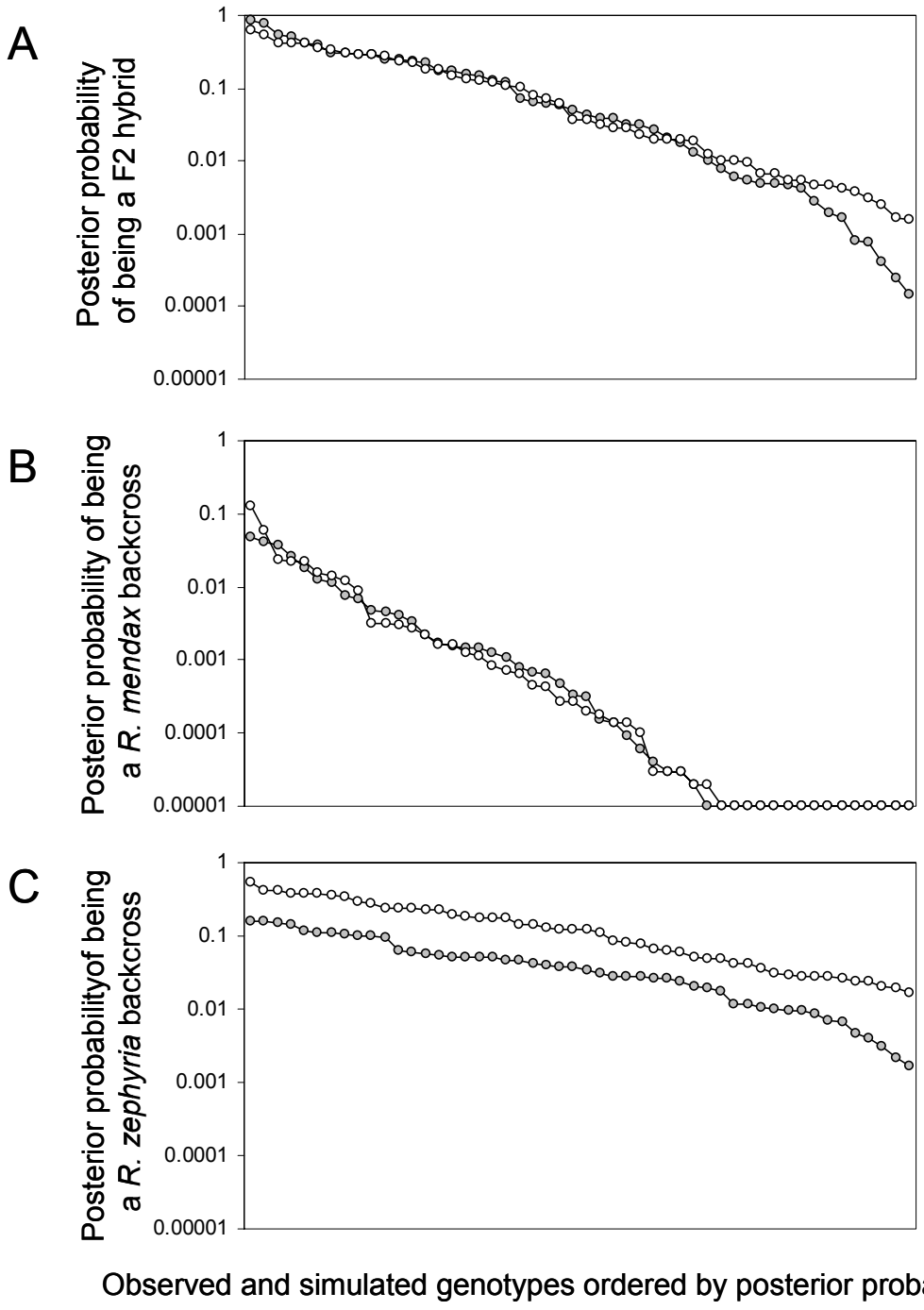

Figure 2-4 Posterior probability for the assignment of observed *Lonicera* Fly individuals to different hybrid classes that result from two generations of hybridisation between *R. mendax* and *R. zephyria* (as implemented in NewHybrids version 1.1beta). Grey = observed *Lonicera* Fly individuals, white = simulated population in Hardy-Weinberg and linkage equilibrium based on observed *Lonicera* Fly allele frequencies. **A** Posterior probability of being an F2 hybrid. **B** Posterior probability of being a backcross with *R. mendax*. **C** Posterior probability of being a backcross with *R. zephyria*. Logarithmic scale.

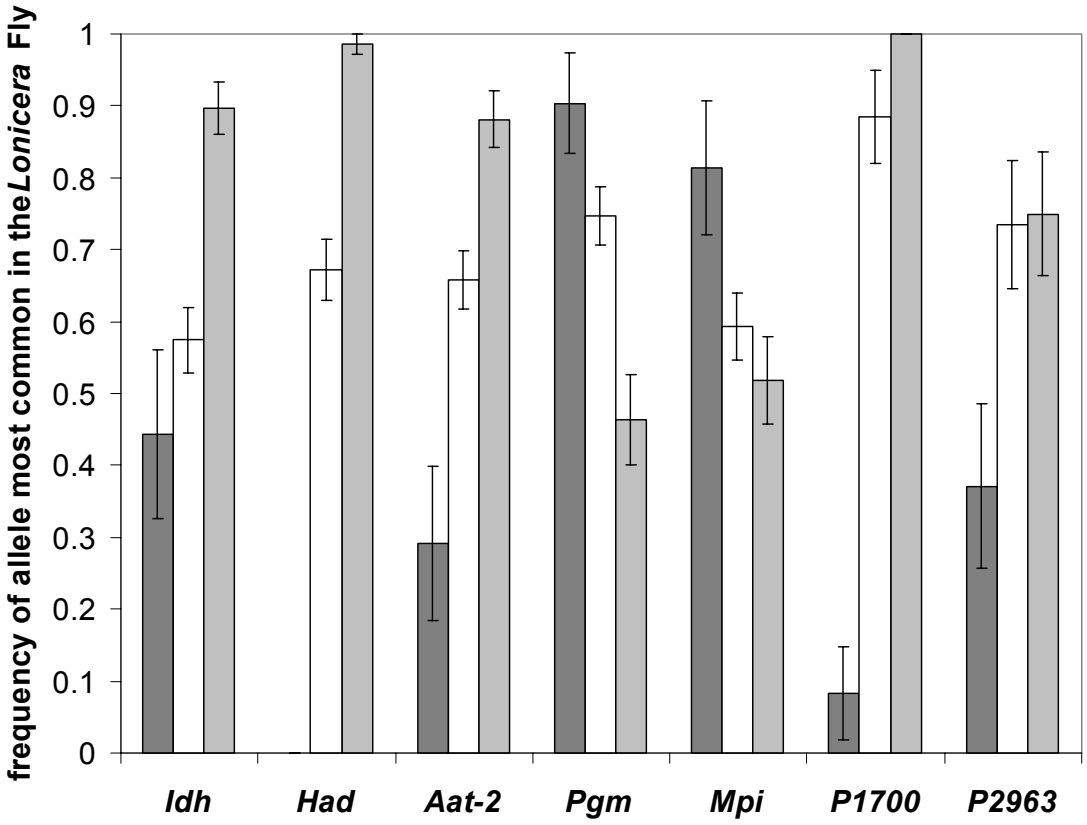

Figure 2-5 Comparison of alelle frequencies for the most common allele in the *Lonicera* Fly between *R. mendax* (dark grey bars), the *Lonicera* Fly (white bars), and *R. zephyria* (light grey bars) at seven nuclear loci. *Fum* has been excluded because of its tight linkage to *Had*. *R. mendax* is the Snyder County population from Table 2-2. The data for the *Lonicera* Fly represents the pooled allele frequencies of all populations from Table 2-2. To obtain the R. zephyria frequencies the populations from State College Old Main and Munson in Table 2-2 have been pooled. See Table 2-2 for sample sizes. The vertical bars represent 95% Confidence intervals (calculated assuming Hardy-Weinberg equilibrium, Weir 1996).

Chapter 3

REPRODUCTIVE AND ECOLOGICAL ISOLATION OF THE LONICERA FLY AND ITS PARENTS

1. Introduction

Host-choice behavior

All taxa in the *Rhagoletis pomonella* species complex are-host plant specialists, each of which attacks the fruit of only a limited number of host species. In this species complex, hosts are used exclusively by only one taxon (Berlocher 2000). Like other specialist herbivores, *Rhagoletis* species have evolved behaviors and sensory adaptations that allow them to find their specific host plants. The ability to discriminate among suitable and unsuitable hosts is critical for specialized insects because these species often possess physiological or life-history adaptations for their preferred host (Filchak et al. 2000). Choosing the wrong host can result in the loss of fitness (Bierbaum and Bush 1990; Rausher 1993). In *Rhagoletis*, host choice is also central to reproductive isolation among the taxa because mating takes place on the host plant (Prokopy et al. 1971; Smith and Prokopy 1980; Smith and Prokopy 1982). It is, therefore, assumed that host choice equals mate choice in the *Rhagoletis pomonella* species complex. This combination results in an epistatic interaction between traits for host-specific fitness and traits for assortative mating that greatly facilitates sympatric speciation (Diehl and Bush 1989; Gavrilets 2003). The behaviors leading to host choice are responsible for this interaction.

The study of host-choice behavior is therefore essential for understanding sympatric speciation in *Rhagoletis* and other host-plant-specific herbivores that mate on the host plant (e.g., Craig et al. 1993).

The *Lonicera* Fly represents a population that was formed by hybridization between two specialized insects. This population of hybrid origin now occupies a novel host plant that has not been previously used by the parental species (see chapter 2). In the previous chapter, I concluded that the *Lonicera* Fly represents an independent species of hybrid origin. Because the *Lonicera* Fly originated by a shift to a new host plant, I suggested that it could have formed by sympatric speciation. In this chapter, I will test the assumption of ecological independence of the *Lonicera* Fly from its parent species by behavioral experiments that study female host choice in the *Lonicera* Fly and its parent species. If I assume that matings in the *Lonicera* Fly system take place only on the host plant (as with other members of the complex), then testing the host-choice behavior will also provide a measure of the reproductive isolation between the *Lonicera* Fly and its parents. I therefore hypothesize that:

1.a. Each fly taxon shows greater preference (or acceptance) for its own host fruit species than for host fruits of other taxa.

In the previous chapter I proposed a mechanism by which hybridization between differentially adapted taxa could facilitate sympatric speciation via host shift. The driving force of sympatric speciation is disruptive selection that acts on populations on different hosts. The greater the selection differential between the new and the old host, the greater the chance of successful speciation (Gavrilets 2003). Specialist taxa that shift to a

resource that requires different adaptations are therefore likely candidates for sympatric speciation (Diehl and Bush 1989). Once populations on different hosts have become reproductively isolated, selection will eliminate mechanisms of host adaptation that are no longer needed on the new host, but that are costly to maintain. We are thus confronted with a paradox: Specialization is a necessary condition for successful sympatric speciation, but at the same time, the evolution of specialization will eliminate the necessary variation for the next host shift (Kondrashov and Mina 1986).

Introgressive hybridization between two different specialist species, as observed in the *Lonicera* Fly, is a mechanism for rapid generation of a large number of new phenotypes that have not been observed in either of the parent taxa (Arnold 1997; Barton 2001; Rieseberg et al. 1999). Among these new phenotypes could be some that represent a pre-adaptation to a new resource like *Lonicera*. These individuals would become the initial colonizers of the new host and could subsequently become reproductively isolated from their parent taxa.

This mechanism represents the mirror image to the hybrid bridge hypothesis proposed by Floate and Whitham (1993). In their scenario, the phenotypic gap between an herbivore‘s ancestral and potential new host is bridged by intermediate plant phenotypes that are the result of hybridization between the hosts (e.g., in a plant hybrid zone). Instead of colonizing the new host taxon directly, the herbivores would undergo a series of shifts to more similar forms, which would allow for gradual adaptation to the new host-plant species.

As a reference to Floate and Whitham's hypothesis, I will term the facilitation of a host shift to and the aquisition of new hosts by herbivore hybridization the "herbivore hybrid bridge model." In this study I will test whether the host-choice behavior of flies in the *Lonicera* Fly system is consistent with a herbivore hybrid bridge model. Two hypotheses can be addressed by conducting behavioral experiments on host choice:

2.a. The Lonicera Fly is less discriminant in its host choice (or, conversely, accepts a greater spectrum of hosts) than its parent taxa. This hypothesis is based on the expectation that introgressive hybridzation will produce hybrid swarms with a variety of host-choice phenotypes.

2.b. Lonicera flies will discriminate less against Lonicera (or more readily accept Lonicera as a host) than will the parental taxa. Hypothesis 2.b. is based on the expectation that the parental taxa will be specialized for finding their own host fruit species and will reject *Lonicera*, whereas some of the hybrid phenotypes will be able to shift hosts.

Not all possible outcomes of the host-choice experiments will be informative for rejecting or accepting these hypotheses. The *Lonicera* Fly could represent a fully isolated and specialized species. In that case, it could have evolved according to the herbivore hybrid bridge model but now be no more variable in its host choice than are its parent species.

Hypothesis 2.b. can also be framed by asking how the *Lonicera* Fly arose. Testing this question will help answer the question of which came first: host shift or hybridization? Under the herbivore hybrid bridge model, hybridization had to precede a

host shift. If the parental taxa do not discriminate against *Lonicera*, then they could have been pre-adapted for choosing *Lonicera* fruit as a host. In this case I cannot exclude that hybridization is the consequence of a host shift. As stated above, host fidelity acts as a mechanism of reproductive isolation in *Rhagoletis* (Prokopy et al. 1971; Smith and Prokopy 1980). If *R. mendax* and *R. zephyria* colonize the same host, i.e. *Lonicera*, then this mechanism of reproductive isolation no longer exists. Unless there are host-independent mechanisms of assortative mating (see below and Smith 1986), *R. mendax* and *R. zephyria* could mate freely on the new host. This would also be the case if both parents met on one of the two parental hosts due to an error in host choice. Whether hybridization preceded a host shift or vice versa depends on which error is more likely to occur – the choice of *Lonicera* or the choice of the other parent's host fruit.

Previous studies have elucidated much of the of the host-choice behavior in *Rhagoletis* (reviewed in Prokopy and Papaj 2000). Most studies have concentrated on *R. pomonella*, and some work has also been conducted on *R. mendax* (Diehl 1986) and the European cherry fruit fly, *R. cerasi* (Boller et al. 1998). Much less is known about *R. zephyria*, but based on the great similarity between *R. pomonella* and *R. mendax*, it appears that the behavior of host choice is conserved in the *Rhagoletis pomonella* species complex. This brief review is limited to female behavior since I did not consider fruit preference of male flies in this study, but male preference for host plants is also of importance for the evolution of host specific taxa (Abrahamson and Weis 1997; Prokopy et al. 1988). Host plants are thought to be located by host fruit odors (Linn et al. 2003) that can be detected from at least 20 m in *R. pomonella* (Prokopy and Papaj 2000). Fruit

odors have been shown to act as cues for discrimination between different host fruit species. *R. pomonella* and *R. mendax* show differences in their antennal sensory response to hawthorn and blueberry (Frey et al. 1992), and the host races of *R. pomonella* can distinguish between apple and hawthorn volatiles (Linn et al. 2003). *Rhagoletis pomonella* can also recognize host trees by visual characteristics (Green et al. 1994). Once inside the architecture of a host plant, flies will use both visual and olfactory stimuli (Aluja and Prokopy 1993), and it has been shown that *Rhagoletis* species are attracted to spherical shapes (reviewed by Prokopy and Papaj 2000). Once on the fruit, flies will walk on the fruit surface, and it is thought that they use their tarsal receptors, antennae and mouthparts to assess fruit quality based on chemical and physical characteristics (reviewed by Prokopy and Papaj 2000).

Another important behavioral element to test fruit quality is the probing of the fruit with the ovipositor. By probing, flies receive information about the chemical and physical properties of the fruit flesh (reviewed by Prokopy and Papaj 2000). In the literature, fruit "acceptance" is often equated with the oviposition of females into the fruit following ovipositor probing (e.g., Prokopy et al. 1988), but one study has also shown that *R. pomonella* and *R. mendax* differ in the intensity of search behaviors when tested on their own or the other species' host fruit (Prokopy and Papaj 2000). Following oviposition, females will mark the fruit surface with oviposition-deterring pheromone. This pheromone will deter oviposition both by the individual that deposited it and by conspecific females (Prokopy 1972). In the only study that compared behavioral traits across more than two taxa in the *R. pomonella* species complex, Prokopy et al. (1976) and Averill and Prokopy (reviewed in Prokopy and Papaj 2000) showed that *R.*

pomonella, *R. mendax*, *R. zephyria*, and – to a lesser extent – *R. cornivora* will respond to heterospecific marking pheromones.

In this study I conducted two different behavioral experiments:

1. A no-choice test that tested host fruit *acceptance*. In this experiment I defined acceptance qualitatively if a fly used her ovipositor to probe or mark (= oviposition) a fruit. I also used the length of the initial search interval as a quantitative measure of acceptance. In the no-choice experiment, I tested the three taxa of the *Lonicera* Fly system and their respective host fruit species. I also included *R. pomonella* and its native host fruit hawthorn as an "outgroup" to assess whether *R. mendax* or *R. zephyria* differed in their behavioral response to *Lonicera* fruit from a closely related species that is not a parent of the *Lonicera* Fly.

2. A three-way choice experiment in which I tested fruit *choice*. As a qualitative measure of choice, I recorded which host fruit species was first probed and marked in an array containing three hosts simultaneously. The quantitative measure of choice in this experiment was the time spent "searching" (defined below) on each of the three host fruit species. Only the *Lonicera* Fly, *R. mendax* and *R. zephyria* and their respective host fruit species were used in the choice experiment.

Host-independent assortative mating

The question of host-independent assortative mating is of great interest in the *Lonicera* Fly system. As discussed above, host fidelity will no longer represent a reproductive barrier if both *R. mendax* and *R. zephyria* individuals switch to *Lonicera*

simultaneously. Do prezygotic behavioral barriers exist that could handicap the formation of hybrids once individuals from different species are on the same host? The same question arises if individuals of either of the two parental species commit an "error" in host choice and attack the host fruit of the other parental species.

While much research has focused on the behavior of host preference in the *Rhagoletis pomonella* species group (Prokopy and Papaj 2000), little research has been devoted to the mating behavior following the initial series of papers by Prokopy (1975), Prokopy et al. (Prokopy et al. 1972; Prokopy et al. 1971), Prokopy and Bush (1972; 1973a), Smith and Prokopy (1980; 1982), and Smith (1986). Some work has been devoted more recently to mating behavior in *R. cerasi* (Jaastad 1998a; Jaastad 1998b) and the role of male sex pheromones in that species (Katsoyannos et al. 1987; Raptopoulos et al. 1995). The most detailed studies on mating behavior in *Rhagoletis* have been conducted for species of the *Rhagoletis suavis* group (reviewed by Prokopy and Papaj 2000). But these results are only of limited value for understanding mating behavior of the *Rhagoletis pomonella* species complex because the *suavis* group has evolved a different mating system (Prokopy and Papaj 2000). Questions of assortative mating among host-specific *Rhagoletis* taxa have received even less attention, even though a theoretical model by (Johnson et al. 1996) predicts that assortative mating in conjunction with host fidelity facilitates the completion of reproductive isolation during sympatric speciation. The only study on assortative mating between taxa of the *Rhagoletis pomonella* species group was conducted by Smith (1986). In a no-choice experiment he tested the propensity of the *Cornus florida* fly (Berlocher 1999), *R. mendax* and *R.*

cornivora to engage in matings with *R. pomonella*. *Cornus florida* flies accepted *R. pomonella* partners to the same extent as conspecifics, but the mating propensity of *R. mendax* was reduced by half and that of *R. cornivora* by ca. 80% when presented with *R. pomonella* instead of conspecific partners (Smith 1986).

Here I present the results of a mate-choice experiment. In this experiment *R. mendax* and *R. zephyria* individuals had the opportunity to choose between conspecific and heterospecific mating partners in the absence of host-plant cues.

2. Materials and Methods

Fly populations

General rearing procedure

I collected individuals of all four taxa studied, *R.mendax*, *R. zephyria*, *R. pomonella*, and the *Lonicera* Fly, in the summer and early fall of 2000 and 2002 by picking infested host fruit (see Table 3-1 for host-plant sampling locations and sampling dates). In the laboratory, mature larvae emerged from the infested fruit and pupated in moist vermiculite. I removed the pupae from the vermiculite by sieving and placed all samples in cold storage at 4°C in the fall, where they spent at least 5 months in diapause. After completion of diapause in the spring of the following year, I incubated the pupae at 22°C to induce eclosion. I staged the incubation and transferred sub-samples at different times to the higher temperature to ensure a constant supply of individuals for the experimental period during the summer. Once eclosion started, I separated the newly eclosed flies by

sex to obtain virgin flies for the mate-choice experiments and to control the mating status in the fruit-acceptance and -choice tests (Opp and Prokopy 1986). I transferred the individuals to 1.6 l rectangular plastic culture cages (similar to a design described in Katsoyannos 1975) where the flies received water and diet (one part yeast hydrolysate and four parts brown sugar). To control for the age of the flies, I separated them into cohorts of individuals that had eclosed during the same week. I kept male and female flies in two separate climate chambers (22°C and 6/18 dark/light photoperiod) to avoid potential habituation to semiochemicals of the other sex. All cages were washed with soap and rinsed thoroughly with water before reuse.

Host choice and acceptance experiments

Flies were treated in identical fashion for both host-choice and acceptance experiments. Females had to reach a maturation time of at least two weeks before I used them in a fruit acceptance or choice experiment. Because I tested each cohort of flies only during one week of experiments, individuals were between 14 - 28 days old at the time of the behavioral assay. I controlled the mating status of the experimental individuals, because mating status influences the propensity for oviposition (Opp and Prokopy 1986). The females were mated in mass crosses with males of the same age. I set up the mating cages one week before the flies entered a host-acceptance or choice experiment. The crosses contained females and males at a ratio of 2:1; in most cases I used 30 females and 15 males. I transferred the flies to a clean cage one day before I used them in a behavioral assay to avoid over-stimulation of tarsal and other receptors by chemical stimuli left by the flies on the cage wall (Gienapp and Heubel personal communication).

Mate-choice experiments

For the mate-choice experiments, I used virgin males and females that were kept

under the same general rearing conditions. Because the mate-choice experiments required many individuals and the supply of flies was limited, I did not control for the age of the males and females that I tested. Males and females ranged between 7 – 35 days of age at the time of the experiment.

Host-choice and acceptance experiments

Outline of experiments

I conducted two different experiments to test for the host specificity of the *Lonicera* Fly, its parent taxa and *R. pomonella* (the latter species was used only in the no-choice experiment).

No-choice host acceptance

In this experiment I released a single female on an array of fourteen fruits of the same host fruit species. This experiment tested the acceptance of four different host fruit species (blueberry, *Lonicera*, snowberry and hawthorn) by four different fly taxa (*R. mendax*, *Lonicera* Fly, *R. zephyria* and *R. pomonella*) in the absence of choice among host fruit species. For this experiment I used only unripe, green snowberry fruit. I tested 14 of the 16 possible fly/fruit combinations (see Table 3-1). The two combinations not included in this study are *R. mendax* on hawthorn and *R. pomonella* on blueberry.

Three-way host choice

In this experiment I tested a single female on an array that consisted of two blueberry, two *Lonicera*, and two snowberry fruits. This experiment tested the host choice of individual *R. mendax*, *Lonicera* Fly and *R. zephyria* females when simultaneously confronted with the three host fruit species of the *Lonicera* Fly system. In the three-way choice experiment, I used white snowberries, except for a second set of

replicates for *R. zephyria* in which the choice consisted of green snowberry, *Lonicera* and blueberry.

Host fruits

I used fruit from the same sources for both the acceptance and host-choice experiments. I collected all host fruit for our experiments at locations in Centre County, Pennsylvania, U.S.A. I collected highbush blueberry fruit, *Vaccinium corymbosum* – one of the hosts of *R. mendax* – from two locations: Bear Meadows Natural Area (Rothrock State Forest) and from cultivated plants in Port Matilda, PA. I obtained *R. zephyria*'s host, snowberry, *Symphoricarpos albus*, from a site at Old Main lawn on the Penn State campus and from a newly established site in Port Matilda, PA. I collected *Lonicera* fruit from plants along the Penn State golf course. The native host of *R. pomonella* – hawthorn, *Crataegus* spp., fruit – was collected from a tree on the Penn State campus (Wagner Building). In the case of the blueberry fruit, I found no evidence for an infestation based on traps and the visual inspection of the fruit. The infestation of the Port Matilda snowberries was so low (1 larva found by visual inspection in five years of observation) that the risk of picking an infested fruit for my experiments was unlikely. All other collection sites for experimental fruit supported heavy infestations by *Rhagoletis*, and I covered the fruit with mesh bags on the host plant before fly emergence in the field. Thereby I prevented oviposition by wild flies in the experimental fruit that could have altered fruit quality through the presence of a larva inside a fruit or oviposition deterring pheromone on the fruit surface (Prokopy 1972).

Shortly before starting a set of experiments, I picked fruit with latex gloves and transferred it into a plastic bag. In order to determine the suitable fruit stage for

oviposition by the flies, I conducted a preliminary study of fly and host-plant phenology (see Appendix B). In the case of *Lonicera* fruit, flies were only present when the fruit was fully ripened. On hawthorn I observed the peak in trap catches while red fruit was starting to soften (Figure Appendix B-1). I therefore used the fruit stages that coincide with maximum fly activity in the field for my laboratory experiments. Because snowberries continually fruit, different fruit stages co-occurred during the time when *R. zephyria* was caught on traps. During field observations of female flies, I found that females will oviposit in both green (diameter > 4 mm) and white (diameter ca. 10 mm) snowberry fruit; therefore, both types of fruit were used in the experiments. The densities of *R. mendax* populations in the vicinity of the Pennsylvania State University are extremely low and flies could not be detected by traps. A previous study had shown that *R. mendax* will oviposit into ripening (turning from green to blue) blueberries in the field (Lathorp and Nickels 1932), but my preliminary experiments showed that *R. mendax* showed a stronger response to freshly ripened, blue and soft fruit in the laboratory. This latter fruit stage was used in all reported experiments.

Observed behaviors

I recorded the behavior that the females displayed in response to fruit with event recording software (The Observer, Noldus, Wageningen, Netherlands). The program generates time event tables that specify when and for how long a predefined behavioral state was observed. The behavioral states that I recorded in the host-acceptance and host-choice experiments were the same and fall into three major categories: General, Search and Oviposition behavior (Figure 3-1). For statistical analysis, I considered only behaviors that the fly performed while on fruit. All other behavior in the experimental

arena I recorded as "off fruit" during the observations (Figure 3-1).

General behaviors include general cleaning, proboscis use, the active state (fly stationary but wings are tilted 45° and lifted over the abdomen), and resting (fly stationary but wings folded on the abdomen).

I classified two behavioral types as **search behavior** (underlined in Figure 3-1). One is searching in which the fly walks over the fruit with its wings tilted 45° and lifted above the abdomen. The other search behavior is ovipositor cleaning, during which the female extends its ovipositor and cleans it with its hind legs.

Oviposition behavior (bold type in Figure 3-1) falls into two distinct behaviors. The first is ovipositor probing during which the female inserts her ovipositor into the fruit. This behavior is part of assessing the fruit quality for oviposition and does not necessarily result in oviposition (Prokopy and Papaj 2000). However, if ovipositor probing is followed by the second behavioral type, marking, the female laid an egg in the host fruit (Prokopy 1972). While marking, the female walks swiftly around the fruit with its everted ovipositor touching the surface and applies oviposition-deterring pheromone that serves as a signal that a specific fruit has received an egg.

Measures of behavioral response and statistical analysis

No-choice fruit acceptance test

The first measure of fruit acceptance is the proportion of females that probed or marked a specific host fruit species at least once during an observation. In the no-choice experiment, each observation is started with "searching" as the default behavioral state (Figure 3-1). I used this first search interval as my second measure of fruit acceptance. I

subdivided the tested individuals into females that searched for less than 5 seconds and females that searched for 5 seconds and longer. This time interval matches the time it took me to start the observation with the event recording software and to obtain accurate recordings of female behavior. I analyzed the counts that I obtained from both measures of fruit acceptance in a contingency table and used a G-test (Rohlf and Slice 1996) to test for differences in the acceptance by a fly taxon of the different fruits. To further explore the results I constructed 2 x 2 contingency tables to compare a fly taxon's response to two different host fruit species or to compare the response of two different fly taxa to the same host fruit species. Strictly speaking, such testing violates proper statistical procedure as it tests hypotheses that I generated after studying the data (Zar 1999). I will therefore refer to this additional analysis as "exploratory" testing.

To survey the data for differences in behavior if individuals accepted a fruit for ovipositor probing, I formed a subset of individuals that had shown ovipositor probing. To avoid the potential confounding influence of multiple ovipositions, I only analyzed the behavior that I observed until the first ovipositor probing event. I further deleted the first search interval from each data file if it was shorter than 5 seconds or deleted the first 5 seconds if the initial search interval was longer than 5 seconds to eliminate any inaccuracy in recording the start of the experiment. The remaining observational information was then converted into three different measures: (1) the total waiting time on fruit until the first ovipositor probing, (2) the mean duration per event during this interval, and (3) the proportion of the total waiting time that a female spent searching or cleaning her ovipositor. The second measure represents a surrogate for the activity level of the females. I analyzed these three measures for statistical significance using Tukey's

multicomparison test (Rohlf and Slice 1996) or a two-sample t-test (Minitab version 12.23) where applicable.

Three-way host choice test

In the three-way choice experiments, I also analyzed both oviposition and search behavior. In the choice experiments I only considered the behavior until the first marking of a fruit. Females will avoid marked fruit (Prokopy 1972), which leads to a skewed ratio of the different host fruit types after the first fruit has been chosen for oviposition. If no fruit was marked during a replicate, I used the entire duration of the observation for data analysis. The first measure I used was the number of females from each fly taxon that probed or marked either blueberry, *Lonicera* fruit or snowberry. I defined an ovipositor probing as all consecutive recordings of fruit probing – without marking – on the same host fruit species. The female could change to other behaviors between the individual probes, but as long as the female did not mark this fruit type or switch to a different host fruit species I considered all probes to be part of the same ovipositor probing bout. I did not distinguish between individual fruits, but only between host fruit species in the behavioral recordings. A single ovipositor probing bout could have therefore included ovipositor probings on two different individual fruits of the same host fruit species. Almost all females probed only one host fruit species before marking it or the observation period ended. I therefore compared the observed number of first ovipositor probings of the three different host fruit species with the expected number of ovipositor probings under the null hypothesis that females are equally likely to probe each host fruit species first (log-likelihood ratio test for goodness of fit, Zar 1999). The same test was conducted for the first marking events. My second measure of host fruit choice was the total time spent searching or ovipositor cleaning on a specific host fruit species until the first mark

or the end of the experiment. I analyzed these data with Tukey's multicomparison test (Rohlf and Slice 1996)

Experimental procedures

No-choice fruit acceptance test

The experimental arena consisted of a glass plate (220 x 220mm) with 14 holes (3mm diameter) arranged in a hexagonal fashion with each hole being equidistant (30 mm) from its six neighboring holes. I inserted fruits of a single host species with the peduncle-side into each hole. By giving each female fly the choice among several fruits, I controlled for differences in individual fruit quality. The glass plate with the fruits was contained in a metal screen cage (300 x 300mm) that was closed on the observer side with clear plastic wrap. I provided light by six fluorescent plant lights (P40PL/AQ 40W, General Electrics, Louisville) and two greenhouse lights (Lucalox 400W high pressure sodium bulbs, General Electrics, Louisville). The center of the glass plate received 4000 lux of light and the temperature at this point was 31°C with the lights on. I moved the cages with the mass crosses out of the climate chamber to the experimental area before the start of the experiment to acclimate them to the conditions of the arena. I performed all experiments between the 7^{th} and 11^{th} hour of the insects' photoperiod (this has been shown to be the period of highest activity in *R. cerasi*, Katsoyannos et al. 1980).

To start the experiment I removed a fly from the culture cage with an aspirator and held the opening of the aspirator in front of a host fruit in the center of the arena. Once the fly walked out of the aspirator onto a fruit and "recognized" the fruit, the experiment started with "search" as the default state (Figure 3-1). According to my definition, a fly "recognized" a fruit if it spent enough time on the fruit to allow us to start the observation

with the event recording software (3-5 s). This definition excludes instances in which the fly just used the fruit as a stepping-stone for flying off to the cage wall. If a fly did not respond with fruit recognition for three consecutive trials, we placed it back in the culture cage to be used in another replicate. I assumed that uncooperative, naïve flies, even though they spent a brief amount of time in the experimental arena, did not acquire experience with fruit that could influence their behavior in a successful replicate (Prokopy et al. 1982). For this latter reason, I excluded flies from any other experiments after they had completed a successful replicate and froze them at –80 C for future genetic analysis. I observed each female for a maximum of 15 min and recorded its behavior (see above). I terminated the observation before the maximum time if the female flew to the cage wall or walked off the glass plate (Figure 3-1). If a fly probed or marked a fruit with its ovipositor, I replaced this fruit after the experiment using new latex gloves to prevent contamination with oviposition-deterring pheromone (Prokopy 1972). After five replicates, I replaced all fruits in the arena and cleaned the glass plate with soap and ethanol and rinsed it with water before reusing it. The fly taxa were alternated during each day of experiments in order to correct for possible differences in environmental conditions or host fruit quality.

Three-way host choice test

For the choice experiments I used a closed rather than an open experimental arena, because preliminary trials and our experience from the no-choice experiments had shown that only a relatively small proportion of flies would search the whole array. The closed arena consisted of a polysterene Petri dish (15 x 100 mm). I cut six holes (diameter 5 mm), which formed the vertices of a hexagon with the centers of the holes 30 mm apart, into the bottom of the dish. Two fruits each of three different host fruit species were

placed in alternating order in the depressions formed by the holes. To avoid effects of fruit order, I changed the sequence of the fruits every five experiments. In addition, I also rotated the dish 45° before each trial to randomize any effects of lighting or temperature in the experimental area. To start a replicate I introduced a mated, naïve female (see above) through a hole in the lid of the Petri dish that represented the center of the hexagon formed by the experimental fruit (Figure 3-1). I recorded the behavior of the females (see above) for a period of 15 minutes. Due to limitations on fly material, individuals that did not touch fruit during the experimental period were re-used in a three-way choice experiment after a rest period of at least one day. Females from successful observations were frozen for future genetic analysis. I conducted the experiments during the 7^{th}-11^{th} hour of their 16-hour photoperiod as in the no-choice acceptance test.

Host independent assortative mating

Summary of Experiment

The experiment tested whether *R. mendax* and *R. zephyria* flies show a preference for conspecific mating partners in a choice situation. I combined males and females of *R. mendax* and *R. zephyria* in a mating cage and recorded the frequency of the four possible mating combinations. The *Lonicera* Fly could not be tested in the mate choice experiments because there were not enough individuals available.

Experimental Procedure

For the mate-choice experiments I converted standard rearing cages described above into an experimental arena by inserting three additional holes for the easy removal of

flies. Like the rearing cages, the arena contained water, food, and two plastic leaves. The experiments were conducted with 10 females and 20 males each of *R. mendax* and *R. zephyria* in the experimental cage. Individuals of the two species were distinguished by a colored mark of enamel paint (Testors) that had been applied at least one day prior to the experiment with a small paintbrush. *Rhagoletis mendax* individuals received a blue and *R. zephyria* flies a red mark. This procedure did not appear to handicap the flies or alter their behavior. I first introduced the female flies to the cage and allowed them to acclimate for some time before starting the observation by adding the male flies, thus ensuring that matings did not result from forced copulations with freshly introduced, "disoriented" females. I surveyed the cages at least every 10 minutes for copulating pairs. Mating pairs will stay in copula for at least 20 min. (Smith and Prokopy 1982), making it unlikely that successful copulations between individuals were missed. I also recorded unsuccessful mating attempts that consisted of events in which males appeared to try aggressively force copulation against the visible resistance of a female. During my observations, none of these unsuccessful mating attempts resulted in successful copulation. Successful mating pairs were removed from the experimental arena, and their live mass was determined with a precision balance.

No mated individuals were reused in another experiment, but were frozen for future genetic analysis. Due to limited fly material, I reused unmated individuals on consecutive days. Because of this procedure I could not obtain the mass of all unmated individuals because it would have been impossible to determine whether a single individual had been weighed repeatedly. Instead I used all unmated flies that were left after a single day of observation as a sample for determining the mass of individuals without mating success.

Experimental sessions lasted 2-3 hours and were conducted during the 7^{th}-11^{th} hour of the 16-hour light phase of the flies' photoperiod (Katsoyannos et al. 1980). I compared the observed number of mating events between conspecific and heterospecific males and females with the expected mating frequencies under the null hypothesis of random mating between the two taxa (log-likelihood ratio test for goodness of fit with Yates correction, Zar 1999). I conducted this comparison using successful matings and the sum of both successful and unsuccessful mating attempts. To test for the potentially confounding effect of sexual selection on this study of sexual isolation, I used two sample t-tests (Minitab version 12.23) to test for differences in the live body mass of mated versus unmated individuals within each sex and species. I then conducted a second set of two-sample t-tests that tested the null hypothesis that individuals of the same sex have the same live body mass in both species. For these tests I pooled both mated and unmated conspecific individuals that were of the same sex and belonged to the same species.

3. Results

No-choice host acceptance experiments

Proportion of females showing oviposition behavior

Not all females that probed fruit during a replicate also oviposited into fruit during the same observation (Figure 3-2). But there was no difference (all p-values > 0.689, G-contingency test) in the ratio of females that probed and marked versus females that probed only when a fly taxon was tested on different host fruit species. Ovipositor

probing is therefore a legitimate measure of host fruit acceptance in the no-choice host acceptance test. Due to the larger sample sizes, I used ovipositor probing as a surrogate of oviposition in the statistical analysis.

The four fly taxa displayed significant differences in the acceptance of the different host fruit species. A G test on a 2 x 3 or 2 x 4 contingency table that compared the proportions of females from one fly taxon probing three or four different host fruit species yielded p-values < 0.001 for all taxa except *R. mendax* for which the test produced $p = 0.027$ (Figure 3-2). *Rhagoletis mendax* (Figure 3-2A) accepted blueberry and *Lonicera* fruit equally, but avoided snowberry fruit. The proportion of *R. zephyria* females that oviposited in *Lonicera* and snowberry fruit is equal ($p = 0.31$, G-contingency test, Yates correction), whereas there was no probing in blueberry or hawthorn (Figure 3-2C). *Rhagoletis pomonella* (Figure 3-2D) probed only hawthorn fruit, displaying no oviposition behavior on *Lonicera* or snowberry fruit. The *Lonicera* Fly (Figure 3-2B) probed *Lonicera* fruit, snowberry fruit and hawthorn, but showed no oviposition behavior on blueberry in the no-choice host acceptance test. The significant differences in the host fruit acceptance of the *Lonicera* Fly were caused not only by the absence of oviposition in blueberry (Figure 3-2B) but also by also by a higher level of *Lonicera* fruit acceptance when compared to the acceptance of snowberry. An exploratory direct comparison of *Lonicera* fruit acceptance to snowberry and hawthorn fruit acceptance by the *Lonicera* Fly (Figure 3-2B) in a 2 x 2 G-contingency test showed significant differences in *Lonicera* vs. snowberry fruit acceptance ($p = 0.003$, Yates correction applied) but no significant differences for *Lonicera* vs. hawthorn fruit acceptance ($p = 0.059$ for, Yates correction applied). The level of snowberry fruit acceptance by the *Lonicera* Fly (Figure

3-2B) was so low that it is not significantly different from the lack of ovipositor probing in 44 *Lonicera* Fly females that were tested on blueberry fruit ($p = 0.126$, see Table 3-2 for sample sizes).

Length of initial search interval

The three described *Rhagoletis* species (Figures 3-3A, C, D) all showed significant differences in the length of the first search interval on the different fruit types, with each taxon favoring their natal host. The *Lonicera* Fly (Figure 3-3B) failed to discriminates among hosts using this metric, which consists of the proportion of females with a long (equal to or longer than five seconds) initial search interval (see Material and Methods).

The proportion of *R. mendax* females with a long initial search interval (Figure 3-3A) was three times greater on blueberry than on either *Lonicera* or snowberry fruit. Most *R. zephyria* females performed long initial searches on snowberry (Figure 3-3C), while only 18 % of *R. zephyria* females on blueberry searched equal to or longer than 5 seconds during their initial search. The proportion of long initial searches by *R. zephyria* on *Lonicera* and hawthorn fruit was intermediate to the proportion of long initial searches I observed for this fly taxon on snowberry and blueberry fruit (Figure 3-2C). The exploratory analysis of differences between the proportion of long initial searches by *R. zephyria* on snowberry and *Lonicera* fruit was significant ($p = 0.015$, Yates correction). The same was true for the comparison of *R. zephyria*'s response to snowberry and hawthorn fruit ($p = 0.005$, Yates correction). The differences between the proportion of long initial searches by *R. zephyria* on either *Lonicera* and hawthorn and blueberry fruit was not significant (*Lonicera* – blueberry: $p = 0.053$ and hawthorn – blueberry: $p =$

0.057, Yates correction). I observed the highest proportion of long initial search intervals in *R. pomonella* females when I tested this fly taxon on hawthorn, its native host fruit species. *R. pomonella* females showed the lowest proportion of long initial search intervals when I exposed them to *Lonicera* fruit. The proportion of long initial searches in the *R. pomonella*/snowberry fruit combination was intermediate to the response of *R. pomonella* females towards hawthorn and *Lonicera* fruit. An exploratory analysis of the pairwise differences between the proportion of long initial searches on snowberry versus hawthorn and the proportion of long initial search intervals on snowberry versus *Lonicera* failed to show significant differences (Figure 3-2D).

As mentioned above, there were no significant differences in the proportions of long initial search intervals by *Lonicera* Fly females that I tested on different fruits (Figure 3-2B). I failed, however, to reject the null hypothesis of equal proportion of long search intervals on all four different host fruit species by only a small margin ($p = 0.077$, Figure 3-2B). While the *Lonicera* Fly showed similar proportions of long initial search intervals on *Lonicera* and snowberry fruit (ca. 60%), the proportion of long initial searches on blueberry and hawthorn was only 40% of all tested individuals (Figure 3-2B). The *Lonicera* Fly's response to blueberry of ca. 40% (Figure 3-2B) was higher than both the proportion of long initial searches by *R. mendax* on snowberry (Figure 3-2A) and *R. zephyria* on blueberry (Figure 3-2C), but neither of these two cross-species comparisons showed significant differences in an exploratory analysis ($p = 0.123$ and $p = 0.052$ respectively).

Differences in behavior preceding the first ovipositor probing

For this analysis, I considered only individuals that probed fruit. I organized the panels in Figure 3-4 by host plant. This helped me to examine whether individuals of different fly taxa behaved differently on the same host fruit species, even though all analyzed females accepted the host fruit species for ovipositor probing. In some cases, the number of females that displayed ovipositor probing on a particular host is small (Table 3-2). Only two *Lonicera* Fly females probed snowberry fruit, and, in this case, statistical significance should be interpreted with caution. Each row of panels in Figure 3-4 (marked A, B, and C) represents one of the three measures that I used to compare female behavior before the first ovipositor probing.

Total waiting time until first ovipositor probing (Figure 3-4A)

There were no differences between *Lonicera* Fly and *R. zephyria* on snowberry fruit or between *Lonicera* Fly and *R. pomonella* on hawthorn fruit (Figure 3-4A, two-sample t-test). On *Lonicera* fruit, *R. mendax* females spent significantly more time on the fruit until the first probe than did *R. zephyria* individuals. While the *Lonicera* Fly's waiting time was not statistically different from that of either parent, it was qualitatively more similar to that of *R. zephyria* (Figure 3-4A, Tukey's test for multiple comparisons). A comparison of *R. mendax* waiting time until first ovipositor probing on blueberry and *Lonicera* fruit showed no significant differences (Figure 3-4A, two-sample t-test)

Search behavior as a proportion of total waiting time until first probe (Figure 3-4B)

As for total waiting time until the first probing event, there were no differences in searching behavior as a proportion of the total waiting time on either snowberry or hawthorn fruit among females that eventually accepted these fruit (Figure 3-4B). On

Lonicera fruit, I again observed significant differences between *R. mendax* and *R. zephyria* (Figure 3-4B, Tukey's test for multiple comparisons). *Rhagoletis zephyria* individuals spent a higher proportion of the time until ovipositor probing in searching and cleaning their ovipositor than did *R. mendax* flies. The proportion of the total waiting time until the first probe spent by *Lonicera* Fly females on search behavior was similar to that of *R. zephyria*, but is not significantly different from *R. mendax* (Figure 3-4B, Tukey's test for multiple comparisons). There was no statistically significant difference between the proportion of search behavior of *R. mendax* on *Lonicera* and blueberry fruit (Figure 3-4B, two sample t-test).

Mean time per event during the total waiting time until the first ovipsitor probe (Figure 3-4C)

The mean time per event presented the same pattern as was seen in the analysis of total waiting time until the first ovipositor probing. There were no statistically significant differences between *Lonicera* Fly and *R. zephyria* on snowberry fruit or between *Lonicera* Fly and *R. pomonella* on hawthorn fruit (Figure 3-4C, two-sample t-test). I used mean time per event as a surrogate for the activity of female flies before ovipositor probing (see Materials and Methods). On *Lonicera* fruit, both *Lonicera* Fly and *R. zephyria* spent significantly less time during each behavioral event than *R. mendax* (Figure 3-4C, Tukey's test for multiple comparisons). But the average length of each behavioral event was not different when *R. mendax* females were tested on blueberries or *Lonicera* fruit (Figure 3-4C, two-sample t-test).

Behavioral patterns of *R. mendax* on blueberry and *Lonicera* fruit (Figure 3-4A, B and C)

Rhagoletis mendax differed in its behavioral pattern before ovipositor probing from both *R. zephyria* and the *Lonicera* Fly. Was the different behavior of *R. mendax* a specific response to *Lonicera* fruit or was the same pattern also observed on its natal host blueberry? An exploratory comparison could not detect statistically significant differences between *R. mendax* behavior on blueberry and *Lonicera* fruit for any of the three metrics (two-sample t-test).

Three-way host choice experiment

Ovipositor probing and oviposition

In almost all cases, females probed a fruit only once before they either marked a fruit or the maximum observation time had expired (Figure 3-5, see Materials and Methods for definition of "probing" in the analysis of the three-way host choice experiment). Only three females probed two different host fruit species before marking or the end of the experiment. The low number of observed probing and marking events that were recorded in this experiment (Figure 3-5), were in part due to a shortage of appropriate fly material (Table 3-2).

Three-way choice tests with white snowberry fruit

Rhagoletis mendax probed all three host fruit types, but it probed its native host blueberry twice as often as other host species (Figure 3-5A). In the replicate in which a *R. mendax* female chose snowberry fruit for its first ovipositor probing, the probing event

did not happen until ca. 50 seconds before the end of the observation period. In another replicate, a *R. mendax* female chose *Lonicera* fruit for its first ovipositor probing, but the individual left the *Lonicera* fruit without marking and performed a second probing bout on blueberry fruit. *Rhagoletis mendax* females marked only blueberry fruit during the first marking event in each replicate (Figure 3-5A), and this invariant preference for their native host was statistically significant ($p = 0.012$, log-likelihood ratio test of goodness of fit). Marking of blueberry fruit occurred in the four cases, in which blueberry was the first choice for oviposition.

The *Lonicera* Fly also probed all three host fruit species, but significantly preferred *Lonicera* fruit over snowberry and blueberry fruit (Figure 3-5B, $p = 0.004$, log-likelihood ratio test of goodness of fit). In contrast to its parent species (Figures 3-5A, C and D), it marked all three host fruit species. The higher number of first markings on *Lonicera* fruit suggests that the *Lonicera* Fly also preferred its natal host for oviposition, but this effect was not statistically significant (Figure 3-5B, $p = 0.121$, log-likelihood ratio test of goodness of fit).

The number of *R. zephyria* individuals that displayed oviposition behavior when the three-way choice included white fruit was very small, so the results were merely anecdotal (Figure 3-5C). Probing by *R. zephyria* females occurred both on snowberry and *Lonicera* fruit, but the only two observed marking events were both performed on snowberry fruit (Figure 3-5C).

Three-way choice test with green snowberry fruit

Rhagoletis zephyria was the only fly taxon that was also given the choice between green snowberry, *Lonicera*, and blueberry fruit. In this case, *R. zephyria* probed both

green snowberry and *Lonicera* fruit as its first choice (Figure 3-5D). The significant deviation from random choice ($p = 0.008$, log-likelihood ratio test of goodness of fit) was caused mainly by a lack of probing in blueberry fruit (Figure 3-5D). An exploratory analysis revealed no significant difference in the number of times *R. zephyria* females would probe green snowberry versus *Lonicera* fruit ($p = 0.833$, log-likelihood ratio test of goodness of fit with Yates correction). I did not observe any marking of blueberry fruit by *R. zephyria*, and the individuals marked *Lonicera* fruit three times more often than they marked green snowberry fruit (Figure 3-5D). The significant deviation from equal choice ($p = 0.014$, log-likelihood ratio test of goodness of fit) was again caused primarily by the absence of blueberry marking in *R. zephyria* females. The direct comparison of *R. zephyria* marking response to green snowberry and *Lonicera* fruit revealed no statistically significant difference ($p = 0.562$, log-likelihood ratio test of goodness of fit with Yates correction). It is, however, noteworthy that two *R. zephyria* females, after first probing green snowberry fruit, switched to *Lonicera* fruit for subsequent ovipositor probing and marking (Figure 3-5D).

Search behavior until first oviposition

A measure of host fruit choice that considered all individuals that I tested in the three-way host choice test (Table 3-2) is the total time a female spent in search behavior on each of the three different host fruit species. I considered both searching and ovipositor cleaning as "search behavior," and the times for both behaviors were summed over the entire observation period or the time interval preceding the first marking event, whichever came first (see Materials and Methods).

Three-way choice tests with white snowberry fruit

Rhagoletis mendax spent, on average, 3-4 x more time engaged in search behavior on blueberry fruit than on either *Lonicera* or snowberry fruit (Figure 3-6A). This difference, however, was not statistically significant (Tukey's test for multiple comparisons). The *R. mendax* response to *Lonicera* and snowberry fruit was nearly identical (Figure 3-6A).

The *Lonicera* Fly showed no statistically significant differences in the total time spent searching on different fruits (Figure 3-6B, Tukey's test for multiple comparisons). The average search time on snowberry fruit was shorter than the average time that the *Lonicera* Fly spent in search behavior on blueberry or *Lonicera* fruit.

Rhagoletis zephyria clearly distinguished between *Lonicera* or snowberry fruit on the one hand and blueberry fruit on the other (Figure 3-6C). There was no difference in the time spent in search behavior on *Lonicera* and snowberry fruit. The mean time *R. zephyria* searched on blueberry fruit is ten times shorter than time spent on the same behaviors on snowberry or *Lonicera* fruit (significant in Tukey's test for multiple comparisons).

Three-way choice test with green snowberry fruit

Substitution of green (unripe) snowberry fruit for white fruit offset the previously observed pattern of *R. zephyria* search under three-way choice conditions (Figure 3-6C and D). *R. zephyria* still searched significantly longer on *Lonicera* fruit than on blueberry fruit (Figure 3-6C, Tukey's test for multiple comparisons). Its searching and ovipositor cleaning times on blueberry were, however, very similar to its response to green snowberry fruit (Figure 3-6D). The difference between total search time that *R. zephyria* spent on *Lonicera* and snowberry fruit was not significant.

Host independent assortative mating

Frequency of con- and heterospecific matings

The most conspicuous feature of the data was the complete absence of successful matings for the *R. zephyria* female x *R. mendax* male mating combination (Figure 3-7). The second major result was the smaller number of successful matings in the *R. mendax* female x *R. zephyria* male mating combination as compared to both conspecific pairings (Figure 3-7). The combined number of conspecific successful matings was nearly 4 x times higher than the number successful heterospecific matings (Figure 3-8, $p < 0.001$, log-likelihood test of goodness of fit). An alternative measure to display the observed assortative mating is to calculate the index of sexual isolation (Stalker 1942). This index takes values from -1 to 1 with -1 indicating the complete preference of males for heterospecific females, 0 no preference, and 1 the complete preference of males for conspecific females. For *R. mendax* this index equals 0.3 and for *R. zephyria* it equals 1 mirroring the observed asymmetry in interspecific matings.

The number of unsuccessful mating attempts in the *R. zephyria* female x *R. mendax* male combination was approximately equal to the number of successful matings in the *R. mendax* female x *R. zephyria* female pairing (Figure 3-7). There was a highly significant difference in the ratio of successful matings to unsuccessful mating attempts among the four possible pairings ($p < 0.001$, 2 x 4 G-contingency test, Figure 3-7). I observed the highest number unsuccessful mating attempts of all comparisons in the *R. zephyria* female x *R. mendax* male pairing (Figure 3-7). If this combination was excluded in an exploratory analysis, the significant difference in the ratio of the two mating events

disappeared (p = 0.124, 2 x 3 G-contingency test). This latter result further indicated that the lack of unsuccessful mating attempts in the *R. mendax* female x *R. zephyria* male combination (Figure 3-7) was not a statistically significant effect. The absence of unsuccessful mating attempts in this heterospecific pairing could have reflected the overall smaller total number of mating events.

The total number of mating attempts, both successful and unsuccessful, was reduced in heterospecific as compared to conspecific mating combinations Figure 3-8). Mating events between conspecific males and females were more than twice as common as mating events between heterospecific individuals of the opposing sex (p = 0.007, log-likelihood test of goodness of fit).

Influence of live body mass on mating success

There was a tendency for females successful in mating to be heavier than unsuccessful females (Figures 3-9A and B). This was the case for both *R. mendax* (Figure 3-9A) and *R. zephyria* females (Figure 3-9B), although this trend is not statistically significant in either species.

Mated *R. mendax* males (Figure 3-9C) were, on average, smaller than males that did not mate (p = 0.02, two-sample t-test). In contrast, there was no difference between mated and unmated males in *R. zephyria* (p = 0.061, two-sample t-test, Figure 3-9D).

The observed differences in mating success between individuals of different live body mass could have potentially confounded the study of sexual isolation between *R. mendax* and *R. zephyria*. This does not seem likely, however, since no significant difference in live mass was detected between females (p = 0.23, Figure 3-10A) or males

(p = 0.47, Figure 3-10B) (two-sample t-test, sample size in table 3-3) of the two species.

4. Discussion

BEHAVIORAL ISOLATION BY HOST ACCEPTANCE AND HOST PREFERENCE

I have subdivided the discussion of the results from the no-choice host acceptance and three-way host-choice experiments into three parts. These correspond to the three hypotheses that I formulated in the introduction to this chapter. The discussion of hypothesis 1.a. addresses the reciprocal behavioral isolation of the *Lonicera* Fly, *R. mendax*, *R. zephyria*, and *R. pomonella*. For hypotheses 2.a. and 2.b., I will discuss whether the results for the behavioral isolation are consistent with a herbivore hybrid bridge model.

Hypothesis 1.a.: Each fly taxon shows greater preference (or acceptance) for its own host fruit species than for host fruits of other taxa

Behavioral isolation among described *Rhagoletis* species – can my study design detect differences in host acceptance and preference?

Described species within the *Rhagoletis pomonella* species complex are distinguished by specialization on different sets of host plants (Berlocher 2000). For most of the species, host specialization is inferred indirectly from population genetic data. If two *Rhagoletis* populations, each of which occurs on a different host plant, are

distinguished by private alleles or unique combinations of alleles, they are regarded as reproductively isolated species. This, in turn, suggests that the two fly species are specialized on the host plants on which they were collected (Berlocher 2000). Extensive experimental studies on the ecological isolation among species of the *R. pomonella* species complex have been conducted only for the *R. pomonella*/*R. mendax* species pair. These experiments showed strong interspecific differences in host acceptance and host choice (Diehl 1986) and in the behavioral and electrophysiological response to chemical stimuli of the host fruit (Bierbaum and Bush 1988; Frey et al. 1992). It has been assumed, but not tested, that similar levels of ecological isolation exist between the other described species of the *R. pomonella* species complex.

Although my main objective was to examine the ecological isolation of the *Lonicera* Fly relative to closely related *Rhagoletis* species, I also collected data on the behavioral isolation of two previously unstudied species pairs in the *R. pomonella* species group: *R. mendax*/*R. zephyria* and *R. zephyria*/*R. pomonella*.

The results of the no-choice host acceptance test are consistent with the postulated ecological isolation of described species in the *R pomonella* species complex (in my study, specifically *R. pomonella*, *R. mendax* and *R. zephyria*). All described taxa accept their native host fruit species for oviposition (Figures 3-2A,B, and D). *Rhagoletis mendax* will neither probe nor mark snowberry fruit (Figure 3-2A), and *R. zephyria* does not probe or mark blueberry fruit (Figure 3-2C). The same is true for *R. zephyria* and *R. pomonella*, neither fly will accept the host fruit species of the other species for probing or marking (Figures 3-2C and D).

My second metric of host acceptance, the length of the initial search interval,

reinforces the observation that *R. mendax* and *R. zephyria* do not select the other fly's host (Figure 3-3). The proportion of *R. mendax* females conducting long initial searches was higher on blueberry than on snowberry fruit (Figure 3-3A). The converse was true for *R. zephyria* individuals on snowberry and blueberry fruit, where *R. zephyria* searched significantly longer on its own host (Figure 3-3C). In an experimental comparison of *R. zephyria* and *R. pomonella*, these flies both showed higher proportions of long initial searches on their native host than on the host of the other fly taxon (Figure 3-3C and D). For *R. pomonella*, this difference was not significant (Figure 3-3D and see Results). One possible explanation is that many *R. pomonella* females departed rapidly from snowberry, indeed, before I could start an observation. This may have biased the *R. pomonella* sample towards individuals predisposed toward snowberry. *Rhagoletis* flies have been shown to discriminate against fruit diameters that are smaller than the diameter of their preferred host fruit (Prokopy and Bush 1973b). Hawthorn fruits are much larger than green snowberry fruits, and the difference in diameter could have led to the rejection of snowberry fruit by some *R. pomonella* females before I was able to start the observation.

In conjunction with previous studies on the ecological isolation of *R. pomonella* and *R. mendax* (Diehl 1986), these results suggest a high degree of behavioral host specialization for *R. pomonella*, *R. mendax* and *R. zephyria*. This result is in accordance with the results of population genetic studies that indicate a nearly complete reproductive isolation of the three fly taxa (Berlocher 2000; Feder et al. 1999). There is evidence for oviposition by *R. pomonella* females in snowberry fruit, but such host-choice errors appear to be extremely rare in the field (Feder et al. 1999).

Under the experimental conditions of this study, *R. mendax*, *R. zephyria* and *R. pomonella* were completely isolated by their host-acceptance behavior. This indicates that my experimental design can detect host specialization as measured by this behavioral metric. It therefore is a suitable tool for studying the behavioral isolation of the *Lonicera* Fly. The response of the described *Rhagoletis* species to their native host fruit species and to the native host of other described species serves as a standard for the following discussion. It will help to answer the following questions: Does the *Lonicera* Fly behave like the described species in terms of host choice? Do the described fly species respond to *Lonicera* as if it were a "host" or a "non-host" fruit species?

No-choice host acceptance versus three-way choice experiment

In the previous section, I did not use the data from the three-way choice experiment to discuss the reciprocal behavioral isolation of the two described species *R. mendax* and *R. zephyria*. While the results of the three-way host choice experiment are suggestive of the reciprocal isolation of the parent taxa (Figures 3-5 and 3-6), it is statistically invalid to use them for a two-way comparison. *Lonicera* fruit, as a third potential choice, can influence a fly's response to the two host fruit species of the parents. This effect could, for example, underlie the lack of differentiation by *R. zephyria* between blueberry and green snowberry fruit in the three-way host choice-experiment (Figure 3-6D). Under these experimental conditions, *R. zephyria* preferred *Lonicera* fruit over both blueberry and green snowberry fruit (Figure 3-6D), but the same fly might have preferred snowberry over blueberry in the absence of *Lonicera* fruit.

In addition to this specific limitation of the three-way host-choice experiment, there are other difficulties associated with choice experiments in general. First, true choice situations like the one that I used in this study are only rarely encountered by *Rhagoletis* under natural conditions. It is more likely that females will find patches of the same host fruit species, such as entire bushes or branches of a single host plant. In that respect, the no-choice host acceptance test represents a more realistic situation. This is especially true because females can leave the patch at any time and are not forced to remain in the experimental arena.

The second problem is the potential confounding influence of individual fruit quality. Although choice experiments are conducted to test an individual's preference for different host fruit species, the actual choice that a female makes is between individual fruits that belong to different host fruit species. Insects not only distinguish among different host-plant species, but also choose among individuals of the same host (Singer and Lee 2000). Despite a general preference for one host fruit species over the other, a female could have chosen a high-quality fruit of the less preferred host fruit species over a low-quality fruit of the preferred host. In the no-choice test, individual fruit quality could still have had an influence on host fruit acceptance. However, the probability of rejection due to inferior individual quality was lowered because in the no-choice experiment females could choose among 14 different fruits of a single host species.

Another potential difficulty with my three-way choice experiment is the size and design of the experimental arena. Females use fruit volatiles as cues to discriminate between hosts (Linn et al. 2003), and individuals might become confused in the small

poorly ventilated space of the Petri dish. In a no-choice test, such "contamination" is not a problem, because only one host fruit species is tested.

In summary, there are fewer potential difficulties associated with the no-choice host acceptance test than with the three-way choice experiment. Even with these caveats, relative differences in the response of the three fly taxa to the same – albeit unnatural – array of three different host fruit species can be informative (Figures 3-5 and 3-6). Despite the potential confounding influence of the experimental design of the choice experiment, the overall results of the no-choice (Figures 3-2 and 3-3) and three-way choice approach (Figures 3-5 and 3-6), which I will discuss below, are very similar.

Is the *Lonicera* Fly ecologically isolated?

How do the described Rhagoletis species respond to Lonicera fruit?

The results of the no-choice fruit acceptance test show a strong difference in the acceptance of *Lonicera* fruit between the parent species of the *Lonicera* Fly (*R. mendax* and *R. zephyria*) and *R. pomonella*. Both *R. mendax* and *R. zephyria* will accept *Lonicera* fruit for probing and marking in proportions similar to their acceptance of their respective native hosts (Figure 3-2A and C). *Rhagoletis pomonella* in contrast, does not accept *Lonicera* for either probing or oviposition (Figure 3-2D). However, data discussing the length of the initial search interval present a different pattern. According to this metric, both *R. mendax* (Figure 3-3A) and *R. zephyria* (Figure 3-3C and Results) accept *Lonicera* fruit at significantly lower proportions than their native hosts.

It is likely that a female will integrate several factors, such as olfactory cues, contact chemoreception, visual and mechanical cues before deciding whether or not to probe a

fruit (Prokopy and Papaj 2000). The length of the initial search time might only be determined by a subset of these factors, the combined effect of which causes a difference in the proportion of long searches between *Lonicera* fruit and the native hosts of *R. mendax* and *R. zephyria*. The two metrics could therefore measure two different aspects of host-acceptance behavior. The cues a fly perceives upon initial contact could be more different between *Lonicera* and the natal hosts of *R. zephyria* and *R. mendax* than the cues a fly encounters when it spends more time on the fruit.

The sample sizes for the three-way choice experiment are too small to draw confident conclusions about the oviposition response of *R. mendax* and *R. zephyria* to *Lonicera* fruit (Figure 3-5). The results for *R. mendax* do indicate its preference for blueberry fruit in a three-way choice situation (Figure 3-5A). Whether the data in Figure 3-5C suggest a preference for white snowberry fruit over *Lonicera* fruit by *R. zephyria* is speculative. When the choice test is conducted with green snowberry fruit, *R. zephyria* does not distinguish between snowberry and *Lonicera* fruit and preferred both over blueberry fruit (Figure 3-5D). The observed numbers even suggest a preference for *Lonicera* fruit as a first choice for host marking (Figure 3-5D). The total time that a female spent engaged in searching behavior on each of the three host fruit species (Figure 3-6) represents a larger sample of tested individuals (Table 3-2). For *R. mendax*, the time spent in search behavior shows a preference for blueberry fruit over *Lonicera* and snowberry fruit in a simultaneous test (Figure 3-6A). In *R. zephyria*, the search and ovipositor cleaning times provide no indication for a preference of white snowberry over *Lonicera* fruit (Figure 3-6C). Although not significant in a three-way comparison, *R.*

zephyria appears to spend somewhat more time in searching behavior on *Lonicera* than on green snowberry fruit (Figure 3-6D).

The behavior of host selection prevents *R. mendax* and *R. zephyria* from ovipositing into the host fruit of other described fly taxa, but it does not serve as a complete barrier to the use of *Lonicera* fruit by *R. mendax* and *R. zephyria*. The response of the two parent species to *Lonicera* is more similar to the incomplete behavioral isolation that has been observed between *R. pomonella* (Prokopy et al. 1988) and *R. cerasi* (Boller et al. 1998) host races.

The results further indicate that *R. zephyria* has a greater propensity than does *R. mendax* for selecting *Lonicera* fruit as a host under no-choice and choice conditions. Snowberry and *Lonicera* both belong to the plant family Caprifoliaceae, whereas blueberry is part of the Ericaceae. A greater similarity in chemical host plant cues between snowberry and *Lonicera* than between blueberry and *Lonicera* could explain the observed asymmetry in host selection between *R. mendax* and *R. zephyria*.

The greater propensity of *R. zephyria* for selecting *Lonicera* fruit does correspond to a greater genetic similarity between the *Lonicera* Fly and *R. zephyria* (see Chapter 2). Based on the current genetic data, it is not possible to determine whether this greater degree of relatedness is due to ongoing gene flow from *R. zephyria* to the *Lonicera* Fly. Because host choice most likely equals mate choice in *Rhagoletis* (Prokopy and Papaj 2000), the observed lack of ecological isolation could translate into incomplete reproductive isolation. This conclusion is consistent with the possibility of ongoing gene flow from *R. zephyria* into the *Lonicera* Fly. In contrast, the genetic study found no evidence for current immigration of *R. mendax* into the *Lonicera* Fly population based

upon current sample sizes (see chapter 2). This result is consistent with the discrimination against *Lonicera* fruit by *R. mendax* in the three-way choice test (Figures 3-5A and 3-6A) and the lower level of *Lonicera* fruit acceptance by *R. mendax* in the no-choice test when compared to *R. zephyria*.

The greater genetic similarity of *Lonicera* Fly and *R. zephyria* could also be the explanation for the fact that the search patterns before the first ovipositor probing (Figure 3-4) in the two taxa are indistinguishable. In contrast, behavior by *R. mendax* on *Lonicera* fruit is statistically different in all three measures that I used to describe the behavioral pattern. This difference appears not to be the result of a lower response of *R. mendax* flies to *Lonicera* fruit, but rather to represent general differences in behavior between *R. mendax* and other taxa.

How does the Lonicera fly respond to the host fruit of described parent species?

The *Lonicera* Fly does not fit the pattern of host fruit acceptance and choice that I observed for *R. mendax*, *R. zephyria*, and *R. pomonella*. In addition to its natal host, the *Lonicera* Fly will also oviposit in snowberry and hawthorn fruit in the no-choice host-acceptance test (Figure 3-2B). In the three-way choice experiment it will probe and mark *Lonicera*, blueberry and snowberry fruit (Figure 3-5B), a broader behavioral response than described above for *R. mendax* and *R. zephyria*. The *Lonicera* fly does appear to show some specificity for *Lonicera* fruit despite this acceptance of multiple host species. The proportion of *Lonicera* Flies that accept *Lonicera* fruit in the no-choice test was significantly higher than the proportion of *Lonicera* Flies that accepted snowberry or blueberry fruit (Figure 3-2B). The *Lonicera* Fly significantly preferred *Lonicera* fruit

over the other two host fruit species for ovipositor probing in the three-way choice test (Figure 3-5B). Though not significant, a preference for *Lonicera* Fruit seems also to be the case for the first marking of fruit in the three-way host-choice test.

However, I did not detect this specialization on *Lonicera* in the two other measures of host fruit acceptance and choice (Figures 3-3 and 3-6). The *Lonicera* Fly did not exhibit longer initial search intervals on *Lonicera* fruit (Figure 3-3B). Likewise, it did not spend more time searching *Lonicera* than it spent on blueberry or snowberry in the three-way choice test (Figure 3-6B).

There are at least two possible explanations for these differences among metrics of host selection. First, searching and oviposition represent two different decisions. Oviposition in a fruit likely represents a greater investment than spending search time on a fruit. Specialization in oviposition behavior is therefore expected to evolve more quickly than would specialization for which hosts to examine. The second explanation is the possibility that the lower probability of oviposition in blueberry and snowberry fruit is an experimental artifact. If there were systematic differences in individual fruit quality among the three host fruit species, I might obtain the results seen in my choice experiment. Lower quality snowberry or blueberry fruit could have biased oviposition in favor of *Lonicera* fruit, even if the *Lonicera* Fly had the same propensity to oviposit in all three host fruit species equally. The low level of blueberry acceptance by *R. mendax* (Figure 3-2A) might suggest a low individual quality of the blueberry fruit. The observation that green snowberry was readily accepted by *R. zephyria* flies (Figure 3-2C) makes suboptimal fruit quality seem a less likely explanation for the low level of acceptance of snowberry fruit by the *Lonicera* Fly.

The *Lonicera* Fly accepted its natal host more readily than either snowberry or blueberry (as measured by oviposition). This pattern differs from the results of host-acceptance tests in *Rhagoletis* host races. Derived host races of both *R. pomonella* and *R. cerasi* retained a higher degree of acceptance for the old host fruit than for the new host fruit (Boller et al. 1998; Prokopy et al. 1988). The observed host selection behavior indicates that the *Lonicera* Fly is an independent population that shows a stronger ecological and (inferred) reproductive isolation than the recognized host races of *Rhagoletis*.

Hypothesis 2.a.: The Lonicera Fly is less discriminant in its host choice (or, conversely, accepts a greater spectrum of hosts) than its parent taxa.

Is the *Lonicera* Fly more variable in its host selection than its parent taxa?

In the discussion of the behavioral isolation of the *Lonicera* Fly, above, I described that the *Lonicera* Fly accepts a unique combination of host fruit species for oviposition (Figure 3-2). The oviposition response of *R. mendax* to hawthorn fruit was not tested under the experimental conditions of this study. Diehl and Prokopy (1986) tested *R. mendax* individuals in a different no-choice host-acceptance test on hawthorn fruit and found that 33% of all tested females would attempt to oviposit in hawthorn fruit. This suggests that the acceptance of hawthorn fruit for oviposition by the *Lonicera* Fly might be the result of the *R. mendax* component of the ancestry of the *Lonicera* Fly and not an emergent property of its hybrid origin (Rieseberg et al. 1999). If oviposition into

blueberry fruit during the three-way choice experiment is considered, the *Lonicera* Fly combines the host spectra of *R. mendax* (blueberry, *Lonicera* and hawthorn fruit; Figure 3-2 and Diehl 1986) and *R. zephyria* (snowberry and *Lonicera* fruit; Figure 3-2). Thus, it shows greater variability in host choice than do either of its parent taxa.

The same is true when I consider the length of the initial search interval in the no-choice host-acceptance experiment (Figure 3-3). The *Lonicera* Fly was the only taxon with intermediate to high proportions of long initial search intervals on all four host fruit species (Figure 3-3B).

The results of the three-way choice experiment provide further support for the hypothesis that the *Lonicera* Fly is more variable in its host-selection behavior. All three host fruit species are used by the *Lonicera* Fly as its first choice for ovipositor probing or marking in the three-way choice experiment (Figure 3-5B). The *Lonicera* Fly was also the only taxon that did not discriminate against any host fruit species in its allocation of searching behavior (Figure 3-6B).

The combined evidence of both experiments supports the hypothesis that the *Lonicera* Fly has a wider host spectrum than its parent taxa and that it is less discriminant in its host selection. These findings are consistent with quantitative genetic models that predict a greater phenotypic variability in hybrid origin populations (Barton 2001). Greater phenotypic variability, like I observed for the *Lonicera* Fly, is a necessary condition for host shifts according to the herbivore hybrid bridge model (see Introduction). Even though there is evidence for some degree of specialization of the *Lonicera* Fly on *Lonicera* fruit, adaptation to *Lonicera* has not progressed far enough to mask the behavioral effects of a hybrid origin. Alternatively, the *Lonicera* Fly could

experience ongoing introgression from the parental taxa that would maintain a greater level of phenotypic variability in the *Lonicera* Fly.

What is the mechanism that generates phenotypic variability in the *Lonicera* Fly?

The behavior of a group of organisms is commonly described by the population mean (for ratio scale data) or the proportion of individuals performing a certain behavior (for nominal data). This practice assumes that all organisms within the population have an identical chance of performing a certain behavior. Any variation around the mean population response is assumed to be stochastic. In a population of hybrid origin these assumptions are almost certainly violated, because quantitative genetic models predict a high phenotypic variability among individuals. It would have therefore been more appropriate to test the hypothesis of greater behavioral variability in the *Lonicera* Fly by repeatedly measuring single individuals (Singer and Lee 2000). Because it has been demonstrated for *R. pomonella* that previous experience will influence host-selection behavior (Prokopy et al. 1982), repeated testing was impractical and (perhaps) biologically invalid.

It is therefore uncertain whether the greater variation in host choice and acceptance of the *Lonicera* Fly is caused by individual differences in host specificity or by reduced host specificity in all *Lonicera* Fly individuals. The first possibility is predicted by quantitative genetic models of hybridization (Barton 2001). New possible cues could, e.g., be received by mixing receptors from the parental species. The second possibility is suggested by preliminary results on the electrophysiology of olfaction in artificial *Rhagoletis* F1 hybrids (Olsson, personal communication). Single receptor recordings

have shown that – compared to pure parentals – laboratory-reared *R. pomonella* x *R. mendax* F1 hybrids have an altered receptor response. This suggests that hybrids could lack host specificity because they lack a functional sensory system to discriminate among host cues.

One possibility to distinguish between these alternatives without repeated testing of females would be to combine genotyping and behavioral testing. A significant correlation between diagnostic alleles and behavioral responses would suggest that individual differences in host specificity exist. This test depends on the availability of informative genetic markers. Previous studies in *R. pomonella* have shown that certain allozyme loci (i.e., *Had* and *Aat-2*) are closely associated with diapause length, which is crucial for host adaptation in *R. pomonella* (Filchak et al. 2000). This suggests that other traits associated with host specificity could also be linked to known allozyme or DNA-based markers, especially if they are located in the extensive regions with strong linkage disequilibrium on chromosomes I, II, and III (Feder et al. 2003, see also chapter 4 for a discussion of inversion polymorphisms in *Rhagoletis*). A quantitative trait locus mapping study is currently in progress for *R. pomonella* to map loci that are involved in host discrimination by olfaction (Roelofs, Feder, and Berlocher, personal communication). This work will yield useful markers for the study of host specificity (or the lack thereof) in the *Lonicera* Fly.

Hypothesis 2.b.: Lonicera flies will discriminate less against *Lonicera* (or more readily accept *Lonicera* as a host) than will the parental taxa.

Does hybridization facilitate a host shift to *Lonicera*?

I hypothesized under the herbivore hybrid bridge model that a hybrid origin generates behavioral pre-adaptations that are necessary for the colonization of a new host. As discussed above, both parental taxa will accept *Lonicera* fruit for oviposition (Figures 3-2A and B). This indicates that no behavioral barriers exist that would have prevented either *R. mendax* or *R. zephyria* to shift to *Lonicera* fruit independently.

But does hybridization generate a relatively greater propensity for the acceptance and choice of *Lonicera* fruit than the phenotypic flexibility that I observed in the parental taxa? The difficulty with answering this question is that the *Lonicera* Fly does not represent a hybrid swarm *in statuo nascendi*, but more likely is an isolated population of hybrid origin (see chapter 2) that appears to have evolved some degree of behavioral specialization for *Lonicera* fruit (see above). In an ideal case, my hypothesis would also be examined with an artificially generated hybrid swarm. However, rearing consecutive generations of crosses and backcrosses in the lab involves many practical difficulties. These include the univoltine life cycle of the flies that would have required two years to generate F2 and backcross individuals. It is therefore impossible to experimentally determine the cause for the lower acceptance of *Lonicera* fruit by *R. mendax* in comparison to the response of the *Lonicera* Fly. A higher degree of *Lonicera* fruit acceptance by the *Lonicera* Fly could be an effect of hybridization or represent adaptation to *Lonicera* fruit after the initial colonization. Despite the inferred adaptation

of the *Lonicera* Fly to *Lonicera* fruit, *R. zephyria* accepts *Lonicera* fruit for oviposition at almost identical proportions as does the *Lonicera* Fly (Figure 3-2B and C). In a three-way choice situation, *R. zephyria* spends equal amounts of time in search behaviors on *Lonicera* and white snowberry fruit (Figure 3-6C). When the three-way choice test contains green snowberry fruit, *R. zephyria* prefers *Lonicera* over snowberry fruit (Figures 3-5D and 3-6D).

These findings reject – at least for the comparison with *R. zephyria* – the hypothesis that the hybrid origin endowed the *Lonicera* fly with a higher degree of behavioral pre-adapatation to *Lonicera* fruit than either of its parents. Both parents are pre-adapted for accepting *Lonicera* fruit as a host. But while the *R. mendax* response to *Lonicera* is marginal, there appear to be no behavioral barriers that prevent *R. zephyria* from colonizing *Lonicera*. One possible explanation is the greater relatedness of snowberry and *Lonicera* fruit—both taxa are members of the Caprifoliaceae. Members of the same plant family are likely to share chemical characteristics, and a host shift by herbivores between plants of the same taxonomic family may be easier than shifts to a plant in a different family (Mitter et al. 1991). That a shift from snowberry to *Lonicera* might be straightforward is suggested by the example of the European cherry fruit fly, *R. cerasi*. This fly's native host is European Fly honeysuckle, *Lonicera xylosteum* (Wiesmann 1937), but it will also attack snowberry, which has been introduced as an ornamental to Europe (Schwarz, unpublished data). *Lonicera* species not only serve as a host for *R. cerasi*, but also for a number of other *Rhagoletis* taxa throughout Eurasia (Smith and Bush 2000). This plant genus could have characteristics that make it a likely host for *Rhagoletis*. Its colonization by *Rhagoletis* in North America is therefore not surprising.

Conclusions

Is the *Lonicera* Fly a likely example of a herbivore hybrid bridge?

The *Lonicera* Fly shows a greater phenotypic variability in its host-selection behavior than either of its two parent taxa. This observation is in accord with the idea that hybridization will result in increased phenotypic variability. The fact that both parent taxa show behavioral preadaptations for the colonization of *Lonicera* fruit contradicts the idea that a hybrid origin is a necessary condition for the development of behavioral adaptations to *Lonicera*. Based upon host-selection behavior, it seems unlikely that hybridization resulted in advantages for the colonization of *Lonicera* fruit. It is conceivable that pure *R. zephyria* flies could have been as successful in parasitizing *Lonicera* fruit as the *Lonicera* Fly.

This latter conclusion is at odds, however, with the preliminary genetic data on geographically dispersed samples of the *Lonicera* fly (see chapter 2). All five samples that have been reared from *Lonicera* at different locations in the Mid-Atlantic states show evidence for a hybrid origin of the *Lonicera* Fly. This result is more consistent with the hypothesis that a hybrid origin is instrumental in the successful colonization of *Lonicera*. My study examined only host acceptance and choice behavior. It is possible that hybridization generated unique physiological adaptations that facilitated *Lonicera* Fly (relative to the parental fly taxa) survival and reproduction on *Lonicera* fruit. Differences in host-specific fitness between the *Lonicera* Fly and its parents should be the subject of a future study.

A model for the initial formation of the *Lonicera* Fly

The finding that both *R. mendax* and *R. zephyria* will accept *Lonicera* as an oviposition substrate (Figures 3-2A and C) provides important information for the reconstruction of the genesis of the *Lonicera* Fly. My data show that *Lonicera* fruit might have been colonized by pure parentals from both species, which subsequently hybridized to form the *Lonicera* Fly ("host shift first" hypothesis). The host shift to *Lonicera* would not have had to occur synchronously in both parental taxa. One taxon, e.g., *R. zephyria*, could have colonized honeysuckle first and then experienced introgression from the other parent species. However, the observation that both *R. mendax* and *R. zephyria* will accept *Lonicera* fruit as a host in the laboratory does not exclude the possibility that invasive brushy honeysuckle was initially colonized by individuals of hybrid origin ("hybridization first" hypothesis). But, given the data on the reciprocal behavioral isolation of the parent taxa (Figures 3-2, 3-3, 3-5, and 3-6), the "host shift first" hypothesis is a more parsimonious mechanism than is the "hybridization first" hypothesis. Since mating takes place on the host plant, hybridization on either parental host would have had to involve errors in host choice. Based on the results of this study (Figures 3-2, 3-3, 3-5 and 3-6), such errors are less likely than the shift of pure parentals to *Lonicera*.

HOST-INDEPENDENT ASSORTATIVE MATING

Partial isolation by assortative mating occurred between *R. mendax* and *R. zephyria* under my experimental conditions (Figure 3-8). The observed level of assortative mating

between *R. zephyria* and *R. mendax* was consistent with the degree of isolation that Smith (1986) observed between *R. mendax* and *R. pomonella*. The results are not directly comparable because Smith used a no-choice experiment to quantify mating propensity, but his work represents the only other study of host-independent sexual isolation in the *Rhagoletis pomonella* species complex. This scarcity of studies is unfortunate. Despite the importance of host fidelity in reproductive isolation, host-independent assortative mating is expected to play an important role in sympatric speciation, especially in the later stages of reproductive isolation (Johnson et al. 1996). There is no host independent assortative mating between the host races of *R. cerasi* (Schwarz 2000) or between *R. pomonella* and the flowering dogwood fly (Smith 1986). These examples suggest that host races, and even more strongly isolated undescribed taxa like the flowering dogwood fly, have not yet evolved host-independent assortative mating. Partial sexual isolation in *Rhagoletis* appears to be associated with later stages of speciation as shown by this study and Smith (1986). It would therefore be very interesting to compare the level of host-independent assortative mating between the *Lonicera* Fly and its parents with the level of sexual isolation between the parent taxa. Such a study would provide further information about the level of reproductive isolation of the *Lonicera* Fly.

Sexual selection versus sexual isolation

I used live body mass to test for the influence of individual fly quality on mating success. This character was chosen in the absence of information about which traits could influence mating success in the *Rhagoletis pomonella* species complex (Prokopy and Papaj 2000). "Forced" copulations constitute about 25% of all matings off the host fruit in *R. mendax* (Smith and Prokopy 1982), and heavier males could be more successful in

"forced" encounters. Contradicting this initial hypothesis, the only significant effect of male body size on mating success was found in *R. zephyria*, in which mated males were smaller than unmated males (Figure 3-9C). It is unclear what biological significance could underlie the greater mating success of lighter males. It is also conceivable that the observed effect is an artifact of the paint marks on the back of the flies (see Materials and Methods). Flies with larger paint marks could have been more handicapped than flies with smaller paint marks. In a similar study, (Jaastad 1998a) did not find a significant influence of body size on male mating success in the European cherry fruit fly, *R. cerasi*.

Why heavier females appear to have been more successful in both taxa is also open to speculation. Heavier females could, for example, have carried a larger egg load that increased their readiness to mate.

The observed differences in mating success are, however, not likely to bias the result for the sexual isolation between *R. mendax* and *R. zephyria*, since there are no statistically significant differences in the mean live mass of the two species (Figures 3-10A and B).

Asymmetry of heterospecific matings

Successful conspecific matings outnumber heterospecific matings by a threefold margin (Figure 3-8). This is the combined effect of two factors. First, individual flies are simply more likely to initiate mating events with partners from the same taxon (Figure 3-8). Second, there were no successful *R. mendax* male x *R. zephyria* female crosses (Figure 3-7).

The observed absence of *R. mendax* male x *R. zephyria* female matings is at odds with the observed mitochondrial haplotype frequencies in the *Lonicera* Fly. DNA-

sequence data confirm that the *Lonicera* Fly displayed haplotypes from both parent taxa (chapter 2). There are two possible explanations for this apparent contradiction. The first is that *R. mendax* male x *R. zephyria* female matings may occur if there are no conspecific mating partners available (Kaneshiro 1990). This was the case when *R. mendax* males and *R. zephyria* females were confined for a long period of time in the same cage. This is an anecdotal observation from a cross that I set up in the summer of 2003 to generate artificial hybrids. The second explanation is the introgression of *R. zephyria* females into a randomly mating population that originated by hybridization between *R. zephyria* males and *R. mendax* females. This population would contain intermediate males (bearing *R. mendax* mtDNA haplotypes) that might successfully mate with *R. zephyria* females.

In the *R. mendax* male x *R. zephyria* female cross, the lack of successful matings is accompanied by a high number of unsuccessful mating attempts (Figure 3-7). One possible explanation for this observation could be asymmetric morphological incompatibilities in the genitalia of the two taxa. The successful matings under no-choice conditions show that such incompatibilities cannot represent an absolute mechanical barrier to the coupling of *R. mendax* males and *R. zephyria* females. Instead, they may reduce the number of successful matings per mating attempt. Also, morphological incompatibilities could help females fend off unwanted mating attempts more successfully. *R. zephyria* is set apart from the other taxa in the *R. pomonella* species complex by morphological differences in its genitalia (Bush 1966). This difference in male genitalia, which is likely to be matched in the genital morphology of *R. zephyria* females, is consistent with the hypothesized mechanism of morphological

incompatibility.

Consequences for hybridization

While there is a substantial degree of sexual isolation between the parental taxa of the *Lonicera* Fly, it is not sufficiently strong to present a significant obstacle to hybridization (at least in the case of *R. zephyria* males x *R. mendax* females). The most crucial prezygotic reproductive barrier is the host fidelity of the parents. Once this isolation mechanism is breached, e.g., by a shift to a new host, *R. mendax* and *R. zephyria* might be expected to hybridize in substantial proportions.

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Collected host fruit	Location	Sample month
Hawthorn	Allen St., PSU Campus, S.C., PA	September 2000
	Forum Bldg., PSU Campus, S.C., PA	September 2000
Blueberry	Fennville, MI	August 2002
	East Wareham, MA	August 2002
Snowberry	Munson, Clearfield Cty., PA	August 2000
	Munson, Clearfield Cty., PA	Aug. and Sept. 2002
Lonicera	Golfcourse, PSU Campus, S.C., PA	August 2000
	Slab Cabin Park, S.C., PA	August 2000
	Waupelani Dr., S.C., PA	Aug. and Sept. 2000
	Golfcourse, PSU Campus, S.C., PA	Aug. and Sept. 2002
	Waupelani Dr., S.C., PA	August 2002
	Slab Cabin Park, S.C., PA	August 2002
	Walnut Pring Park, S.C., PA	August 2002
	Scotia Rd, S.C., PA	September 2002
	Valley Vista Rd., S.C., PA	September 2002
	Sunset Park, S.C., PA	Aug. and Sept. 2002

Table 3-1 Sampling locations and sampling dates of flies used in the experiments. *R. mendax* was collected from blueberry, *R. zephyria* from snowberry, the "*Lonicera* Fly" from *Lonicera*, and *R. pomonella* from hawthorn S.C. = State College.

Experiment	Fly taxon	Host fruit species	n	n (probe)
No-choice fruit acceptance	**R. mendax**	blueberry	27	5
		Lonicera fruit	30	4
		snowberry (green)	27	
	***Lonicera* Fly**	blueberry	44	
		Lonicera fruit	33	18
		snowberry (green)	31	3
		hawthorn	30	6
	R. zephyria	blueberry	36	
		Lonicera fruit	29	15
		snowberry (green)	41	15
		hawthorn	38	
	R. pomonella	*Lonicera* fruit	29	
		snowberry (green)	24	
		hawthorn	42	22
3-way fruit choice	**R. mendax**	white snowberry	25	
	***Lonicera* Fly**	white snowberry	28	
	R. zephyria	white snowberry	15	
	R. zephyria	green snowberry	23	

Table 3-2 Number of females tested in the experimental combinations of the no-choice fruit acceptance and 3-way fruit choice experiments. n (probe) = Number of females displaying ovipositor probing in the no-choice host acceptance test.

Comparison	Species	Sex	mating success	n
R. mendax* vs. *R. zephyria	*R. mendax*	female		27
	R. zephyria	female		17
	R. mendax	male		29
	R. zephyria	male		35
mated vs. unmated flies	*R. mendax*	female	unmated	9
			mated	18
	R. zephyria	female	unmated	10
			mated	7
	R. mendax	male	unmated	21
			mated	8
	R. zephyria	male	unmated	19
			mated	16

Table 3-3 Sample sizes in the comparison of live masses of individuals in the mate choice test between *R. mendax* and *R. zephyria*.

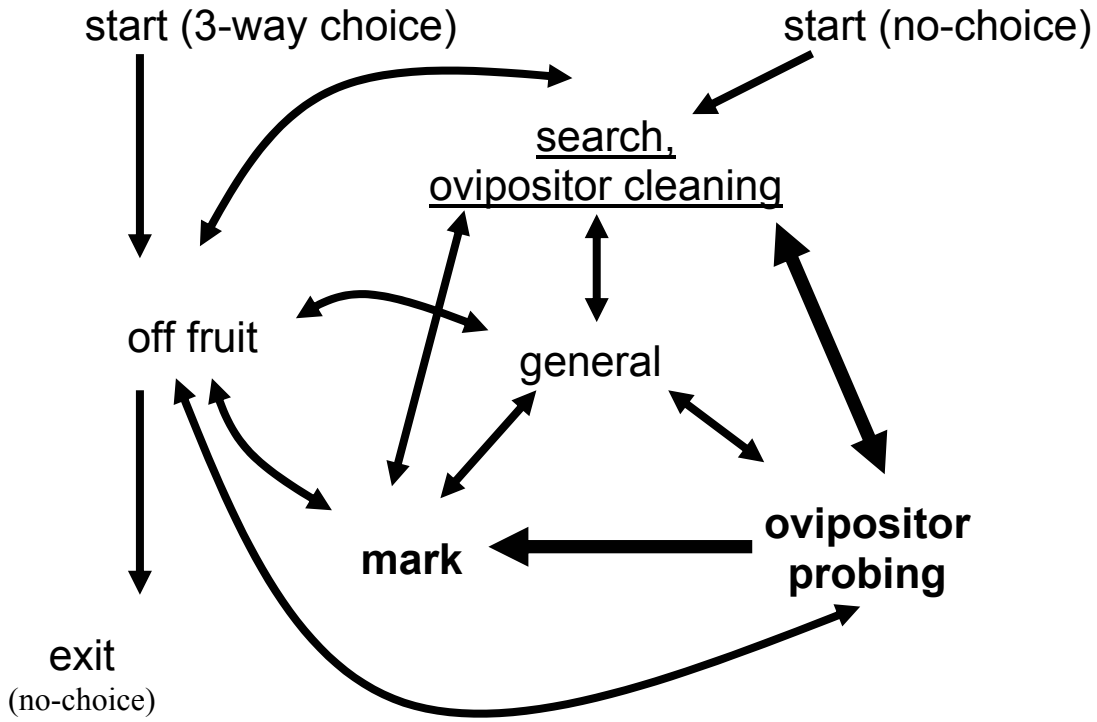

Figure 3-1 Flow chart of behavioral transitions during the no-choice fruit acceptance and three-way fruit choice tests. Underlined type: Searching behavior. Bold type: Oviposition behavior. Bold arrows: Oviposition sequence. The default behavior for the start of no-choice test is "search". The default behavior for the start of the three-way choice test is "off fruit." All observations were terminated after 15 min. In the no-choice tests observations could also be ended by a female leaving the experimental arena.

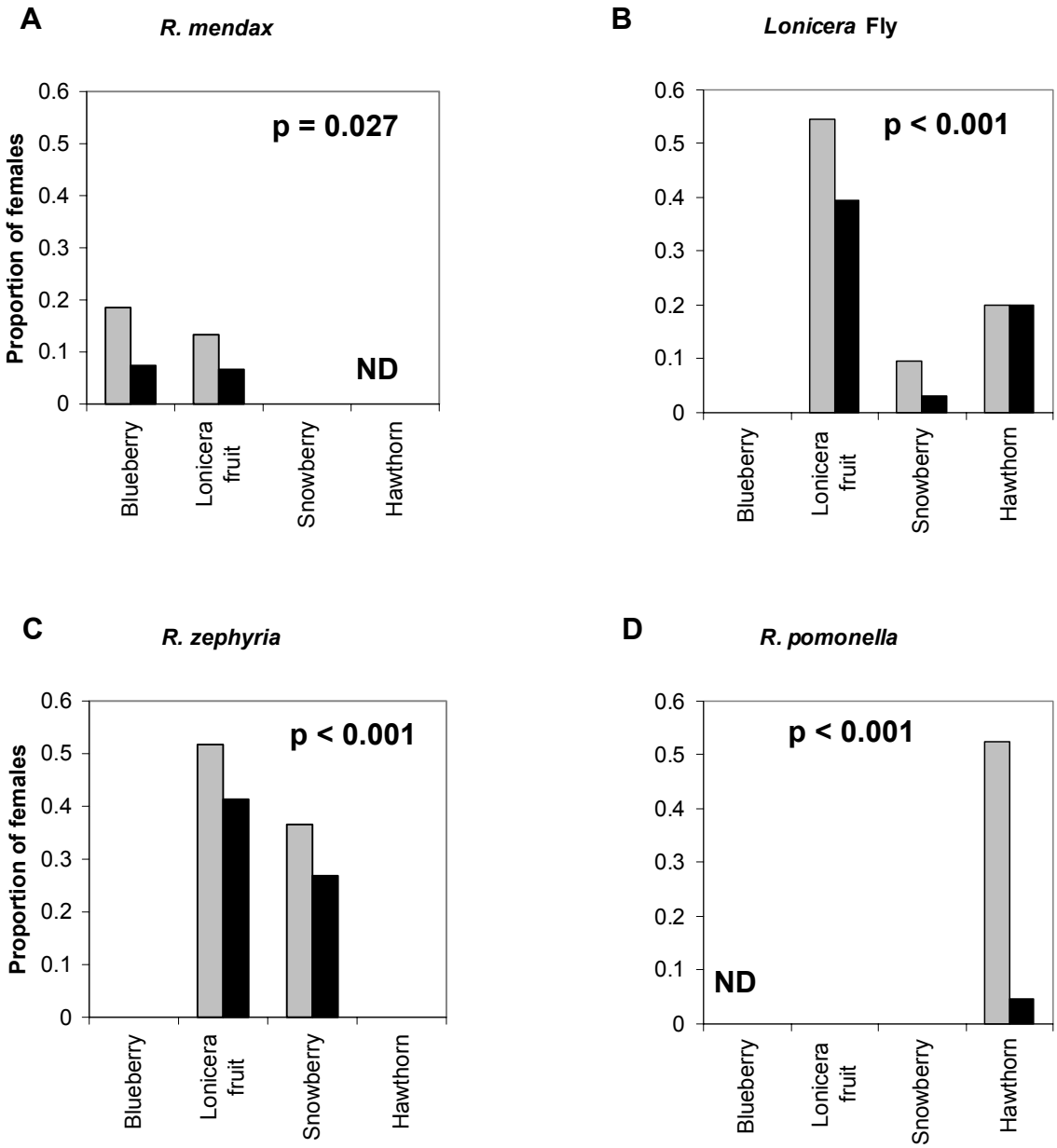

Figure 3-2 Proportion of females showing oviposition behavior in a no-choice test. Grey = Proportion of females probing fruit. Black = proportion of females marking fruit. P-values shown in each panel were obtained by conducting a G-contingency test for the number of females displaying ovipositor probing. A: *R. mendax*, B: *Lonicera* Fly, C: *R. zephyria*, D: *R. pomonella*. ND = No data.

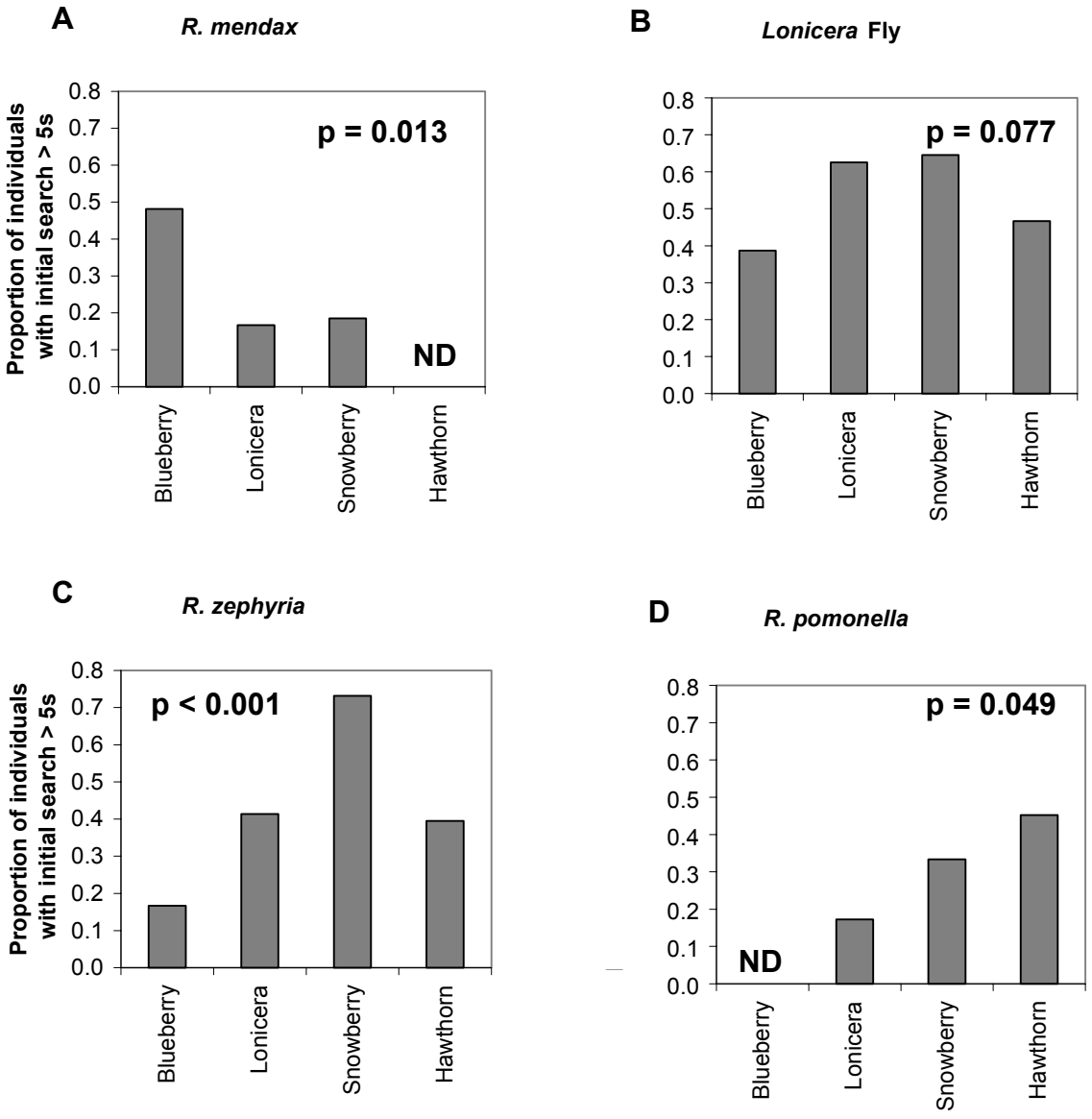

Figure 3-3 No-choice host acceptance experiment. Proportion of females with an initial search interval equal to or longer than five seconds. The p-values in each panel represent the results of a G test on a 2 x 3 or 2 x 4 contingency table. A: *R. mendax*, B: *Lonicera* Fly, C: *R. zephyria*, D: *R. pomonella*. ND = No data.

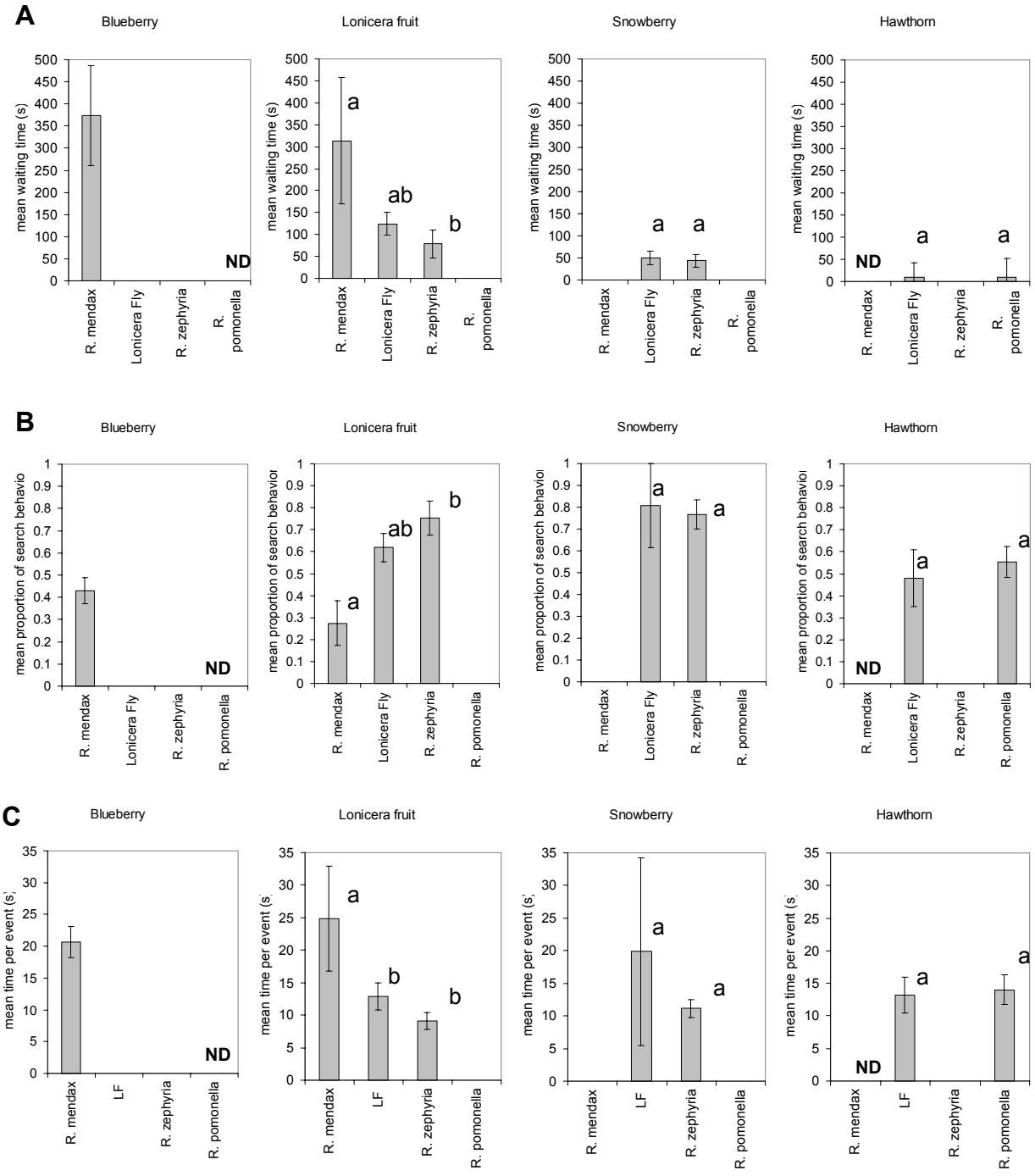

Figure 3-4 Differences in behavior leading to the first ovipositor probing (subset of replicates with ovipositor probing). A: Mean total waiting time until first probe (s). B: Proportion of total waiting time until first probe spent searching and ovipositor cleaning. C: Mean time per behavioral event during total waiting time until first probe (s). Error bars: SE of the mean. Different letters indicate statistically significant differences (α = 0.05) in Tukey's or two sample t-test. ND: No data.

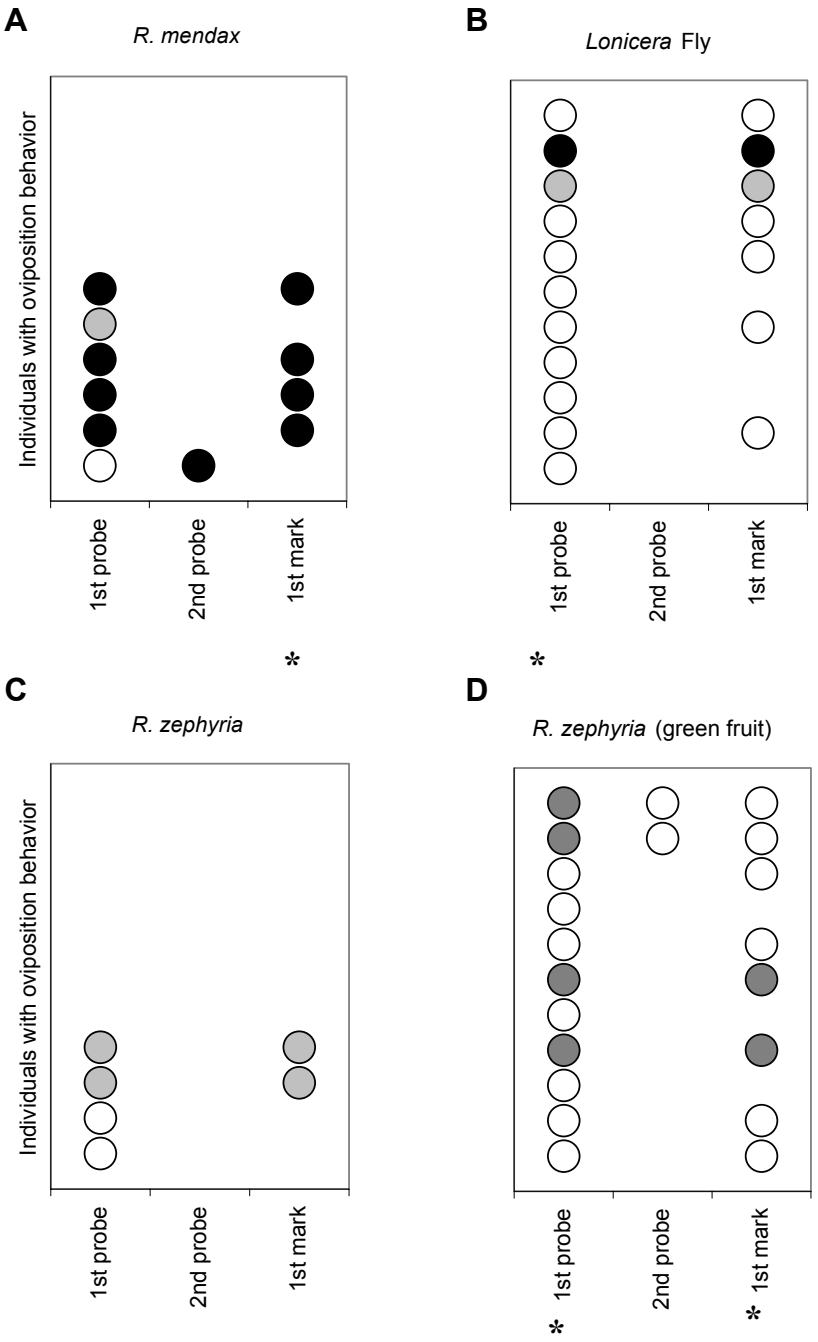

Figure 3-5 Oviposition behavior of females in the three-way choice test (only individuals that probed fruit are shown). Each row represents the probing and marking sequence of a single female. Columns represent the first two choices for probing until marking and the first choice for fruit marking. Black: Blueberry. White: *Lonicera* Fruit. Light gray: white snowberry. Dark gray: green snowberry. Asterisk: Significant deviation ($\alpha = 0.05$) from expected values under random choice (goodness of fit test).

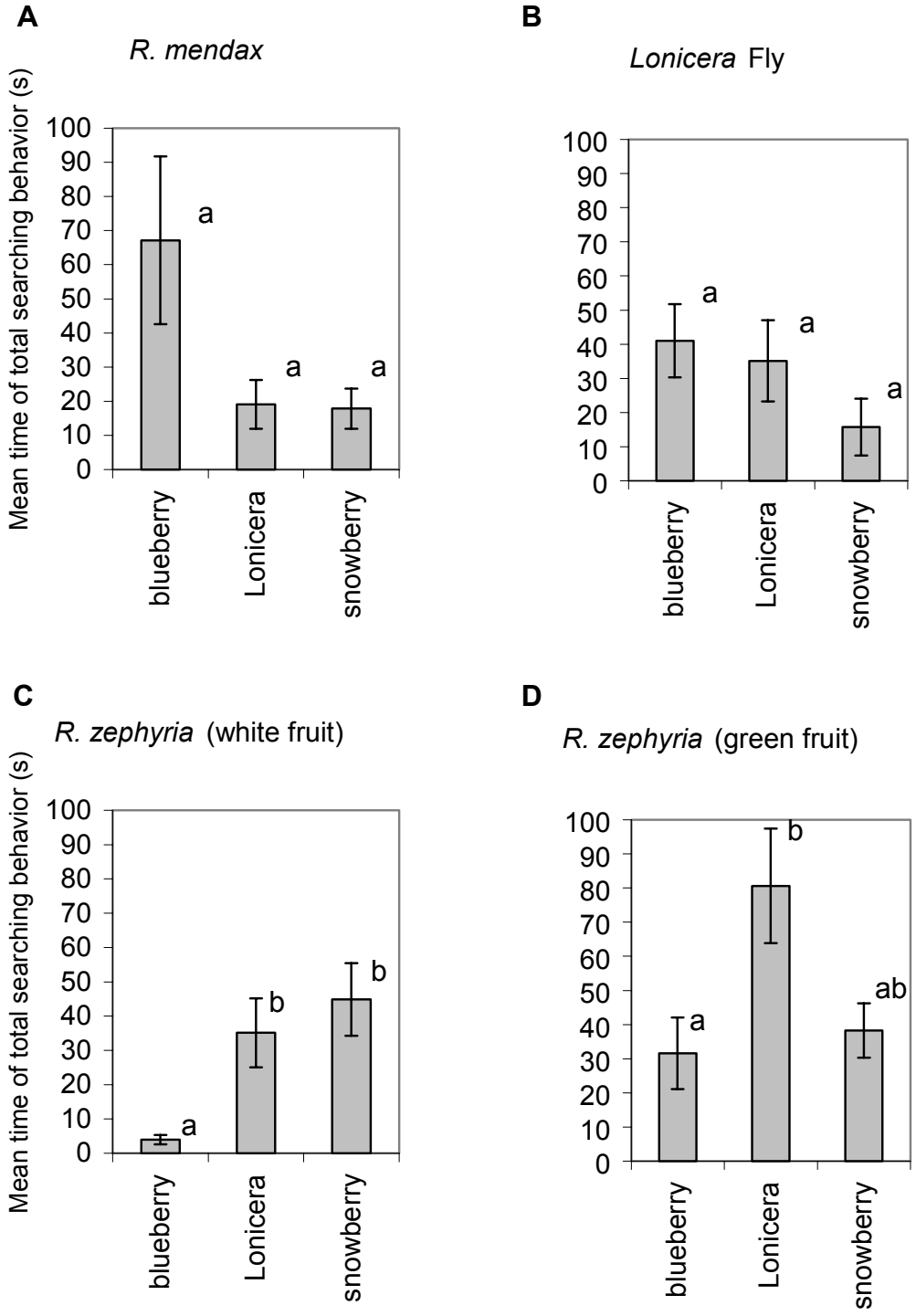

Figure 3-6 Mean total search and ovipositor cleaning time until the first marking or the end of the three-way choice replicate. Green fruit: Choice between green snowberry, blueberry and *Lonicera* fruit. Error bars: SE of the mean. Different letters indicate statistically significant differences ($\alpha = 0.05$) in Tukey's multicomparison test.

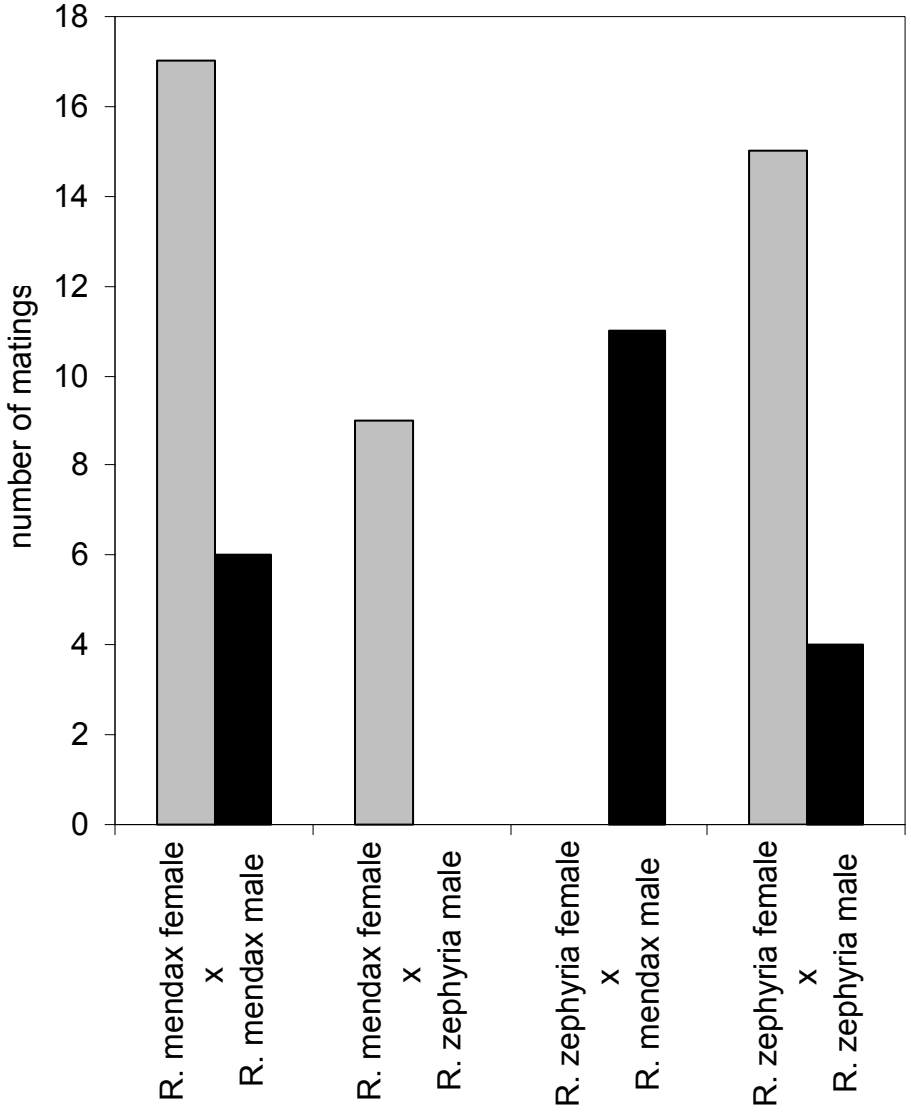

Figure 3-7 Number of observed mating events in mate choice test between *R. mendax* and *R. zephyria*. Gray: Successful matings. Black: Unsuccessful mating attempts.

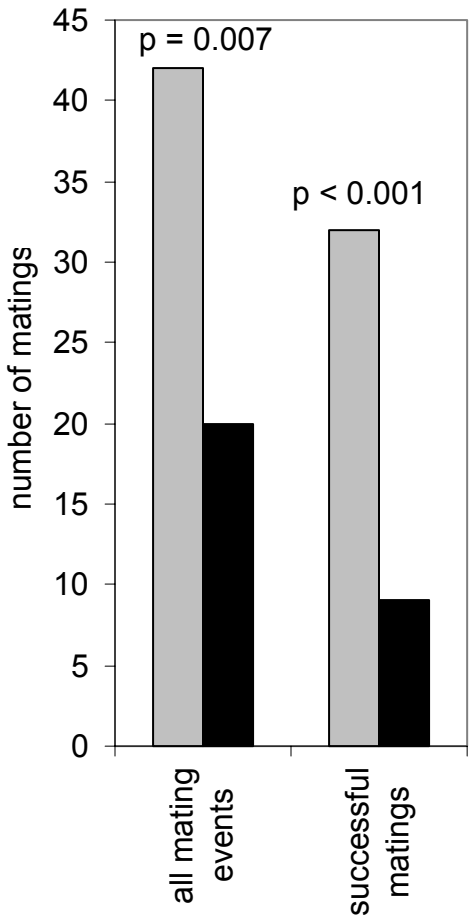

Figure 3-8 Number of conspecific and heterospecific mating events between *R. mendax* and *R. zephyria* in mate choice test. Gray: Conspecific matings. Black: Heterospecific matings. All mating events = successful matings + unsuccessful mating attempts. P-values from goodness of fit test against expected number of matings under random choice.

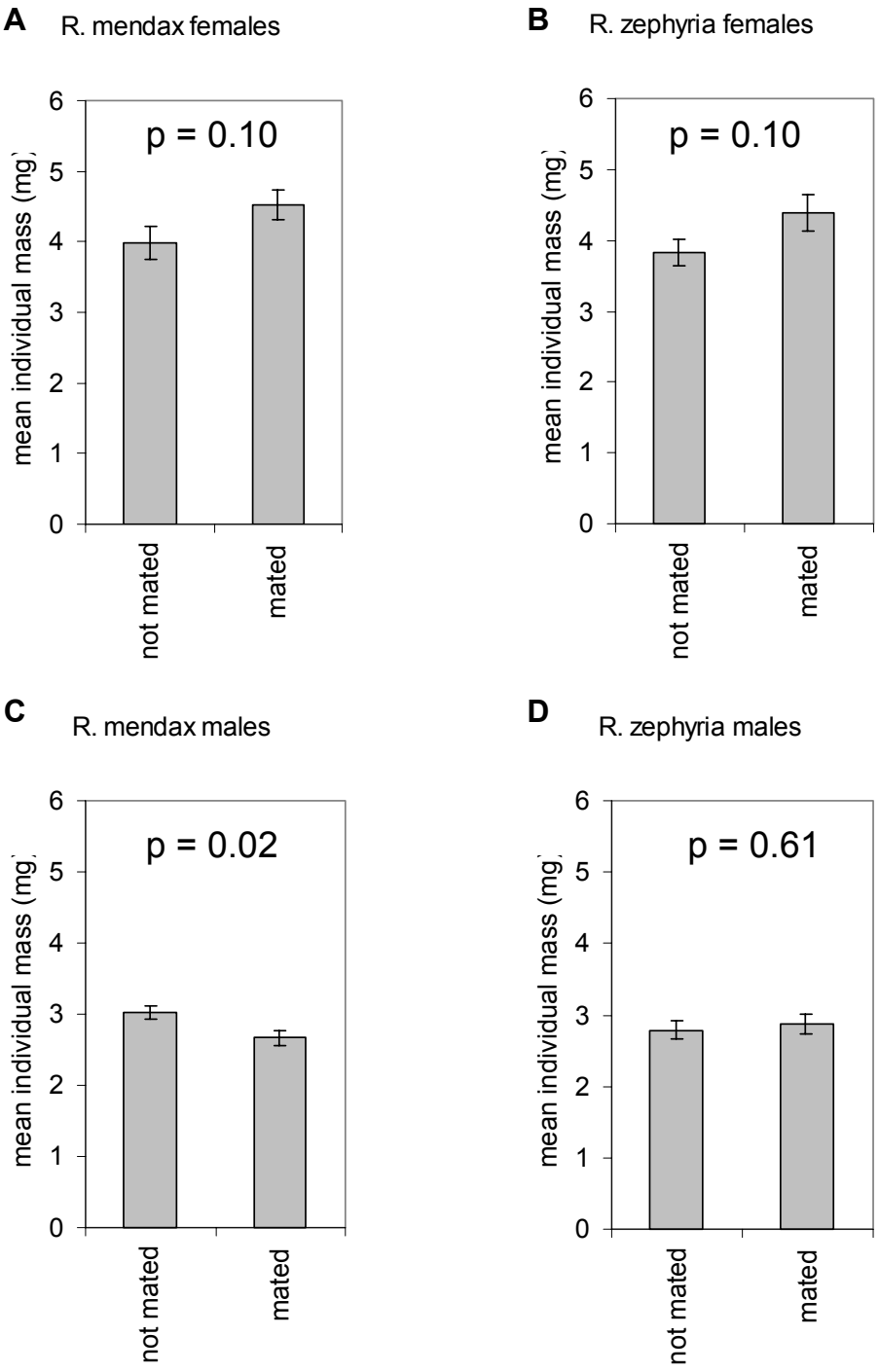

Figure 3-9 Comparison of live mass between mated and unmated males and females in both species. Error bars: SE of the mean. P-values from two sample t-test.

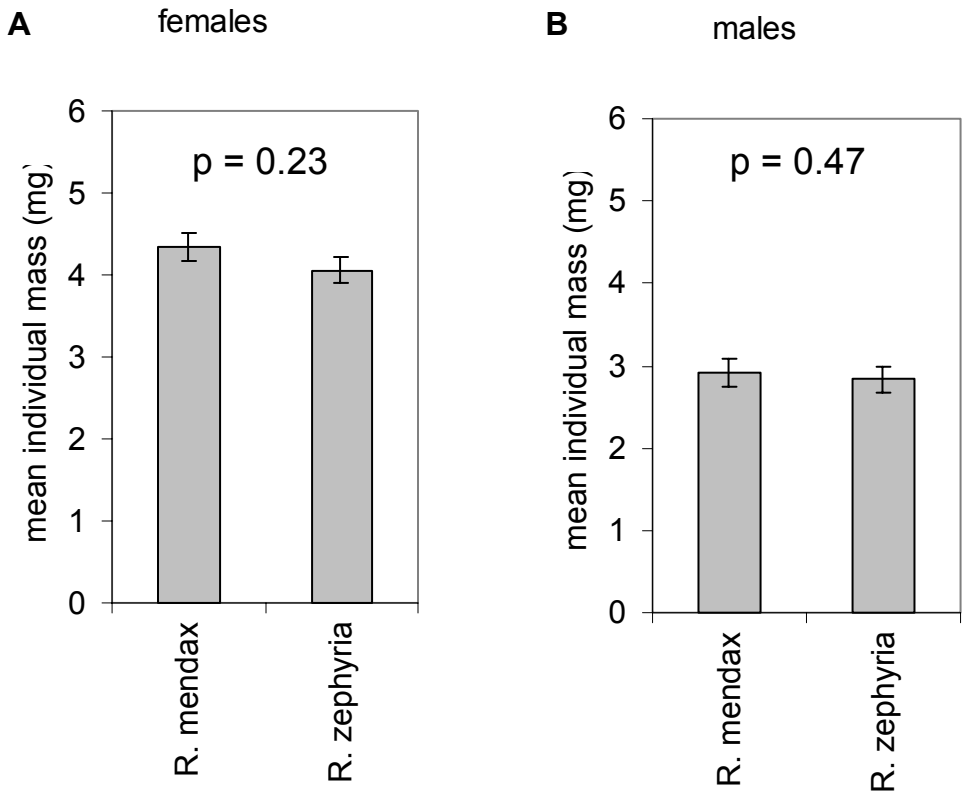

Figure 3-10 Comparison of live mass between *R. mendax* and *R. zephyria* by sex. Error bars: SE of the mean. P-values from two sample t-test.

Chapter 4

IS THE *RHAGOLETIS POMONELLA* SPECIES GROUP A "BIOLOGICAL METASPECIES?"

Species are largely reproductively isolated populations, but occasional migration (in its population genetic sense) between related species (= hybridization) is common in many taxa (Arnold 1997). This description closely resembles the definition of a metapopulation, which is described as "the assemblage of spatially delimited local populations that are coupled by some degree of migration" (Hanski and Gaggiotti 2004). In analogy I propose a new term, "biological metaspecies," that describes a group of sibling species that are connected by some degree of hybridization. One could argue that a biological metaspecies is a special case of a metapopulation, and this is, to some extent, true for a group of species with geographically isolated distributions. But for a species group in which the taxa have sympatric distributions (like the *R. pomonella* species group), the taxa are not geographically isolated like the subpopulations of a metapopulation. In this case, different ecological niches become the equivalent of different patches, and mechanisms of reproductive and ecological isolation, such as host fidelity, take the role of geographical distance as an isolating factor. Speciation following the acquisition of a new niche would consequently be the analogy to the colonization of an empty patch in a metapopulation. I use the qualifier "biological" in biological metaspecies to distinguish it from the use of "metaspecies" in taxonomy (Donoghue 1985). I do not intend to introduce the biological metaspecies as yet another species

concept, but, rather, as a description of an evolutionary process. In the following, I propose that the *Rhagoletis pomonella* species complex is a biological metaspecies, and I discuss the evolutionary consequences of the metaspecies process using the example of the *Lonicera* Fly.

All members of the *R. pomonella* species group occupy different sets of host plants and most of the taxa have overlapping ranges (Berlocher 2000). It has been further hypothesized that the group formed by sympatric speciation following a shift to a new host (Bush 1966). There are two examples in which a host shift by members of the *R. pomonella* species group in historic time has resulted in reproductively isolated populations: the host races of *R. pomonella* (Berlocher and Feder 2002) and the *Lonicera* Fly (Chapters 2 and 3). In addition to the hybridization between *R. mendax* and *R. zephyria* that I demonstrated in Chapter 2, low levels of introgression have also been described between *R. pomonella* and *R. zephyria* (Feder et al. 1999; McPheron 1990). There is also evidence for historic gene flow between the now geographically isolated "Mexican *pomonella*" and the remainder of the *R. pomonella* species group (except for the basal *R. cornivora*; Feder et al. 2003a). These three descriptions of gene flow among otherwise largely isolated taxa (Berlocher 2000 and previous chapters) suggest that the *R. pomonella* species group could represent an example for a biological metaspecies.

What is the significance of gene flow among the members of the *R. pomonella* species group? It is possible that low levels of gene flow (Feder et al. 1999; McPheron 1990) lead to the introgression of neutral alleles among the taxa of the *Rhagoletis pomonella* species complex (Navarro and Barton 2003; Noor et al. 2001). The high

degree of shared polymorphism among the taxa of the *R. pomonella* species complex (Berlocher 2000; Feder et al. 2003a; this study) could be an effect of introgression. However, it is difficult to distinguish introgression from incomplete lineage sorting (Avise 2000). Species diagnostic differences are only found in a certain subset of allozyme loci. This intra-genomic difference in interspecific differentiation suggests that the diagnostic markers are under host-specific selection. This has, indeed, been demonstrated for the host races of *R. pomonella* (Feder and Filchak 1999; Feder et al. 1997a; Feder et al. 1997b; Filchak et al. 1999; Filchak et al. 2000). A recent study has shown that in *R. pomonella* these diagnostic allozyme loci are part of chromosomal inversions (Feder et al. 2003b) that span substantial parts of three chromosomes. Inversions greatly reduce recombination rate and could effectively link alleles that confer adaptations for the same host plant, thereby creating "pre-packaged"(Feder et al. 2003b) host specialization. Hybrid offspring with the "wrong" set of alleles for host specificity are therefore expected to be selected against on the parental hosts, whereas hybrid individuals with the "right" allelic combination could still serve as conduits for the introgression of neutral alleles (Wu 2001). This study found, e.g., that the *Lonicera* Fly did not serve as a conduit for the introgression of diagnostic alleles between the parents (see data for *Had* and *Fum* in Chapter 2). In the majority of cases, hybridization in *Rhagoletis* is therefore not expected to be advantageous and produce evolutionary novelty.

The rarity of plant hybrid individuals had been explained in a similar manner by Anderson (Anderson 1948). He noted that hybrid individuals are most commonly encountered in disturbed or intermediate habitats in which the competition from the

parental taxa is weak. This observation also fits the hybrid origin of the *Lonicera* Fly. It is found on a new host plant, which has not been previously used as a host by *Rhagoletis* in North America. *Lonicera* could be a novel habitat in which hybrid origin genotypes can escape the competition of their parent taxa. This hypothesis is drawn into question by the data from the host-selection experiments (Chapter 3), because both *R. mendax* and *R. zephyria* will accept *Lonicera* as an oviposition substrate. In the case of *R. zephyria* there appear to be no behavioral barriers that would keep this parent species from utilizing *Lonicera* fruit as a host (Chapter 3). At least one *R. zephyria* larva was able to complete its development in *Lonicera* fruit after a *R. zephyria* female had oviposited in a *Lonicera* fruit during the behavioral experiments. This demonstrates that at least *R. zephyria* could successfully use *Lonicera* as a host. However, in all five analyzed geographical samples of the *Lonicera* Fly, I have found evidence for a hybrid origin. In combination, the results suggest that *Lonicera* fruit as a resource is not free from potential competition with at least one parent species. The *Lonicera* Fly might instead experience a selective advantage on *Lonicera* fruit.

In Chapters 1 and 2, I referred to the paradoxical situation that models of sympatric speciation both require and eliminate genetic variation (Kondrashov and Mina 1986). I proposed that hybridization in association with sympatric speciation could provide a solution to this problem (Chapter 2 and 3). While the evolution of specialization to a particular host is likely to lead to a loss of variation in a single host-specific species, the necessary variation for the successful colonization could be maintained at the metaspecies level. Hybridization between taxa is the process by which this variation

becomes available. *Rhagoletis pomonella* (Berlocher and Feder 2002) and the *Lonicera* Fly (Chapters 2 and 3) are the two known cases in which a host shift in historic times has resulted in reproductive isolation. Quite curiously, both examples are associated with gene flow between taxa of the *R. pomonella* species group. In the *Lonicera* Fly system, hybrid origin genotypes are only found on the new host plant *Lonicera* (Chapter 2). The historic gene flow between Mexican *pomonella* and the northern taxa of the *R. pomonella* group appears to be the source for the genetic variation that is now under divergent selection within *R. pomonella* host races (Feder et al. 2003a). Both cases argue that gene flow between the taxa of a biological metaspecies could be instrumental in the acquisition of new habitats and subsequent speciation.

The increased variation in host selection behavior that I observed in the *Lonicera* Fly is consistent with the expectation that introgressive hybridization will result in greater phenotypic variability (Barton 2001). Some of the newly generated phenotypes could be adapted to the new habitat and be favored by selection (Arnold 1997). Because *R. zephyria* was as efficient in its acceptance of Lonicera Fruit as the *Lonicera* Fly itself, I could not find evidence for a better adaptation to *Lonicera* fruit as a function of hybridization (see discussion of Chapter 3). To date I have tested only host acceptance and preference and not other ecological aspects of the *Lonicera* Fly system, such as survival on different host plants. It therefore remains unclear whether hybridization plays a functional role in the speciation of the *Lonicera* Fly.

It is unknown whether chromosomal incompatibilities between *R. mendax* and *R. zephyria* that could have initiated homoploid speciation similar to the example of

sunflower hybrids (Rieseberg et al. 1996; Rieseberg et al. 1995). But one type of chromosomal rearrangements, inversions, are likely to differ between the parent taxa and could play a role in the speciation of the *Lonicera* Fly. A recent model suggests that inversions promote reproductive isolation by reducing the recombination rate (Navarro and Barton 2003). This reduces the homogenizing effect of geneflow and facilitates the evolution of allelic combinations that are favorable in a particular environment. As mentioned above, allozyme loci that are under host specific selection in *Rhagoletis* are most likely located in inversions (Feder et al. 2003b).

Inversions have never been physically observed in *Rhagoletis*. Unlike *Drosphila*, *Rhagoletis* lacks polytene chromosomes that can be successfully stained (Berlocher, personal communication). Instead, Feder et al. (2003b) deduced inversion polymorphism in *R. pomonella* from linkage maps for single pair crosses. They observed extensive linkage disequilibrium and reversion of gene order among single pair crosses. Is there evidence for inversion polymorphism in *R. mendax* and *R. zephyria* and in the *Lonicera* Fly itself? Feder et al. (2003a) concluded that the gene flow from Mexican *pomonella* introduced novel inversions into the northern population of *R. pomonella*. The same haplotypes that are indicative of this introgression of new inversions are also shared by *R. mendax* and *R. zephyria*. It is therefore likely that *R. mendax* and *R. zephyria* are polymorphic for inversions as well, although this has not been confirmed by linkage mapping (Feder et al. 2003a). The enzyme locus for Fumarase (*Fum*) is polymorphic in *R. mendax* (and the *Lonicera* Fly) and the allele Fum^{158} is species-diagnostic for *R. mendax* (Berlocher 2000). In *R. tabellaria*, *Fum* has been mapped to the same linkage group as *Had* (McPheron and Berlocher 1985). Berlocher (1995) had found complete

linkage disequilibrium among *Had* and *Fum* in some populations of *R. mendax*. This is consistent with the hypothesis that both loci are contained in the same inversion. In the *Lonicera* Fly I have observed strong linkage between the two loci as well. I did not observe any *Lonicera* Fly individual that combined a Had^{111}/Had^{111} genotype with a Fum^{100}/ Fum^{158} or a Fum^{158}/ Fum^{158} genotype. Combined, both observations make it highly unlikely that the *Lonicera* Fly contains a Had^{111} and Fum^{158} chromosome. This means that *Had* and *Fum* are likely contained in the same chromosomal inversion and that the *Lonicera* Fly has retained the inversion polymorphism of the parent taxa.

Let us imagine that the combination of Had^{111} and Fum^{158} was favorable on *Lonicera* fruit. In the present scenario *Lonicera* Fly individuals would have to be heterozygous for two different inversions (Had^{111} and Fum^{100}; Had^{100} and Fum^{158}) to enjoy the advantage of this allelic combination. Due to random mating, the offspring of these inversion heterozygotes would also produce homozygotes that would be less fit on *Lonicera* and that might show a higher propensity for accepting a parental host fruit. A rare double exchange or gene conversion event could produce an inversion containing both Had^{111} and Fum^{158}. The offspring of individuals homozygous for such a combination would all be well adapted to *Lonicera* and lack individuals with a preference for the host fruits of the parent taxa. This would promote the reproductive isolation of the *Lonicera* Fly. Under a quantitative genetic model inversions will limit the number of possible multilocus genotypes that can be generated in a randomly mating population of hybrid origin, thereby reducing the potential benefits of hybridization. But, at the same time, they could also capture and preserve new favorable hybrid allele combinations.

The example of the *Lonicera* Fly and its parents demonstrates that animal species are not isolated evolutionary units. Closely related species could instead act as biological metaspecies that at certain points in space and time undergo reticulate evolution. The metaspecies could serve as reservoir for adaptive variation and hybridization serves as the process by which pre-existing variation becomes recombined. This new variation could serve as the raw material for adaptive evolution, e.g., during the colonization of a new habitat.

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Appendix A:

SUPPORTING MATERIAL FOR CHAPTER 2

This appendix provides additional information on the methodology that I used in the population genetic analysis of the *Lonicera* Fly system. It also contains additional allozyme data, a test for cytonuclear disequilibria, a haplotype network for the COII sequences, and alignments of haplotypes of the nuclear markers P1700 and P2963 and the mitochondrial COII locus.

Materials and Methods

PCR conditions

The PCR reactions for the three different DNA markers (P1700, P2963 and COII) were identical. I added 1µl of template to 50 or 30 µl of master mix. The master mix consisted of the 10x buffer (15µM), $MgCl_2$ (15µM), forward and reverse primers (10 nM), dNTPs (10 nM) and *Taq*-polymerase (250 units/l, Qiagen GmbH, Hilden). The reactions were run under the following conditions on a Perkin Ellmer Applied Biosystems GeneAmp PCR System 9700 thermocycler.

P1700: 3 min at 94 C; 39 cycles (1 min at 94 C; 1 min starting at 60 C, with a decrease of 0.2 C in the annealing temperature during each cycle; 1 min at 72 C); 10 min at 72 C.

P2963: 3 min at 94 C; 39 cycles (1 min at 94 C; 1 min at 55 C; 1 min at 72 C); 10 min at 72 C.

COII: 3 min at 94 C; 39 cycles (1 min at 94 C; 1 min at 50 C; 1 min at 72 C); 10 min at 72 C.

Additional allozyme data

Test for allele frequency differences between generations and stages of the Lonicera Fly

I collected different generations and stages for allozyme analysis at the *Lonicera* Fly sampling site State College Waupelani. I trapped adult flies in the summer of 2000 and collected the offspring of this generation from infested fruit. In the next summer I trapped another sample of imagines that represents the adult stage of the larvae that I had collected in the previous year. A G-contingency test over all three sub-samples shows no significant allele frequency differences (Table Appendix A-1). This result is consistent with the conclusion of Chapter 2 that the *Lonicera* Fly represents an isolated and self-sustaining population.

Preliminary allozyme data for Lonicera Fly samples from New York and Maryland

In addition to the three samples of the *Lonicera* Fly for which I present population genetic data in Chapter 2, I obtained preliminary data for samples from Corning, New York, and Woodlawn, Maryland. Like the three samples from central Pennsylvania, the New York and Maryland samples show the unique combination of *R. mendax* and *R. zephyria* specific private allozyme alleles at *Had* (Figure Appendix A-1). This indicates that the samples from Corning and Woodlawn are of hybrid origin as well.

Test of cytonuclear disequilibria

One possible consequence of hybridization is the evolution of disequilibria between cytoplasmic and nuclear markers. Cytonuclear disequilibria will arise when one direction of matings between parent taxa is more common than the other (e.g., Lamb and Avise

1986). I tested cytonuclear disequilibria in the *Lonicera* Fly with an exact test using the algorithms of Asmussen and Basten (1994) and Basten and Asmussen (1997) as implemented in the program CND for multi-allelic systems. I pooled *Lonicera* Fly samples from State College Waupelani and Munson to conduct a more powerful test. I limited the test of cytonuclear disequilibria to *Had* and COII because *Had* represents the most promising nuclear marker for detecting such equilibria in the *Lonicera* Fly. I designated alleles Had^{100} and Had^{122} as specific to *R. mendax* and Had^{111} as specific to *R. zephyria*. I defined all COII haplotypes that I found in *R. mendax* and the *Lonicer*a Fly only as specific to *R. mendax* and all COII haplotypes that I detected in *R. zephyria* and the *Lonicera* Fly only as specific to *R. zephyria*. I defined all other haplotypes, including those found only in the *Lonicera* Fly, as shared between the parental taxa. Cytonuclear equilibrium was rejected for none of the nine possible nuclear genotype/cytotype combinations (Table Appendix A-2). The number of sampled individuals is low, but the result of this test is consistent with the lack of detectable hybridization between *R. mendax* and *R. zephyria* (see Chapter 2).

Haplotype network of COII sequences

An additional way to evaluate the hypothesis of a hybrid origin for the *Lonicera* Fly against the competing hypotheses of single parent host race formation and incomplete lineage sorting is the phylogenetic analysis of sequence information. I used a 423-bp-long subsection of mitochondrial COII gene (see Materials and Methods in Chapter 2) to construct a haplotype network using an algorithm developed by (Templeton et al. 1992) as implemented in TCS version 1.18 (Clement et al. 2000). In addition to the sequences

that I generated in this study I added sequences of *R. mendax* and its close relative sparkleberry fly, and *R. zephyria* from Gavrilovic (2001) that have been sampled from multiple locations from within the range of the *Lonicera* Fly's parent taxa. While sharing some haplotypes *R. mendax* and *R. zephyria* each show a large number of mutations that are not shared by the other parent taxon (Figure Appendix A-2). In contrast, the *Lonicera* Fly is spread over the entire network and shares the parental haplotypes with only two exceptions (Figure Appendix A-2). There is no indication that the *Lonicera* Fly forms independent clades or that it is related to haplotypes from one of the two parent taxa only. The former would be consistent with the hypothesis of the *Lonicera* Fly as an extant, undescribed taxon, while the latter would indicate a single-parent origin. The two haplotypes that I found in the *Lonicera* Fly only are separated each by a single mutational step from haplotypes that were found in *R. zephyria* or the sparkleberry fly. They could have arisen by single mutation after the formation of the *Lonicera* Fly, but insufficient sampling of *R. mendax* and *R. zephyria* haplotypes (see Chapter 2) is also a likely explanation for the occurrence of these *Lonicera* Fly "specific" sequences.

References

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locus	allele	adults 2000	larvae 2000	adults 2001	
	N	50	84	40	
Idh	100	0.330	0.393	0.425	
	118	0.660	0.607	0.563	
	128	0.010	0.000	0.013	**p = 0.432**
	N	49	90	40	
Had	100	0.173	0.150	0.238	
	111	0.704	0.666	0.588	
	122	0.122	0.175	0.175	**p = 0.336**
	N	48	90	37	
Aat-2	21	0.677	0.666	0.676	
	25	0.000	0.011	0.000	
	50	0.302	0.311	0.297	
	75	0.021	0.011	0.027	**p = 0.803**
	N	45	90	38	
Pgm	80	0.022	0.028	0.013	
	92	0.033	0.072	0.000	
	100	0.800	0.711	0.803	
	111	0.144	0.189	0.184	**p = 0.081**
	N	50	140	29	
Mpi	33	0.190	0.117	0.103	
	66	0.250	0.281	0.241	
	100	0.560	0.602	0.655	**p = 0.441**

Table Appendix A-1 *Lonicera* Fly State College Waupelani. Comparison of allozyme allele frequencies between two generations and two life stages within one generation. Adult flies were collected from yellow sticky traps. Larvae 2000 consists of larvae that were either directly extracted from fruit or imagines that were reared from infested fruit that was collected in 2000. N = number of individuals. P-values were obtained from a G-contingency test over all three sub-samples.

Nuclear genotype	Cytotype	Joint cytonuclear counts	Disequilibria	Normalized disequilibria	Probability of exact test
M/M	Z	3	0.0068	0.0962	0.961
	M	1	-0.0140	-0.3974	0.813
	S	2	0.0072	0.0784	0.946
M/Z	Z	5	-0.0647	-0.3783	0.185
	M	5	0.0005	0.0027	0.999
	S	8	0.0643	0.3767	0.128
Z/Z	Z	13	0.0579	0.2540	0.279
	M	7	0.0136	0.0962	0.917
	S	3	-0.0715	-0.5284	0.090

Table Appendix A-2 Test for cytonuclear disequilibria between COII and *Had* in the *Lonicera* Fly (State College Waupelani and Munson samples pooled). M = *R. mendax* nuclear alleles and haplotypes (Had^{100} and Had^{122} and COII haplotypes private to *R. mendax*), Z = *R. zephyria* nuclear allele and haplotypes (Had^{111} and COII haplotypes private to *R. zephyria*), S = shared COII haplotypes (includes haplotypes only observed in the *Lonicera* Fly).

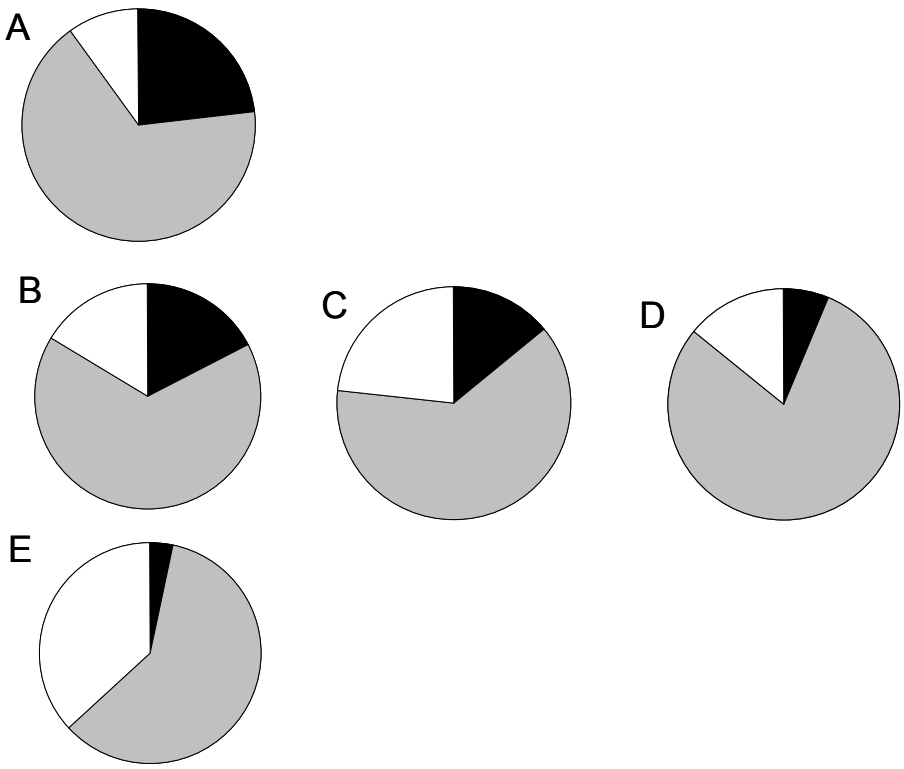

Figure Appendix A-1 Allele frequencies for *Had* in five populations of the *Lonicera* Fly. **A**: Corning, NY (n = 15). **B**: State College Waupelani, PA (n = 179). **C**: State College Slab Cabin Park, PA (n = 34). **D**: Munson, PA (n = 32). **E**: Woodlawn, MD (n = 32). Black = *Had*100. Grey = *Had*111. White = *Had*122.

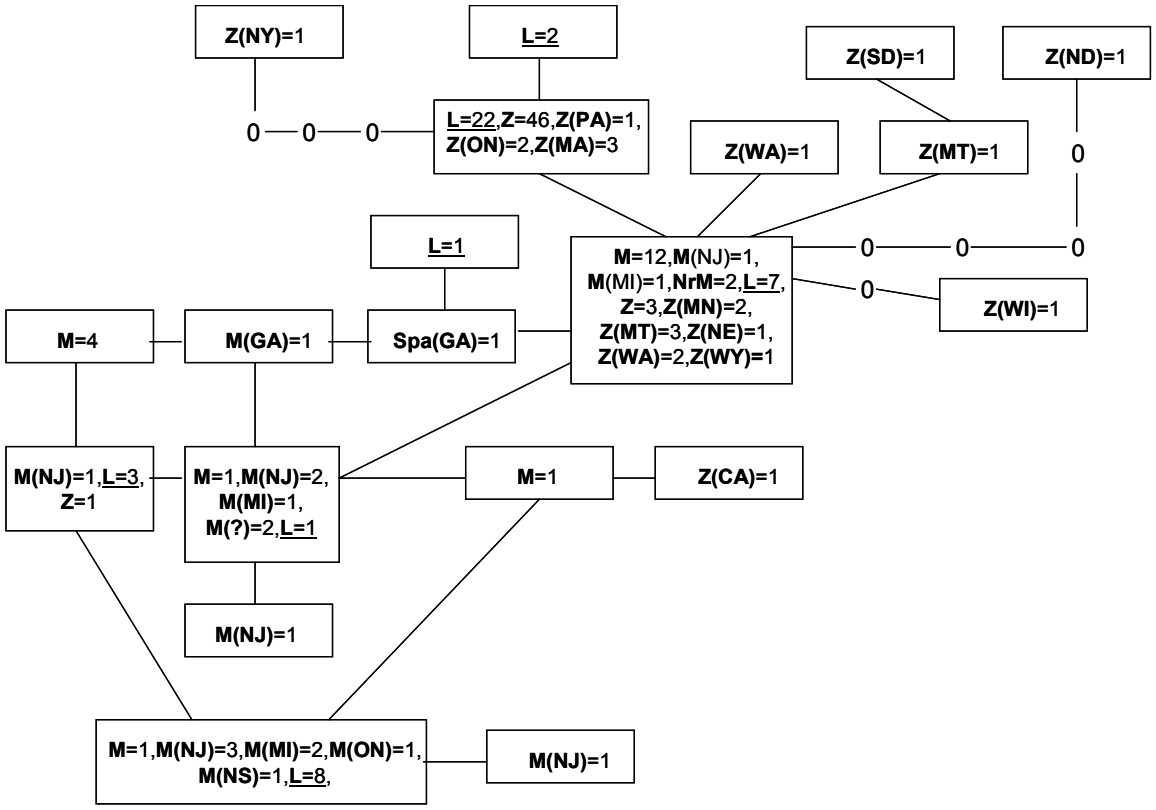

Figure Appendix A-2 Haplotype network of a 423-bp-long subsection of the mitochondrial COII gene. Each square represents a separate haplotype and lines indicate a single mutational step. Missing intermediate haplotypes are presented by "0." M = *R. mendax*, L = *Lonicera Fly*, Z = *R. zephyria*, Spa = sparkleberry fly. United States state and Canadian province abbreviations in brackets indicate the sampling locations for haplotype sequences that are reported in Gavrilovic (2001) with the exception of M(NJ) that represent individuals collected in New Jersey as part of this study. Taxon abbreviations without sampling location in brackets are from Snyder Cty, PA (R. mendax) and State College and Munson, PA (*Lonicera* Fly and *R. zephyria*). The integers indicate the number of individuals with a particular haplotype from each population.

Appendix A: Alignment of P1700. Consensus sequences of two haplotypes for each individual. Four clones of P1700 were sequenced for each individual. Singleton mutations are indicated by a lower case letter. If cloning P1700 from a single individual yielded more than two haplotypes that are distinguished by a sequence difference (other than a singleton mutation), all haplotypes are reported. Lines delimited cloned sequences from individual flies. L = *Lonicera* Fly State College Waupelani; RZ = *R. zephyria* Munson; RM = *Rhagoletis mendax* Snyder County.

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                  1 1111111112 2222222223 3333333334 4444444445 5555555556 6666666667
            1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
L020523-01  ACAAACTAGT -AGATACAAA TTTCCTTGGT AGGTCTGAAA TTGTACTAAA GAGTTAGAAA ATAACGAAAA
L020523-01  ........TA Ca........ .......... .......... .......... .......... ..........
L020523-02  .......... -......... .........t .......... .......... .......... ..........
L020523-02  .......... -......... .......... .......... ...C...... .......... ..........
L020523-02  .......... -......... .......... .......... .......... .......... ..........
L020523-03  .......... -......... .......... .......... ...C...... .......... ..........
L020523-03  .......... -......... .......... .......... ...C...... .......... ..........
L020523-04  ........TA C......... ........g. .......... .......... .......... ..........
L020523-04  ........TA C......... ........g. .......... .......... .......... ..........
L020523-05  ...a...... -......... .......... .......... ...C...... .......a.. ..........
L020523-05  ...a...... -......... .......... .......... ...C...... .......a.. ..........
L020523-10  .......... -......... .......... .......... ...C...... .......... ..........
L020523-10  .......... -......... .......... .......... ...C...... .......... ..........
RM020923-01 .......... -......... .......... .......... .......... .......... ..........
RM020923-01 .......... -......... .......... .......... .......... .......... ..........
RM020923-02 ........TA C......... .......... .......... .......... .......... ..........
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RM020923-03 .......... -......... .......... .......... .......... .......... ..........
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RM020923-05   ..a..... -......... .......... .......... .......... .......... ..........
RM020923-05 .......... -......... .......... .......... .......... .......... ..........
RZ020516-01 .......... -......... .......... .......... ...C...... .......... ..........
RZ020516-01 .......... -......... .......... .......... ...C...... .......... ..........
RZ020516-02 .......... -......... .......... .......... ...C...... .......... ..........
RZ020516-02 .......... -......... .......... .......... ...C...... .......... ..........
RZ020516-03 ......t... -......... .......... .......... ...C...... .......... ..........
RZ020516-03 .......... -....a.... .......... .......... ...C...... .a........ ..........
RZ020516-04 .......... -......... .......... .......... ...C...... .......... ..........
RZ020516-04 .......... -...t..... .......... .......... ...C...... ....t..... ..........
RZ020516-05 .......... -......... .......... .......... ...C...... .......... ..........
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RZ020517-01 .......... -......... .......... .......... ...C...... .......... ..........
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RZ020517-02 .......... -......... .......... .......... ...C...... .......... ..........
RZ020517-02 .......... -......... .......... .......... ...C...... .......... ..........
RZ020517-02 .......... -......... .......... .......... ...C...... .......... ........a.
RZ020517-03 .......... -......... .......... .......... ...C...... .......... ..........
RZ020517-03 .......... -......... .......... .......... ...C...... .......... ..........
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           1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
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L020523-01 .......... .......... .......... .......... .......... .......... ..........
L020523-02 .......... .......... .......... .......... .......... .......... .........t.
L020523-02 .......... .......... ........A. .......... .......... .......... ..........
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L020523-10 .......... .......t.. c......... .......... .......... .......... ..........
L020523-10 .......... .......... .......... .........a .......... .......... ........C.
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RZ020517-05 .......... .....a.t.. .......... G......... .......... .......... ..........
RZ020517-05 .......... .....a.t.. .......... G......... .......... .......... ..........
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            1111111112 2222222223 3333333334 4444444445 5555555556 6666666667 7777777778
            1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
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L020523-01  .......... .......... .......... .......... .......... .......... .T.......
L020523-02  .......... .......... .......... .......... .......... .....---.. .T.......
L020523-02  .......... .......... .......... .......... .......... .....---.. .T.......
L020523-02  .......... .......... .......... .......... .......... .....---.. .T.......
L020523-03  .......... .......... .......... .......... .......... .t...---.. .T.......
L020523-03  .......... .......... .......... .......... .......... .....---.. .T.c......
L020523-04  .......... .......... .......... .......... .......... .......... .T.......
L020523-04  .......... .......... .......... .......... .......... .......... .T.......
L020523-05  .......... .......... .......... .......... .......... .....---.. .T.......
L020523-05  .......... .......... .......... .......... .......... .....---.. .T.......
L020523-10  ..t....... .......... .......... .......... .......... .....---.. .T.......
L020523-10  .......... .......... .......... .......... .......... .....---.. .T.......
RM020923-01 .......... .......... .......... .......... .......... .....---.. .T.......
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RM020923-02 .......... .......... .......... .......... .......... .....---.. .T.......
RM020923-03 .......... .......... .......... .......... .......... .....---.. .T.......
RM020923-03 .......... .......... .......... .......... .......... .......... .T.......
RM020923-04 .......... .......... .......... .......... .......... .....---.. .T.......
RM020923-04 .......... .......... .......... .......... .......... .....---.. .T.......
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RM020923-04 .......... .......... .......... .......... .......... .....---.. .T.......
RM020923-05 .......... .......... .......... ..c....... .......... .......... .T.......
RM020923-05 .......... ....t..... .......... .......... .......... .....---.. .T.......
RZ020516-01 .......... .......... .......... .......... .......... .....---.. .T.......
RZ020516-01 .......... .......... .......... .......... .......... .....---.. .T.......
RZ020516-02 .......... .......... .......... ...g...... .......... .....---.. .T......G.
RZ020516-02 .........t .......... .......... .......... .......... .....---.. .T.......
RZ020516-03 .......... .......... .......... .......... .......... .....---.. .T.......
RZ020516-03 .......... .......... .......... .......... .......... .....---.. .T.......
RZ020516-04 .......... .......... .......... .......... .......... .....---.. .T.......
RZ020516-04 .......... .......... .......... .......... .......... .....---.. .T.......
RZ020516-05 .......... .......... .......... .......... .......... .....---.. .T.......
RZ020516-05 .......... .......... .......... .......... .......... .....---.. .T.......
RZ020517-01 .......a.. .......... .......... .......... .......... .....---.. .T.......
RZ020517-01 .......... .......... .......... .......... .......... .....---.. .T.......
RZ020517-02 .......... .......... .......... .......... .......... .....---.. .T.......
RZ020517-02 .......... .......... .......... .......... .......... .....---.. .T.......
RZ020517-02 .......... a......... .......... .......... .......... .....---.. ..........
RZ020517-03 .......... .......... .......... .......... .......... .....---.. .T.......
RZ020517-03 .......... .......... .......... .......... .......... .....---.. .T.......
RZ020517-04 .......... .......... .......... .......... .......... .....---.. .T......T.
RZ020517-04 .......... .......... .......... .........a .......... .....---.. .T.......
RZ020517-05 .......... .......... .......... .......... .......... .....---.. .T.......
RZ020517-05 .......... .......... .......... .......... .......... .....---.. .T.......
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            2222222222 2222222223 3333333333 3333333333 3333333333 3333333333 3333333333
            8888888889 9999999990 0000000001 1111111112 2222222223 3333333334 4444444445
            1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
L020523-01  CATAACGATC TTCCAGTTTT CTTAAAGGGA GGACCAGTAG ACAAAGTATT GTTTGGTTTG ACGGTTGGGC
L020523-01  .......... .......... .......... .......... .......... .......... ..........
L020523-02  .......... .......... .......... .......... .......... .......C.. ..........
L020523-02  .......... .......... .......... .......... .......... .......... ..........
L020523-02  .......... .......... ..t....... .......... .......... .......... ..........
L020523-03  .......... .......... .......... .......... .......... .......... ..........
L020523-03  .......... .......... .......... .......... .......... .......... ..........
L020523-04  .......... .......... .......... .......... .......... .......... ..........
L020523-04  .......... .......... .......... .......... .......... .......... ..........
L020523-05  .......... .......... .......... .......... .......... .......... ..........
L020523-05  .......... .......... .......... .......... .......... .......... ..........
L020523-10  .......... .......... .......... .......... .......... .......... ..........
L020523-10  .......... .......... .......... .......... .......... .......... ..........
RM020923-01 .......... .......G.. .......... .......... .......... .......... ..........
RM020923-01 .......... .......G.. .......... .......... .......... .......... ..........
RM020923-02 .......... .......... .......... .......... .......... .t........ ..........
RM020923-02 .......... .......Gt. .......... .......... .......... .......... ..........
RM020923-03 .......... .......... .......... .......... .......... g......C.. ..........
RM020923-03 .......... .......... .......... .......... .......... .......... ..........
RM020923-04 .......... .......G.. .......... .......... .......... .......... ..........
RM020923-04 .......... .......... .......... .......... .......... .......C.. ..........
RM020923-04 .......... .......G.. .......... .......... .......... .......... ..........
RM020923-04 .......... .......... .......... .......... .......... .......... ..........
RM020923-05 .......... .......... .......... .......... .......... .......... ..........
RM020923-05 .......... .......G.. .......... .......... .......... .......... ..........
RZ020516-01 .......... .......... .......... .......... .......... .......... ..........
RZ020516-01 .......... .......... .......... .......... .......... .......... ..........
RZ020516-02 .......... .......... .......... .......... .......... .......... ..........
RZ020516-02 .......... .......... .......... .......... .......... .......... ..........
RZ020516-03 .......... .......... .......... .......... .......... .......... ..........
RZ020516-03 .......... .......... .......... .......... .......... .......... ..........
RZ020516-04 .......... .......... .......... .......... .......... .......... ..........
RZ020516-04 .......... .......... .......... .......... .......... .......... ..........
RZ020516-05 .......... .......... .......... .......... .......... .......... ..........
RZ020516-05 .......... .......... .......... .......... .......... .......... ..........
RZ020517-01 .......... .......... .......... .......... .......... .......... ..........
RZ020517-01 .......... .......... .......... .......... .......... .......... ..........
RZ020517-02 .......... .......... .......... .......... .......... .......... ..........
RZ020517-02 .......... .......... .......... .......... .......... .......... ..........
RZ020517-02 .......... .......... .......... .......... .......... .......... ..........
RZ020517-03 .......... .......... .......... .......... .......... .......... ..........
RZ020517-03 .......... .......... .......... .......... .......... .......... ..........
RZ020517-04 .......... .......... .......... .......... .......a.. .......... ..........
RZ020517-04 .......... .......... .......... .......... .......... .......... ..........
RZ020517-05 .......... .......... .......... .......... .......... .......... .......g..
RZ020517-05 .......... .......... .......... .......... .......... .......... .......g..
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```
            3333333333 3333333333 3333333333 3333333333 3333333334 4444444444 4444444444
            5555555556 6666666667 7777777778 8888888889 9999999990 0000000001 1111111112
            1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
L020523-01  TTTGCGGCAT AGGACTTGCA GGCATAGTCC AAATGATCTA TGCATTGGGT TTCAAGAAGA AGTCGGCTTA
L020523-01  .......... .......... .......... .......... .......... .......... ..........
L020523-02  .......... .......... .....CA..G .....G.... .......... .......... ..........
L020523-02  .......... .......... .......... .......... .......... .......... ..........
L020523-02  .......... .......... .......... .......... .......... .......... ..........
L020523-03  .......... .......... .......... .......... .......... .......... ..........
L020523-03  .......... .......... .......... .......... .......... .......... ..........
L020523-04  .......... .......... .......... .......... ....t..... .......... ..........
L020523-04  .......... .......... .......... .......... ....t..... .......... ..........
L020523-05  .......... .......... .......... .......... .......... .......... ..........
L020523-05  .......... .......... .......... .......... .......... .......... ..........
L020523-10  .......... .......... .......... .......... .......... .......... ..........
L020523-10  .......... .......... .......... .......... .......... .......... ..........
RM020923-01 .......... .......... .......... .......... .......... .......... ..........
RM020923-01 .......... .......... .......... .......... .......... .......... ..........
RM020923-02 .......... .......... .......... .......... .......... .......... ..........
RM020923-02 .......... .......... .......... .......... .......... .......... ..........
RM020923-03 .......... .......... .....CA..G .....G.... .......... .......... ..........
RM020923-03 .......... .......... .......... .......... .......... .......... ..........
RM020923-04 .......... .......... .......... .......... .......... .......... ..........
RM020923-04 .......... .......... .....CA..G .....G.... .......... .......... ..........
RM020923-04 .......... .......... .......... .......... .......... .......... ..........
RM020923-04 .......... .......... .......... .......... .......... .......... ..........
RM020923-05 .......... .......... ......A... .......... .......... .......... ..........
RM020923-05 .......... .......... .......... .......... .......... .......... ..........
RZ020516-01 .......... .......... .......... .......... .......... .......... ..........
RZ020516-01 .......... .......... .......... .......... .......... .......... ..........
RZ020516-02 .......... .......... .......... .......... .......... .......... ..........
RZ020516-02 .......... .......... .......... ......t... .......... .......... ..........
RZ020516-03 .......... .......... .......... .......... .......... .......... ..........
RZ020516-03 .......... .......... .......... .......... .......... .......... ..........
RZ020516-04 .......... .......... .......... .......... .......... .......... ..........
RZ020516-04 .......... .......... .......... .......... .......... .......... ..........
RZ020516-05 .......... .......... .......... .......... .......... .......... ..........
RZ020516-05 .......... .......... .......... .......... .......... .......... ..........
RZ020517-01 C......... .......... .......... .......... .......... .......... ..........
RZ020517-01 .......... .........a .......... .......... .......... .......... ..........
RZ020517-02 .......... .......... .......... .......... .......... .......... ..........
RZ020517-02 C......... .......... .......... ......t... .......... .......... ..........
RZ020517-02 .......... .......... .......... .......... .......... .......... ..........
RZ020517-03 .......... .......... .......... .......... .......... .......... ..........
RZ020517-03 .......... .......... .......... .......... .......... .......... ..........
RZ020517-04 .......... .......... .......... .......... .......... .......... ..........
RZ020517-04 .......... ......t... .......... .......... .......... .......... ..........
RZ020517-05 .......... .......... .......... .......... .......... .......... ..........
RZ020517-05 .......... .......... .......... .......... .......... .......... ..........
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```
            44
            22
            12
L020523-01  AA
L020523-01  ..
L020523-02  ..
L020523-02  ..
L020523-02  ..
L020523-03  ..
L020523-03  ..
L020523-04  ..
L020523-04  ..
L020523-05  ..
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L020523-10  ..
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RZ020517-01 ..
RZ020517-02 ..
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RZ020517-02 ..
RZ020517-03 ..
RZ020517-03 ..
RZ020517-04 ..
RZ020517-04 ..
RZ020517-05 ..
RZ020517-05 ..
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Appendix A: Alignment of 2963. Consensus sequences of two haplotypes for one *Lonicera* Fly individual. Four clones of P2963 were sequenced from this individual. LM = *Lonicera* Fly Munson

```
                  1 1111111112 2222222223 3333333334 4444444445 5555555556 6666666667
            1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
LM021030-02 TAGTCAACGA CCTGCTTATT TCACATTCAG CATTTGATAG TATAACTAAG TTTTCTTAAT GAACAATCAT
LM021030-02 .......... .......... .......... .......... .......... .......... ..........

                                     1 1111111111 1111111111 1111111111 1111111111
            7777777778 8888888889 9999999990 0000000001 1111111112 2222222223 3333333334
            1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
LM021030-02 TTCTTATCGT TACCACTCTG CAAAAGGATA ATAAAAATAT TTGTAAGTAA ATACAATTTA TATATGTATG
LM021030-02 .......... .......... .......... .......... .......... .......... ..........

            1111111111 1111111111 1111111111 1111111111 1111111111 1111111112 2222222222
            4444444445 5555555556 6666666667 7777777778 8888888889 9999999990 0000000001
            1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
LM021030-02 TATGCGATAA ATTTTCGCTC TACTTTTAGG ATTTATGCAT TT-------- -AGC-----G CAAATACAAA
LM021030-02 .......... .......... .......... .......... ..TATTTATT T..GATTAT. ..TT......

            2222222222 2222222222 2222222222 2222222222 2222222222 2222222222 2222222222
            1111111112 2222222223 3333333334 4444444445 5555555556 6666666667 7777777778
            1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
LM021030-02 ACAAAAACTT CCACAGATTG TGTTTTATGT AGTATTTTAT GTTTGCAAAT ACATTAATTT TAATAGATTT
LM021030-02 .......... .......... .......... ......G... .......... ..C....... ..........

            2222222222 2222222223 3333333333 3333333333 3333333333 3333333333 3333333333
            8888888889 9999999990 0000000001 1111111112 2222222223 3333333334 4444444445
            1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
LM021030-02 TTATAGGTTA TATTTAATTA ATGAATAAAT GTGTACTAAG TTGATATAGA AAGGATTTTC GTGAATTAAG
LM021030-02 .......... .......... .......... .......... .......... .......... ..........

            333333
            555555
            123456
LM021030-02 GTGCA
LM021030-02 .....
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Appendix A: Alignment of COII. L = *Lonicera* Fly State College Waupelani; LM = *Lonicera* Fly Munson; Z = *R. zephyria* Munson; RZO = *R. zephyria* Old Main; RMS = *Rhagoletis mendax* Snyder County.

```
                    1 1111111112 2222222223 3333333334 4444444445 5555555556 6666666667
           1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
L011101-07 CTTAAAACTT CATTCTTCCA TGATCATACA CTTATAATTT TAGTAATAAT TACTACTTTA GTTGGTTATT
L011101-06 .......... .......... .......... .NN....... ...N...... .......... ..........
L011101-08 .......... .......... .......... .......... .......... .......... ..........
L011101-09 ...N.N.... .......... .......... .......... .......... .......... ..........
L011101-10 ...N.N.... .......... .......... .N........ .......... .......... ..........
L020207-06 .....N.... .......... .......... .......... .......... .......... ..........
L020207-07 N.N.NN.... .......... .......... .......... .......... .......... ..........
L020207-08 ......N... .......... .......... .......... ....G..... .......... ....N.....
L020207-09 ..N....... .......... .......... .......... ....G..... .......... ..........
L020207-10 NNNNNN.... .......... .......... ....N..... .......... .......... ..........
L020413-06 ..N....... .......... .......... .......... .......... .......... ..........
L020413-07 NNNNNNN... .......... .......... .......... .......... .......... ..........
L020413-08 N.NN.N.... .......... .......... .......... .......... .......... ..........
L020413-09 NNNNNNNN.. .......... .......... .......... .......... .......... ..........
L020413-10 NNN....... .......... .......... .......... .......... .......... ..........
L020514-01 NNNNNN.... .......... .......... .......... .......... .......... ..........
L020514-02 NNNNNNN... .......... .......... .......... .......... .......... ..........
L020514-03 NNNNNN.... .......... .......... .......... .......... .......... ..........
L020514-04 NNNNNN.... .......... .......... .......... .......... .......... ..........
L020514-05 NNNNNNN... .......... .......... .......... .......... .......... ..........
L020523-01 N..N...... .......... .......... .......... .......... .......... ..........
L020523-02 ...N.N.... .......... .......... .......... .......... .......... ..........
L020523-03 ....N..... .......... .......... .......... .......... .......... ..........
L020523-04 .....N.... .......... .......... .......... .......... .......... ..........
L020523-05 ...N.N.... .......... .......... .......... .......... .......... ..........
L020523-06 NNN..N.... .......... .......... .......... .......... .......... ..........
L020523-07 N....N.... .......... .......... .......... ...N...... .......... ..........
L020523-08 N....N.... .......... .......... ....N..... .......... .......... ..........
L020523-09 ....NN.... .......... .......... .......... .......... .......... ..........
L020523-10 ...N.N.... .......... .......... ....N..... .......... .......... ..........
Z020207-11 ......NN.. .......... .......... N..NNNN... ...N...... .......... ..........
Z020207-12 ....NN.... .......... .......... .....N.... .......... .......... ..........
Z020207-13 ...N.N.... .......... .......... .......... .......... .......... ..........
Z020207-14 .....N.... .......... .......... .......... .......... .......... ..........
Z020413-11 ...NNNN... .......... .......... .......... .......... .......... ..........
Z020413-12 .NNN.N.... .......... .......... .......... .......... .......... ..........
Z020413-13 ...N.N.... .......... .......... .......... .......... .......... ..........
Z020413-14 NNNNNNN... .......... .......... .......... .......... .......... ..........
Z020413-15 ..NNNNN... .......... .......... .......... .......... .......... ..........
Z020516-01 NNNNNN.... .......... .......... .......... .......... .......... ..........
Z020516-02 ..A.NN.... .......... .......... .......... .......... .......... ..........
Z020516-03 NNNNNN.... .......... .......... .......... .......... .......... ..........
Z020516-04 ..NN.N.... .......... .......... .......... .......... .......... ..........
Z020516-05 ..NN.N.... .......... .......... .......... .......... .......... ..........
Z020517-01 ....NN.... .......... .......... .......... .......... .......... ..........
Z020517-02 ....NN.... .......... .......... .......... .......... .......... ..........
Z020517-03 ..NN.N.... .......... .......... .......... .......... .......... ..........
Z020517-04 .....N.... .......... .......... ....N..... .......... .......... ..........
Z020517-05 ..N..N.... .......... .......... .......... .......... .......... ..........
Z020603-09 NNN..N.... .......... .......... .......... .......... .......... ..........
Z020603-10 ....NN.... .......... .......... .......... .......... .......... ..........
Z020603-11 ....NN.... .......... .......... .......... .......... .......... ..........
Z020603-12 .....N.... .......... .......... .......... .......... .......... ..........
Z020603-13 .....N.... .......... .......... .......... ...N...... .......... ..........
Z020605-01 ...N.N.... .......... .......... .......... .......... .......... ..........
Z020605-02 N...NN.... .......... .......... .......... .......... .......... ..........
Z020605-03 NN.N.N.... .......... .......... .......... .......... .......... ..........
Z020605-04 NN...N.... .......... .......... .......... .......... .......... ..........
Z020605-05 ....NN.... .......... .......... .......... .......... .......... ..........
Z020617-01 ...N.N.... .......... .......... .......... .......... .......... ..........
Z020617-02 .....N.... .......... .......... .......... .......... .......... ..........
Z020617-03 .....N.... .......... .......... .......... .......... .......... ..........
Z020617-04 ...N.N.... .......... .......... .......... .......... .......... ..........
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Z020617-05   ..NN.N.... .......... .......... .......... .......... .......... ..........
RMS020923-01 NNNNNNNNNN NNNNNNNNNN NNNN.....N .......... ....G..... .......... ..........
RMS020923-02 NNNN.N.... .......... .......... .......... .......... .......... ..........
RMS020923-03 NNNNNN.... .......... .......... .......... .......... .......... ..........
RMS020923-04 NNNNNNNNNN NNNNNNN... .......... .......... .......... .......... ..........
RMS020923-05 NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN N......... ..........
RMS020923-06 N.NNNN.... .......... .......... .......... .......... .......... ..........
RMS020923-07 NNNNNN.... .......... .......... .......... .......... .......... ..........
RMS020923-08 NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNN... .......N.. .......... ..........
RMS020923-09 N.NN.N.... .......... .......... .......... .......... .......... ..........
RMS020923-10 NNNNNNNNNN NNNNNNNNNN N......NNN .......... .......... .......... ..........
RMS020924-01 NNNNNN.... .......... .......... .......... .......... .......... ..........
RMS020924-02 NNNNNN.... .......... .......... .......... .......... .......... ..........
RMS020924-03 NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNN... .......... .......... ..........
RMS020924-04 NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN N....N.N..
RMS020924-05 NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN ...N...... .......... .......... ..........
RMS020924-06 NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNN... .......... ..........
RMS020924-08 NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN ...N..N... .N..NN.N.. .......... ..........
RMS020925-01 NNNNNN.... .......... .......... .......... .......... .......... ..........
RMS020925-02 NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNN.... ......N...
RMS020925-03 N.NN.N.... .......... .......... .......... .......... .......... ..........
RMS020925-04 NNNNNNNNNN NNNNNNNNNN NNNNN..... ..N....... .......... .......... ..........
RMS020925-05 NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN .......... .......N.. .......... ..N..N....
RMS020925-06 NNNNNNNNNN NNNNNNNNN. .......... .......... .......... .......... ..........
RMS020925-07 NNNNNNNNNN NNNN..... .......... .......... .......... .......... ..........
RMS020925-09 NNNNNN.... .......... .......... .......... .......... .......... ..........
RMS020925-11 ...N.N.... .......... .......... .......... .......... .......... ..........
RMS020925-12 NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNN..... ..........
RMS020925-13 .....N.... .......... .......... .......... .......... .......... ..........
RMS020925-14 ...NNN.... .......... .......... .......... .......... .......... ..........
RMS020925-15 NNNNNNNNNN .......... .......... .......... ....G..... .......... ..........
RMS020925-16 NNNNNN.... .......... .......... .......... .......... .......... ..........
RMS020925-17 NNNNNN.... .......... .......... .......... .......... .......... ..........
RMS020925-18 NNNNNN.... .......... .......... .......... .......... .......... ..........
LM021030-01  NNNNNNNNNN NNNNNNNNNN NN........ .......... .....N.NN. .......... ..........
LM021030-02  NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN N....N.... ....N...NN .......... ..........
LM021030-03  NNNNNN.... .......... .......... .......... .......... .......... ..N.......
LM021030-04  NNNNNNNNNN N......... .......... .......... .......... .......... ..........
LM021030-05  NNNNNNNNNN .......... .......... .......... .......... .......... ..........
LM021030-06  NNNNNNNNNN .......... .......... .......... .......... .......... ..........
LM021030-07  NNNNNNNNNN NNNNNNNNNN .......... .......... .......... .......... ..........
LM021030-08  NNNNNNNNNN .......... .......... .......... .......... .......... ..........
LM021030-09  NNNNNNNNNN NN........ .......... .......... .......... .......... ..........
LM021030-10  NNNNNN.... .......... .......... N......... .......... ....N..... ..........
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LM021030-12  NNNNNNNNNN .......... .......... .......... .......... .......... ..........
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L011101-06   ....N..N.. .......... ...N.N.... .......... .......... N......... A.........
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LM021030-18  .......... .......... .......... .......... .......... .......... ..........
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RZO030130-18 .......... .......... .......... .......... .......... .......... ..........
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L011101-06   ....NNNN.. .......N.. .N....N.N. ....NN.... N....N.... N....N.N.. ....N.NN..
L011101-08   .......... .......... ........NN N.N....... .......... .......... .C........
L011101-09   .......... .......... .......... .......... .......... .......... .C........
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RMS020923-04 .......... .......... .......... .......... .......... .......... ..........
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RZO030130-14 .......... .......... .......... .......... .......... .......... .C........
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RZO030130-19 .......... .......... .......... .......... .......... .......... ..........
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L011101-07   CAGTTTTATG CCAATTGTAA TTGAAAGTAT CCCTATTAAT CATTTTATTA AATGAATTAC AAATANAGTA
L011101-06   ...NN.N.NN ..N..N.-.. ......NN.. .N......NN .N.N.NN... .N..N-NN.. .N.N..N.N.
L011101-08   .......N.. .........N NNN....... .......NNN NN.NN.N.NN NNNN..N... ......NNNN
L011101-09   .......... .......... .......... .......... .......... .......... .....T....
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L020413-08   .......... .......... .......... .......... .......... .......... .....T....
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L020523-04   .......... .......... .......... .......... .......... ....N..N.. ........N.
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RMS020923-07 .........A .......... .......... .......... .......... ..........  ..N..T....
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RMS020924-02 .......... .......... .......... .......... .......... ..........  .....T....
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RZO030130-03 .......... .......... .......... .......... .......... ..........  .....T..NN
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RZO030130-06 .......... .......... .......... .......... .......... ..........  .....T..N.
RZO030130-07 .......... .......... .......... .......... .......... ..........  .....T....
RZO030130-08 .......... .......... .......... .......... .......... ..........  .....TN.N.
RZO030130-09 .......... .......... .......... .......... .......... ..........  .....T...N
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RZO030130-15 .......... .......... .......... .......... .........N ..........  .N.N.T..N.
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RZO030130-19 .......... .......... .NNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNN.NNNN
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RMS020923-04 NNNNNNNN
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Appendix B

SUPPORTING MATERIAL FOR CHAPTER 3

Host fruit and fly phenology

To determine which fruit stages are used by the *Lonicera* Fly, *R. zephyria* and *R. pomonella* for oviposition in the field, I monitored fruit and fly phenology on the respective host plants. For both the no-choice host acceptance and the three-way choice experiment it is important to use fruit that is at an optimal stage for oviposition, because systematic differences in fruit quality among host fruit species can lead to a bias in the response of the fly taxa. There is no *R. mendax* population in the vicinity of State College that is detectable by trap catches and I had to revert to information from the literature. No such information was available for the *Lonicera* Fly and *R. zephyria* and I had to generate it as part of this study.

For each of the three host plant species I selected three individual plants (or plant clusters) for a phenology study in the summer of 2001. All study sites were located on the Pennsylvania State University Campus at University Park (*Lonicera*: Golfcourse; snowberry: Old Main lawn; hawthorn: Wagner and Forum Buildings, Hastings Road). To collect the host plant phenology five branches were haphazardly chosen on each host plant (or cluster of plants). Counting from the tip of each branch, I chose the first seven nodes and all associated side branches for the monitoring of host fruit phenology. In each of these sections I counted all flower buds, flowers and fruit. The fruit was further classified according to color (green and white for snowberry, and green and red for

Lonicera and hawthorn) and firmness (hard and soft). Decayed fruit formed an additional sub-category. I term the sum of flower buds, flowers and fruit "bud sites". The panels in Figure 1 (Appendix Chapter 3) display the counts for a particular fruit stage as a proportion of all bud sites. The same set of branches was sampled bi-weekly throughout the field season. On the same sampling dates I recorded the trap catches of flies on five yellow sticky traps (Pherocon AM, Trece Inc., Salinas). I placed the traps in the same plant or cluster of plants for which I was recording the host fruit phenology. All sites were spatially separated from other potential hosts of *Rhagoletis*. I therefore assumed that the adult individuals that I caught represent the *Rhagoletis* taxon that is specialized on the host plant in which the traps had been placed.

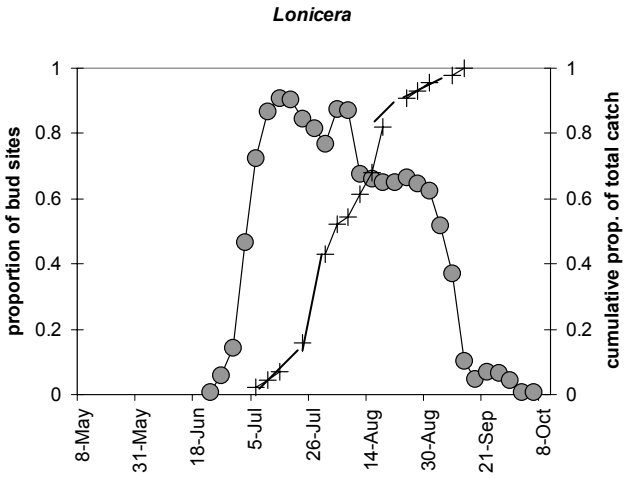
Lonicera fruit ripened at the end of June 2001 and ripe fruit, which is red and soft, remained on the branches until the end of August. I only trapped *Lonicera* Flies during this time period, indicating that red and soft *Lonicera* fruit is the preferred host fruit stage for oviposition by the *Lonicera* Fly (Figure 1A).

Snowberry has an unusual phenology because it flowers and fruits continuously throughout the summer (Figure 1B). I started catching *R. zephyria* at the end of June, before fruit turned white (Figure 1B). This matches observations of oviposition into green fruit by females in the field. The trap catches do not represent the entire time period during which flies would have been present under natural conditions. The site at Old Main lawn was sprayed with a broad spectrum insecticide at the end of July to control vectors of dutch elm disease. After the treatment trap catches of *R. zephyria* declined dramatically. At a different snowberry site in Munson, Clearfield County, I was able to

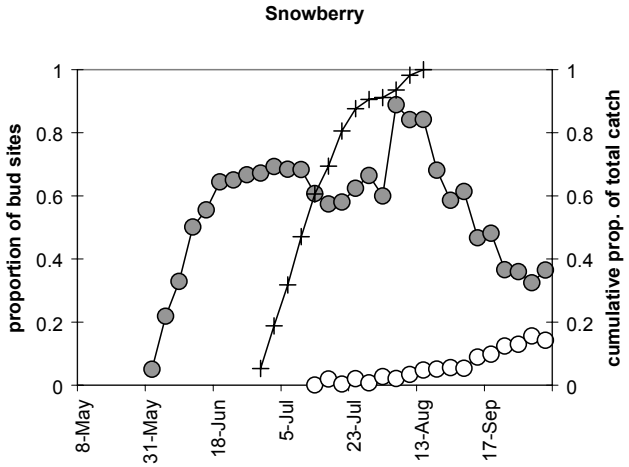
observe adult flies until the end of September. At this site I observed oviposition into white snowberry fruit by *R. zephyria* females. Both green and white fruit stages of snowberry are therefore used by *R. zephyria* in the field.

On hawthorn trap catches of *R. pomonella* closely match the phenology curve of red fruit. This fruit stage therefore likely represents the preferred stage for oviposition by *R. pomonella* (Figure 1C).

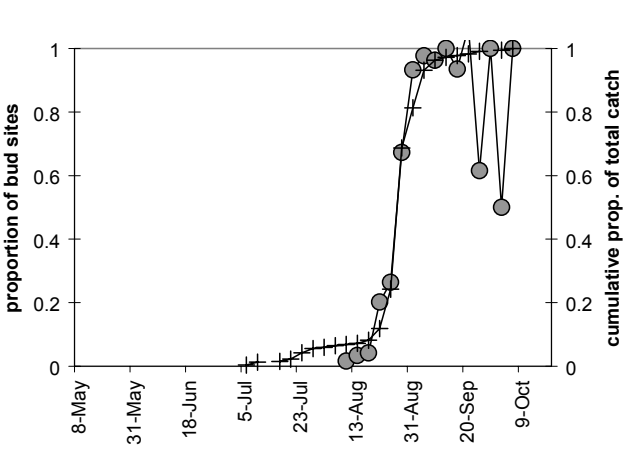
Figure Appendix B-1 Fruit and fly phenology



A. Phenology of *Lonicera* fruit and the *Lonicera* Fly at Golfcourse East. Grey circles: proportion of bud sites (see text) with red, soft fruit. +: cumulative proportion of total *Lonicera* Fly catch.



B. Phenology of snowberry fruit and *R. zephyria* at Old Main South. Grey circles: proportion of bud sites (see text) with green fruit. White circles: proportion of bud sites with white fruit +: cumulative proportion of total *R. zephyria* catch. Note: patch was sprayed with insecticide in mid-July (see text).



C. Phenology of hawthorn fruit and *R. pomonella* at Wagner Building. Grey circles: proportion of bud sites (see text) with red fruit. +: cumulative proportion of total *R. pomonella* catch.

VITA

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EDUCATION

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GRANTS

1999: First Draft of methods section and budget for a grant from the Deutsche Forschungsgemeinschaft (German Science Foundation) to Thomas Hoffmeister for my diploma thesis on the host race differentiation of *Rhagoletis cerasi* (HO 1251/6-1).

2003: First draft of "Ecologically Mediated Hybrid Speciation in a Diploid, Bisexual Animal", awarded by the National Science Foundation to Bruce McPheron, Ottar Bjornstad and Wendell Roelofs (DEB-0343771).

PUBLICATIONS

Schwarz, D., B. A. McPheron, G. B. Hartl, E. F. Boller, T. H. Hoffmeister. 2003. A second case of genetic host races in *Rhagoletis*? – A population genetic comparison of sympatric host populations in the European Cherry Fruit Fly, *Rhagoletis cerasi*. Entomologia Experimentalis et Applicata 108, pp. 11-17.

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Schwarz, D. 2000. Wirtsrassendifferenzierung in der Kirschfruchtfliege *Rhagoletis cerasi* (L.). Diploma thesis. Christian-Albrechts Universität Kiel, Kiel.

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