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PRE-MATING REPRODUCTIVE ISOLATION BETWEEN THREE SYMPATRIC VARIETIES OF A SPECIALIST INSECT

by

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CERTIFICATE OF APPROVAL

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MASTER'S THESIS

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This is to certify that the Master's thesis of

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ABSTRACT

Ecological interactions can play a major role in driving the process of speciation when they lead to a decrease in gene flow between diverging lineages. Various pre- and post-zygotic ecological barriers to gene flow are known to be important in speciation, but the specific barriers that cause the initiation of speciation are often unknown. Phytophagous (plant feeding) insects are powerful systems for evaluating ecologically based reproductive barriers because these organisms generally have a history of traits such as host shifting and host mediated sexual selection associated with speciation. Previous work on the sunflower maggot fly (*Strauzia longipennis*) indicates that three genetically distinct, diverging varieties co-occur on the host plant *Helianthus tuberosus*. In this work, I 1) confirm the existence of three diverging varieties by genotyping microsatellite loci, 2) evaluate the presence and strength of three pre-zygotic barriers to reproduction - habitat isolation, pre-mating sexual isolation, and allochronic isolation - between the varieties, and 3) measure the impacts of allochronic isolation on resource partitioning by evaluating host preference, oviposition location, and larval location between diverging *Strauzia* varieties sharing the same host plant species. I find evidence of pre-mating sexual isolation and allochronic isolation between the three varieties, indicating that these may be reproductive barriers that arise during early stages of divergence. These barriers may have occurred without (or before) the host-shift that is typical of many other diverging phytophagous insect systems. I also find evidence that allochronic isolation leads to resource partitioning of the single host plant resource, which may help the three varieties share the same host plant.

PUBLIC ABSTRACT

New biological diversity is the result of speciation – when one species becomes two or more new species. Because it can take millions of years for speciation to be completed, the overall process of speciation can be difficult to study. Many different factors may contribute to speciation, including genetics, behavior, and ecology of the organisms involved. Though all of these factors are important, ecological interactions - how organisms interact with their local habitats - may be especially important in the beginning stages of speciation, especially for specialist insects. Small differences in the ways that different populations interact with their habitats can reduce their contact with one another. Such a decrease in contact between groups of organisms can initiate the formation of new species.

In this work, I study interactions between three varieties of the sunflower maggot fly (genus *Strauzia*) that are in the early stages of speciation. I measure the importance of three ecological interactions, habitat isolation via habitat choice, sexual isolation (i.e. mate choice), and allochronic isolation (i.e. differences in life cycle timing) in reducing mating between the three varieties. I find that sexual isolation and allochronic isolation both decrease the amount of contact between the varieties. I also evaluate how these three varieties are able share the same host plant habitat without displacing or outcompeting one another. This work provides us with new and valuable information about the early stages of speciation.

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Chapter I

INTRODUCTION

Understanding speciation – the formation of new species – is critical to the study of evolutionary biology, but it continues to be a challenging area. Due to the amount of time that can be required for speciation to take place, we can rarely observe the entire process. Though speciation is defined by its end result, it typically involves a series of interacting evolutionary changes, making it a continuous process. This series of accumulating changes and the resulting genetic divergence between groups is commonly referred to as the "speciation continuum" (Nosil 2012). One way to gather information about the process of speciation is to study several related species at different points along the continuum. During speciation, a variety of reproductive barriers may act to reduce the exchange of genetic information between diverging populations (Nosil 2012). The strength of each of these barriers can be measured between pairs of taxa across a continuum of speciation. Evaluating which barriers arise at different stages of the speciation process helps us to better distinguish the particular types of barriers that are important to the initiation of divergence versus those that add to a speciation event already in progress (Via et al. 2000).

Comparative studies of speciation in insects that utilize a series of taxa at different stages in the continuum are not new. Barriers to reproduction have been measured extensively between diverging *Drosophila* species, providing evidence of a general pattern of barrier acquisition and strength (Coyne and Orr 1989, 1997). Reproductive barriers are generally divided into two categories: pre-zygotic - barriers occurring prior to egg fertilization, and post-zygotic - barriers occurring following egg fertilization. Together, pre-zygotic and post-zygotic barriers contribute to total reproductive isolation between diverging lineages. Comparative studies in *Drosophila* find that both pre and post-zygotic barriers increase in strength with genetic distance, but pre-zygotic barriers evolve more quickly than post-zygotic barriers when the diverging species are in sympatry (Coyne and Orr 1989, 1997). However, intrinsic post-zygotic barriers are predicted to

arise first in the early stages of divergence in *Drosophila* which drives an increase in pre-zygotic barriers to prevent fitness disadvantages due to the production of sterile or partially sterile hybrid offspring (Coyne and Orr 1989). The barriers that form early in speciation may lead to the accumulation of additional reproductive barriers (Hendry 2001) and additional barriers may arise after speciation is complete. Determining the order in which different reproductive barriers evolve is necessary to understand the process of speciation, as well as the ecological interactions driving speciation.

Speciation may be the result of divergent natural selection between environments leading to the formation of reproductive barriers between diverging populations (Schluter 2001). The impact of ecological interactions on species divergence, termed ecological speciation, has an understudied role in the process of speciation (Nosil 2012). Through comparative analysis, ecological factors have been found to be strong contributors to reproductive isolation and consequently, speciation, across a variety of taxa (Funk et al. 2006). Though the role of ecological factors on divergence has been measured in many taxa, most studies compare speciation events across a single pair of diverging taxa that differ by habitat (Feder et al. 1994, Nosil et al. 2006, Via 1991a). Many of these studies indicate that pre-zygotic barriers are more important than post-zygotic barriers in the early stages of speciation (Funk 1998, Filchak et al. 2000, Nosil 2004), but this may be because pre-zygotic isolation is already complete, making post-zygotic reproductive barriers impossible to evaluate though they may have been present earlier in the speciation process (Coyne and Orr 2004). Measuring the contributions of ecological factors to divergence at multiple time points along the speciation continuum using a single genus would provide a broader understanding of the relative importance of pre- and post-zygotic barriers across ecological speciation.

Specialist phytophagous (plant feeding) insects can be useful systems for studying ecological speciation because they often have a history of host shifting, host mediated sexual selection, and other ecologically-based traits that are associated with speciation (Berlocher and Feder 2002).

Specialist phytophagous insects have tight life history associations with one or several host plants - they tend to feed, lay eggs and find mates on a specific plant species. This close relationship between specialist insects and their ecological environment make it possible to measure ecologically driven reproductive barriers at various points along the speciation continuum. In contrast to previous comparative studies in more generalist insect species (e.g., Coyne and Orr 1989, 1997), ecologically-based reproductive barriers may be especially important in the early stages of speciation for specialist insects.

In the pea aphid (*Acyrthosiphon pisum* Harris 1776), two variants are in the process of specializing on different host plant species, leading to habitat isolation between variants (Via 1991a, 1991b). In addition, ecologically-based hybrid inviability serves as a post-zygotic barrier to gene flow between lineages in the early stages of speciation between these variants (Via et al. 2000). Ecological interactions play a similar role in the formation of different ecotypes in the stick insect species, *Timema cristinae (*Vickery 1993), which feeds on two different host plants, *Ceanothus spinosus* Nutt. and *Adenostoma fasciculatum* Hook. and Arn. (Sandoval 1994). Maintaining crypsis drives divergent selection between the two morphs found on different host plants (Nosil et al. 2007). In addition to differential host choice (Nosil et al. 2006), sexual isolation (Nosil et al. 2002) and hybrid inviability (Nosil et al. 2004) also contribute to reproductive isolation between *T. cristinae* ecotypes. Finally, in the apple maggot fly (*Rhagoletis pomonella*) host fidelity to two different hosts contributes to reproductive isolation between diverging races by isolating by mate preference and emergence timing (Feder et al. 1994). Hybrid offspring experience a fitness disadvantage on both hosts indicating the presence of extrinsic post-zygotic barriers that further limit gene flow between races (Dambroski et al. 2005). In *A. pisum*, *T. cristinae*, and *R. pomonella*, as well as additional examples (Craig et al. 1993, Funk et al. 1998), ecological interactions play a critical role in speciation.

Ecological barriers are clearly prominent in specialist insect speciation, but still missing is an evaluation of the change in presence and strength of different reproductive barriers across the continuum of speciation. Flies in genus *Strauzia* provide an unusual opportunity to address this gap in knowledge. Previous work in *Strauzia* indicates that some *Strauzia* are in the late stages of divergence and other taxa are more closely related, with the most recent speciation event occurring less than one million years ago (Lisowski 1979, Forbes et al. 2013). These differences in divergence time across the genus can be used to represent many different stages across the speciation continuum. Previous work in *Strauzia* found evidence of ecological speciation in the form of allochronic isolation between diverging varieties within *Strauzia longipennis* (Wiedemann, 1830) (Forbes et al. 2013). The presence of allochronic isolation between three varieties in the genus *Strauzia* is that other reproductive barriers may be present among these diverging varieties and that ecological speciation may be the primary driver of speciation across the *Strauzia* genus. Ultimately, *Strauzia* provides a system in which to evaluate both pre- and post-zygotic reproductive barriers across the speciation continuum. This larger work will provide a broader understanding of the impacts of ecological interactions on divergence in specialist phytophagous insects.

In this thesis, I focus on studying pre-zygotic isolation among three *Strauzia* varieties in the early stages of divergence, a major contribution to the larger comparative study described above. In Chapter II, I develop and apply microsatellite loci to test the hypothesis of Forbes et al. (2013) that the species *Strauzia longipennis* consists of three diverging varieties. I measure the impact of three pre-zygotic reproductive barriers among these diverging varieties: habitat isolation, pre-copulatory sexual isolation, and allochronic isolation. In Chapter III, I evaluate two competing hypotheses that might explain how the three *S. longipennis* varieties partition resources. I evaluate both of these hypotheses by comparing fly oviposition behavior, larval feeding within plant stems, and larval position inside stems for the three varieties of *S. longipennis*.

Chapter II

DIVERGENCE BEFORE THE HOST SHIFT? PREZYGOTIC REPRODUCTIVE ISOLATION AMONG THREE VARIETIES OF A SPECIALIST FLY ON A SINGLE HOST PLANT[1]

Introduction

Ecological speciation occurs when divergent natural selection in different environments drives the evolution of reproductive barriers (Nosil 2012) – the organismal traits that restrict gene flow between populations (Coyne and Orr 2004). Specialist phytophagous (plant-feeding) insects are among the most widely studied examples of ecological speciation, primarily because new reproductive barriers often arise after insects shift to new host plants (Craig et al. 1993, Via 1999, Hardy and Otto 2014). Widespread host-associated genetic differentiation across many insects (Dres and Mallet 2002, Stireman et al. 2005) and genus-level phylogenetic patterns (Smith and Bush 1997, Borghuis et al. 2009) support a pervasive role for host shifting in diversification.

When phytophagous insects adopt novel host plants, differences between the selective environments of the novel and ancestral plants can directly or incidentally result in the evolution of reproductive barriers between populations on different plants (Caillaud and Via 2000). Habitat isolation – genetically based habitat preferences that reduce gene flow between populations (Coyne and Orr 2004) – is often an important barrier: behaviors that cause insects to choose particular hosts automatically result in assortative mating if insects also find their mates on those hosts (Funk et al. 2002). Examples of habitat isolation evolving as part of a host shift are found across many insect taxa and much emphasis has been placed on its importance in divergence (Funk et al. 2002, Matsubayashi et al. 2010).

Besides the "automatic" barrier of habitat isolation, insect host shifts are frequently associated with other forms of reproductive isolation. One common barrier associated with shifts to new

[1] This chapter is adapted from Hippee et al., 2016. Ecological Entomology.

plant hosts is allochronic (temporal) isolation, wherein individuals in different populations search for mates at different times of the day or year. Different host plants can differ in their life history phenologies, and selection for insect developmental schedules to correspond to their respective host plants may result in host-plant associated insect populations that become sexually mature and seek mates at different times of the year (e.g., Wood and Keese 1990, Feder et al. 1993).

Precopulatory sexual isolation can also contribute to speciation of insects that use different host plants, but the evolution of this barrier may not always have an ecological basis. Sexual isolation evolves due to the evolution of mate choice and the resulting failure of individuals from different populations to recognize each other as potential mates. Precopulatory sexual isolation has been documented between specialist insects (e.g., Nosil and Crespi 2004), but does not necessarily depend on ecological differences between habitats (though see Cocroft et al. 2012).

Such direct and indirect evidence for reproductive isolation evolving in concert with host shifts strongly suggests that host-shifting is important in the speciation of some specialist insects, but it is not yet clear if host shifts are usually the source of primary isolation or if instead some reproductive isolation typically predates host shifting events. Certainly shifts to a new host plant are not necessary for speciation in all specialist insects (e.g., Joy and Crespi 2007, Condon et al. 2008, 2014) – but among groups for which host shifts are commonly correlated with divergence, do the host shifts themselves initiate speciation?

True fruit flies in genus *Strauzia* (Diptera: Tephritidae) generally fit a pattern of historical speciation coinciding with host shifting. *Strauzia* are endemic to North America, are associated exclusively with plants in family Asteraceae, and are specialists - most species feed on different plant hosts (Foote et al. 1993). Adult flies emerge from their puparia in late spring to mid-summer (Westdal and Barrett 1960), after which males stake out and defend territories on leaves of host plants where they meet and mate with females. A short courtship dance (2 - 10s) usually precedes copulation (Stoltzfus 1988), and then females oviposit one or more eggs into the growing tip of

the plant. *Strauzia* eggs hatch after about 8 days (Westdal and Barrett 1960) and larvae grow as they feed on the pith of the host plant. Larvae pupate in late summer and early fall, either in the tuber of the host plant or in the soil directly around the base of the plant (Steyskal 1986). Most species in the *Strauzia* genus are associated with a single host plant species (Stolzfus 1988).

In an exception to the one *Strauzia*-one plant rule, genetic evidence shows that three sympatric but genetically isolated varieties of *Strauzia longipennis* (Wiedemann) all feed on the pith of the same plant: Jerusalem artichoke (*Helianthus tuberosus*) (Axen et al. 2010, Forbes et al. 2013). Taxonomists have long suspected *S. longipennis* was composed of two or more reproductively isolated varieties (Loew 1873; Foote et al. 1993) and genetic methods have more recently confirmed this view. Axen et al. (2010) used mitochondrial sequence data to identify two haplotype groups within *S. longipennis*, each closely associated with a particular thoracic striping pattern – but this association between haplotype and morphology was imperfect. Later, Forbes et al. (2013) used AFLP markers to demonstrate that the *S. longipennis* associated with *H. tuberosus* is composed of three genetically distinct clusters. Here, we refer to the three genetic clusters of *S. longipennis* associated with *H. tuberosus* plants as *S. longipennis* var. *longipennis*, *S. longipennis* var. *longitudinalis*, and *S. longipennis* var. *vittigera*. These names derive from species names suggested by Lisowski (1979), who used allozyme loci to distinguish between the same three varieties among flies collected in Illinois. We provide a key in Supplementary Table A1 to other nomenclature previously applied to these flies. The divergence of these three varieties appears to be recent: mitochondrial COI haplotypes are shared among all three varieties (Axen et al. 2010), and apparent hybrids between varieties are found in nature (Forbes et al. 2013), suggesting that reproductive isolation among varieties is incomplete.

The three *S. longipennis* varieties sharing the *H. tuberosus* host present an opportunity to ask questions about speciation of specialist insects in the apparent absence of a host shift. Here, we hypothesize that the three varieties are reproductively isolated by a combination of prezygotic

barriers. Because allochronic and precopulatory sexual isolation have been identified as important early-evolving reproductive barriers for other specialist insects, we test the specific hypothesis that both of these specific barriers reduce gene flow between *S. longipennis* varieties. To evaluate this hypothesis, we develop and score new microsatellite loci for *Strauzia* (necessary to distinguish between flies in two of the varieties), make field observations of adult flies, conduct controlled studies of fly eclosion timing and lifespan, and perform no-choice mating assays.

Materials and Methods

Collection sites and methods

We collected adult and pupal *Strauzia longipennis* flies from *H. tuberosus* plants at several sites in Iowa, Wisconsin, and Illinois from 2011-2014 (see Supplemental Figure A1 for map and site abbreviations). For microsatellite work and mating trials, we also collected representatives of four additional fly species that were captured on different host plant species, *Strauzia noctipennis* Stoltzfus and *Strauzia arculata* Steyskal (hosts are associates of *Helianthus grosseserratus*), *Strauzia perfecta* Steyskal (host is *Ambrosia trifida*) and *Strauzia intermedia* Steyskal (host is *Rudbeckia laciniata*). The distributions of all flies and their host plants overlap broadly across the entire collection region (Heiser et al. 1969, Foote et al. 1993).

We collected adult *Strauzia* flies directly off of their host plants by visually scanning host plants for flies and capturing flies individually in small plastic cups. Some flies were captured *in copulo*, and these events were noted. We identified adult flies using morphology or, when varieties were morphologically cryptic (see results), by genotyping microsatellite loci.

We also collected pupal-stage flies. *S. longipennis* var. *longitudinalis* and *S. longipennis* var. *vittigera* pupate in host plant tubers, so we dug up tubers of *H. tuberosus* in late fall after *Strauzia* pupariation and in the early spring when *Strauzia* begin to emerge from diapause. Tubers and upper stems were bisected carefully with a sharp knife and any puparia inside were extracted. Because *S. longipennis* var. *longipennis* pupates primarily in the soil (Stoltzfus 1988), puparia

were also occasionally collected by digging up and sifting the top 10 cm of soil in a 20 cm diameter around individual plants. All puparia were placed in individual cups of moistened vermiculite.

Development and scoring of microsatellite markers

We developed a set of microsatellite markers to distinguish among flies of different varieties. We collected three *S. longipennis* var. *longitudinalis* and eight *S. longipennis* var. *longipennis* flies in Mount Vernon, IA in 2011, and destructively extracted DNA from these flies using Qiagen Blood and Tissue Kits (Qiagen Sciences, Germantown, MD). We pooled DNA extractions into one sample, which we sent to the Savannah River Ecology Laboratory (Aiken, SC) where a primer library of several thousand microsatellite loci was prepared. We tested 52 primer pairs, 12 of which amplified target loci consistently and for which target loci proved to be polymorphic within and between varieties (See Supplementary Table A2 for primer sequences and additional details).

We genotyped 132 individual *S. longipennis* flies from collections made in Iowa in 2011, 91 of which had been previously genotyped using AFLPs (Forbes et al. 2013), as well as 40 *S. longipennis* and 9 male *S. noctipennis* flies from 2012 collections. We PCR-amplified target loci using fluorescently-labeled forward primers and genotyped amplicons on an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). We scored raw reads automatically using panels designed in the program GeneMarker (SoftGenetics LLC, State College, PA), and then checked all allele calls visually. We used the program STRUCTURE v.2.3.4 (Pritchard et al. 2000) to perform a Bayesian clustering analysis on microsatellite data. We used a model of admixture with a burn-in of 500,000 and 1,000,000 Markov Chain Monte Carlo replications. We used the method of Evanno et al. (2005) to estimate the correct number of populations represented by the sample.

Because STRUCTURE analysis of genotypes (see results) revealed that certain morphological characters (thoracic striping and wing markings) were an accurate and dependable method for discriminating between male *Strauzia* flies of all three varieties and for distinguishing female *S. longipennis* var. *longipennis* flies from other females, we subsequently only genotyped female flies (N=114, all from 2013 collections) of the morphologically cryptic varieties (*S. longipennis* var. *vittigera* and *S. longipennis* var. *longitudinalis*). We did not genotype females from the 2014 collection year, so the 2014 female flies of these two cryptic varieties were not used for allochronic isolation or sexual isolation studies.

Allochronic Isolation

Differences in Eclosion timing

For insects with short-lived adult stages, differences in emergence (eclosion) time under standardized conditions provide strong evidence for allochronic isolation (Filchak et al. 2000). We measured differences in eclosion timing of adult flies from each variety by collecting puparia and allowing adult flies to eclose under controlled laboratory conditions. *Strauzia longipennis* puparia were extracted from tubers of *H. tuberosus* plants at three sites near Iowa City, IA (MV, IC, and WC; see Supp. Fig. 1) in the early spring of 2011. We collected more puparia from the same sites in fall of 2011 and 2012. In the fall of 2013 and spring of 2014, we again collected puparia from the tubers of *H. tuberosus* as well as from soils directly around the plants at the same sites. We overwintered puparia collected in the fall in individual cups of moistened vermiculite in a refrigerator at 4˚C for four months for the 2012 and 2013 eclosions and for six months in 2014. In the spring, we removed cups from the refrigerator and allowed them to sit at room temperature (approximately 18˚C) for one week. We then moved all pupae to an incubator with controlled light and temperature conditions (16:8 light:dark cycle; 25˚C). The warm temperature and extended light cycle act as cues to induce post-diapause development in flies, such that differences in eclosion timing between varieties reflect innate differences in post-

diapause development (diapause break and morphogenesis). Puparia collected in early spring were not overwintered, and were transferred directly to the incubator. Puparia were checked daily and the date of eclosion was recorded for each adult fly.

We sorted eclosing flies by variety using morphological characters and microsatellite markers. Differences in mean eclosion time between *S. longipennis* varieties were calculated using t-tests. Collections from different years and seasons were not pooled. We calculated the degree of temporal isolation between *S. longipennis* var. *vittigera* and *S. longipennis* var. *longitudinalis* as the reduction of overlap between varieties due to differences in their emergence times (*S. longipennis* var. *longipennis* was excluded due to small numbers). The degree of temporal isolation (I) was calculated using the Equation 1 (from Hood et al. 2015):

$$\text{Equation 1: } I = 1 - \left(\frac{\sum x_i y_i}{\sqrt{\sum x_i^2 \sum y_i^2}} \right)$$

where x_i and y_i are the numbers of flies from *S. longipennis* var. *vittigera* (x) and *S. longipennis* var. *longitudinalis* (y) alive on day *i* as predicted by their actual eclosion dates and probabilities of survival to day *i* calculated from fly longevity measurements. Hood et al. (2015) used this equation to compare the amount of overlap between emerging adults of two populations of parasitic wasps. Using data on adult longevity and emergence times, they found that differences in emergence timing resulted in allochronic isolation between populations. We used the average lifespan of 96 flies that eclosed and died in the laboratory from 2012-2014 as a conservative measure of fly longevity (17.6 ± 1.2SE days). Temporal isolation was calculated for each collection, for within-season collections combined, and for all collections combined. To combine

datasets, peak emergence days for each variety were aligned without altering the number of days between peak emergences for the two different varieties.

Temporal Differences in Field Collections of Adults

Previously (Forbes et al. 2013) we published evidence of putative allochronic isolation between the three *S. longipennis* varieties based on overlap of adult flies collected in the field during the summer of 2011. To this we now add an additional three years of collections (2012-2014). All field observations are from the same three Iowa sites from which pupal collections were made. The utility of field collection data is limited because they are affected by differences in effort and time spent collecting across each season and between years, and total number of flies caught from one time period to the next vary will based on field conditions. Nevertheless, because collections spanned four years, some general comparisons are possible. To generate comparable units between years, we divided each collecting year into five time periods (May 20-June 3, June 4-June 18, June 19-July 3, July 4-July 18, and July 19-August 3), totaled the files of each variety collected during each period, and performed goodness of fit G-tests using an expectation of 1/3 proportions for each variety. This evaluates the null hypothesis that flies of all three varieties emerge and seek mates at the same time during the season, and assumes that the total annual population size for each variety is the same. Proportions were calculated for each time period independently and were not compared with the abundance of flies at any other time period over the summer because collection efforts and sizes differed. We also calculated temporal isolation (I) across the pooled 4-year dataset using the equation above, but where *i* represented time periods instead of days.

Precopulatory Sexual Isolation

We measured precopulatory sexual isolation using no-choice mating trials. We paired male and female flies of different varieties and species (a mixture of field collected adults and flies eclosed

in the lab), based on the availability of insects on any given date. Each trial was an independent, no-choice pairing between one male and one female fly. Trials included the three *S. longipennis* varieties, as well as *S. noctipennis*, *S. arculata,* and *S. perfecta*. We did not combine data between reciprocal sex pairings for each species combination because of possible differences in the degree of mate discrimination between sexes. Some pair-wise comparisons were not possible because of low collection numbers or differences in life history timing between species.

We observed each fly pair twice daily and recorded presence or absence of mating. *Strauzia* mate frequently and remain *in copulo* for long durations (Westdal and Barrett 1960), so two daily observations are sufficient to measure the presence and relative frequency of copulation. Observations occurred as long as the pair remained alive (3 – 40 days), with the total number of observations ranging from 5 to 80. To standardize observation number between trials, we used just the first five observations for each pair. We calculated the proportion of observations during which each pair was seen to be mating during these first five observations, and used pairwise t-tests to determine if flies were less likely to mate with a fly of a different species or variety than with a fly of the same species or variety. Each treatment was compared to the conspecific control pairs of both the male and female parent. We used an uncorrected alpha of 0.05 as well as a Bonferroni-corrected alpha.

We also estimated the degree of sexual isolation between each pairwise combination of varieties by calculating an I_{PSI} value for each pairing using Equation 2 (from Coyne et al. 2005):

$$\text{Equation 2: } I_{PSI} = \frac{(PSI_{SS}+PSI_{YY})-(PSI_{SY}+PSI_{YS})}{(PSI_{SS}+PSI_{YY}+PSI_{SY}+PSI_{YS})}$$

Pairwise Sexual Isolation (PSI) coefficients were calculated for each pair type following Rolán-Alvarez and Caballero (2000). S and Y coefficients refer to the two varieties being compared. PSI_{SS} and PSI_{YY} represent same-variety pairings and PSI_{SY} and PSI_{YS} represent different-variety pairings. The PSI values represent the proportion of matings that occurred out of all possible

matings. The final I_{PSI} values are a standardized measure of the number of same-variety matings that occurred compared to different-variety crosses. Values for I_{PSI} were determined using JMATING software (Carvajal-Rodriguez and Rolán-Alvarez 2006) with 100,000 bootstrap replicates. To be counted as a mating pair in calculation of PSI coefficients, flies had to be observed mating at least once. An I_{PSI} value of 0 indicates no evidence of sexual isolation and an I_{PSI} value of 1 indicates complete sexual isolation.

Results

Collections

We collected 1426 adult *Strauzia* on *H. tuberosus*, including 342 *S. longipennis* var. *vittigera*, 600 *S. longipennis* var. *longitudinalis*, and 479 *S. longipennis* var. *longipennis*. The remaining five flies were other *Strauzia* species (Supplementary Table A3). We collected 281 *Strauzia* on *H. grosseserratus*, including 171 *S. noctipennis*, 108 *S. arculata*, one *S. longipennis* var. *vittigera*, and one *S. intermedia* (Supplementary Table A4). We also collected 156 *S. perfecta* flies, and 120 *S. intermedia* flies as adults from their respective host plants. All 141 *Strauzia* collected as puparia from *H. tuberosus* tubers and surrounding soils belonged to one of the three *S. longipennis* varieties. Flies reared from puparia were primarily *S. longipennis* var. *vittigera* (n = 66) and *S. longipennis* var. *longitudinalis* (n = 72). Collections of *S. longipennis* var. *longipennis* (n = 3) were much smaller, likely because this variety pupates primarily in the soil (Stoltzfus 1988) and soil collections were limited in number (Supplementary Table A5).

Microsatellites

STRUCTURE analysis of microsatellite genotypes from flies collected in 2011 and 2012 supported four distinct genetic clusters (Figure 2.1), corresponding to *S. noctipennis* males (yellow cluster, Figure 2.1B) and the three varieties of *S. longipennis* (blue, green, and red clusters) previously identified by Forbes et al. (2013). Microsatellite data revealed that the morphology of flies in the three *S. longipennis* varieties does not overlap for male flies. Male *S.*

longipennis varieties can be distinguished from one another using variety-specific combinations of thoracic striping and wing pigmentation: *S. longipennis* var. *longipennis* males lack dark thoracic stripes and have a distinct "F" marking on their wings. *S. noctipennis* males, which do not share a host plant with *S. longipennis,* also lack thoracic stripes, but have what Axen et al. (2010) describe as a "coalesced" wing pattern. *Strauzia longipennis* var. *vittigera* males have dark thoracic stripes and "F" wing markings, while *S. longipennis* var. *longitudinalis* males have dark thoracic stripes and coalesced wings. Our data therefore show that genetic markers are unnecessary for distinguishing between male *S. longipennis* varieties.

It is less straightforward to distinguish between female *S. longipennis* flies because all female flies have "F" patterned wings. *Strauzia longipennis* var. *longipennis* females can be distinguished from others due to their lack of thoracic stripes, but STRUCTURE analysis showed that the *vittigera* and *longitudinalis* varieties (intermixed green and red bars in Figure 2.1A) cannot be distinguished from one another using morphology. Three microsatellite loci, ST34, ST42 and ST49, were useful in distinguishing between varieties (Supplementary Tables A6, A7, and A8), and those loci were used to identify female flies with striped thoraxes in 2013 collections.

Allochronic Isolation

Allochrony in eclosion timing

Strauzia longipennis var. *vittigera* flies eclosed significantly earlier than *S. longipennis* var. *longitudinalis* in all five independent collections (t-tests; Table 2.1). Temporal isolation between *S. longipennis* var. *vittigera* and *S. longipennis* var. *longitudinalis* within individual collection seasons ranged between I=0.26 and I=0.88 (Table 2.2). Across all years combined, temporal isolation between these two varieties was 0.30. Few *S. longipennis* var. *longipennis* flies were collected because pupal collections were small, so it was not informative to compare eclosion

times of this fly to the other two varieties. One *S. longipennis* var. *longipennis* fly eclosed in 2013 (after 39 days), and four emerged in 2014 (26.3 ± 6.5 [SE] days).

Allochrony in field collections

Field collections of the three varieties showed significant deviation from a null expectation of even proportions during all five time periods (Table 2.3), with proportions of each variety changing throughout the summer. Adult *S. longipennis* var. *vittigera* flies accounted for the greatest proportion of flies captured during the earliest time period (May 20-June 3), but dropped off to lower proportions in all subsequent periods. During time periods 2 and 3, *S. longipennis* var. *longitudinalis* accounted for the highest proportion of captures, with *S. longipennis* var. *longipennis* having the highest proportions in time periods 4 and 5. Temporal isolation between *S. longipennis* var. *vittigera* and *S. longipenni*s var. *longitudinalis* calculated from field collection data was 0.18, lower than all values calculated for these two varieties from pupal eclosion data (see Table 2.2). Temporal isolation was 0.37 between *S. longipennis* var. *longipennis* and *S. longipennis* var. *vittigera*, and 0.08 between *S. longipennis* var. *longipennis* and *S. longipennis* var. *longitudinalis*.

Precopulatory Mating Isolation

Most crosses between *Strauzia* of different varieties or species had significantly lower mean mating frequencies compared with conspecific or same-variety crosses, regardless of whether comparisons were made to the male or female parent's conspecific cross (Table 2.4). One exception was the cross between *S. longipennis* var. *longitudinalis* females and *S. longipennis* var. *vittigera* males: mean mating frequency between these flies was 0.54, which was lower, but not significantly lower, than the same-variety crosses of both partners (*vittigera-vittigera*, 0.72; *longitudinalis-longitudinalis*, 0.59). In general, results did not differ based on the identity of the male vs. female partner. Most crosses showed significantly reduced mating frequencies

irrespective of the male and female partner (Table 2.4). Mating frequencies were significantly higher for crosses between different *S. longipennis* varieties (overall mean mating frequency = 0.21, n = 127) than for crosses between different *Strauzia* species (mean mating frequency = 0.06; n = 48, t-test, $P < 0.001$).

I_{PSI} (sexual isolation) values were high across the board, with I_{PSI} values for crosses between *S. longipennis* varieties (mean=0.69, n=3) significantly lower than crosses between *Strauzia* species (mean=0.89, n=17, T-test, $P < 0.001$). I_{PSI} values between different *S. longipennis* varieties ranged from 0.67 to 0.71. I_{PSI} values between different species of *Strauzia* ranged from 0.70 to 1.00 (Supplementary Table A9).

Because flies collected as adults were sometimes found mating at the time of capture, we also analyzed our data on mating partners in nature. We collected 145 pairs of actively mating *Strauzia* flies off of host plants from 2011-2014 (Supplemental Table A10). Among the *S. longipennis* varieties (n = 69 mating pairs), significantly more pairs were same variety-pairings than different variety pairings compared to an expectation of 1/3 same variety: 2/3 different variety ($\chi^2_1 = 54.8$; $P =< 0.0001$). No inter-species mating pair was captured on any plant in this study, including between *S. arculata* and *S. noctipennis*, which use the same host plant species.

Discussion

Microsatellites confirm that *S. longipennis* associated with *H. tuberosus* in Iowa consists of three reproductively isolated varieties and there is little evidence for substantial levels of gene flow among the three varieties. AFLPs previously showed several fixed differences between *S. longipennis* var. *longitudinalis* and the other two varieties (Forbes et al. 2013), and microsatellites in the current study show a similar signal of strong isolation. The concordance between genetic clusters and morphological traits adds further support to the conclusion that the three varieties are real biological units.

Though the varieties are not isolated by host plant use, allochrony in life history timing partially isolates the three *S. longipennis* varieties from one another. Measures of temporal isolation between *S. longipennis* varieties were comparable to other insects in the early stages of divergence (e.g., Wood and Guttman 1982; Forbes et al. 2009). Measures of isolation calculated from adult fly collections were generally lower than those calculated from pupal collections, but may also be less precise as they were calculated from across four years of collections that differed in collection effort. All data are consistent in suggesting that the three varieties emerge in a sequence, with *S. longipennis* var. *vittigera* emerging in the early summer, followed by *S. longipennis* var. *longitudinalis*, and with *S. longipennis* var. *longipennis* emerging towards the end of the summer. This agrees with previous observations of temporal isolation among these varieties (Stoltzfus et al. 1988, Forbes et al. 2013), and interestingly the degree of allochronic isolation previously reported between "good" *Strauzia* species may be greater than between the *S. longipennis* varieties (Stoltzfus et al. 1988). Sunflowers continue to grow until they flower in August, and *S. longipennis* oviposit into the upper nodes of the plant (A.H., unpublished data), so the allochrony between varieties may reflect partitioning of the stem resource, with the three varieties ovipositing and feeding at different plant growth stages. Such "host age-associated divergence" in the absence of host shifting has recently been described in another specialist insect system, though one that lacked allochronic isolation as all host ages were available to all diverging lineages (Zhang et al. 2015). Future work should focus on making additional efforts toward collecting large numbers of *S. longipennis* var. *longipennis* puparia from soils around plants in order to more accurately compare its eclosion timing with the other varieties.

We also show the first evidence for precopulatory sexual isolation among the three *S. longipennis* varieties. Sexual isolation (I_{PSI}) was relatively stronger between more distantly related species of *Strauzia* than it was between the three varieties, suggesting that sexual isolation is important early in divergence but also continues to increase in degree with time. This pattern is similar to that

seen in *Rhagoletis* flies, a tephritid genus with a well-documented history of speciation via host shifting (Smith and Prokopy 1982, Hood et al. 2012). Speciation driven by mate choice evolution has been invoked as an example of non-ecological speciation (e.g., as an example of mutation-order selection; Schluter 2009) but can also have an ecological component (Nosil et al. 2003). Indeed, ecology could play an indirect role in mate choice for *Strauzia*. Consistent with the thermal melanism hypothesis, which says that dark colored individuals may have a selective advantage over light colored individuals because they can warm more quickly when radiation is lower (Clusella-Trullas et al. 2007), *Strauzia* species with darker striping patterns tend to emerge in the early part of the summer when days are cooler (*S. intermedia*, *S. longipennis* var. *vittigera*, *S. longipennis* var. *longitudinalis*, *S. arculata*), while species with lighter stripes emerge later when days are warmer (*S. longipennis* var. *longipennis*, *S. noctipennis*, *S. perfecta*). If notal striping is relevant to mate recognition in *Strauzia*, then divergent selection for darker vs. lighter notal stripes may also drive sexual isolation in these flies.

While allochronic isolation and precopulatory mating isolation are both important barriers between the sympatric *S. longipennis* varieties, the origin of these barriers and the responsible mechanism(s) remains an open question. One possibility is that barriers originated during previous periods of allopatric isolation, and that flies are currently experiencing secondary contact. For instance, differences in local or regional climates may have selected for faster or slower developmental schedules that now temporally isolate the three varieties. A second (non-mutually exclusive) possibility could involve adaptation to different plant developmental stages, such that eggs are deposited in host plants only during temporal windows when conditions are benign to each coevolving fly population. Many plants, including sunflowers, alter the dosage and composition of protective chemicals as they progress through developmental stages (Chou and Mullin 1993), so independent exploitation of qualitatively different plant life stages by different fly varieties may result in temporal isolation. A third hypothesis should also be addressed:

historical shifts of *Strauzia* to different host plants may have occurred, with subsequent shifts back to *H. tuberosus* after a period of host-associated adaptation and the evolution of reproductive isolation. Though we have not reared these varieties from hosts beyond *H. tuberosus*, other authors describe flies of similar morphology reared from *Helianthus annuus* and *Helianthus decapetalus* (Stoltzfus 1988, Lisowski 1979). Future work should focus on collection and genetic characterization of *S. longipennis* flies from across their geographic and ecological ranges so that they may be compared morphologically and genetically to the three varieties described here.

These three reproductively isolated *Strauzia* varieties beg the following question: do host shifts *cause* speciation for phytophagous insects, or do they occur after divergence has already begun? This is not a trivial point; the study of how speciation progresses from one panmictic population to two or more completely isolated species includes the identification of the reproductive barriers that initially isolate populations from one another. These are the barriers that can be said to be "causal" in speciation, and that promote the evolution of other barriers later in the process (Coyne and Orr 2004). The assertion that host shifts initiate speciation for *Strauzia* and other insects relies on the premise that shifts to new plants drive the evolution of primary reproductive isolation, when in fact preexisting reproductive barriers may instead be responsible (and/or necessary) for the host shift.

Evidence from another North American tephritid offers some complementary insight into these questions. In the early days of the colonization of North America by Europeans, *Rhagoletis pomonella* flies shifted from their ancestral host (fruit of the downy hawthorn, *Crataegus mollis*) into introduced apples (*Malus domestica*), an event that triggered the evolution of reproductively isolated apple and hawthorn fly "host races" (Feder et al. 1994, Linn et al. 2004). Like *S. longipennis*, *R. pomonella* on hawthorns appear to have harbored some variation in life history timing before their shift to apples, and this variation may have "preadapted" them to the earlier-

fruiting apple host (Feder et al. 2003). However, unlike *Strauzia*, there is little evidence that this variation was manifested in multiple reproductively isolated varieties of *R. pomonella* sharing the same hawthorn host. In fact, much contemporary reproductive isolation between "races" of *R. pomonella* on hawthorns is associated with use of different hawthorn species (Powell et al. 2014), implying that host shifts followed by divergent host plant-mediated selection is the primary source of reproductive isolation for *Rhagoletis* flies. *Strauzia longipennis*, then, presents an unusual case of reproductive isolation without an apparent host shift, warranting more investigation into the biogeographic and ecological history of this complex of flies.

Figure 2.1. Bar plot of STRUCTURE results. STRUCTURE results for (K = 4) for 100 male and 63 female *Strauzia longipennis* flies and 9 male *S. noctipennis* flies. Colors indicate variety or species membership: Blue = *S. longipennis* var. *longipennis*; Green = *S. longipennis* var. *longitudinalis*; Red = *S. longipennis* var. *vittigera*; Yellow = *S. noctipennis*. Thoracic and wing morphologies of flies are shown above each section of the plot. Males and females were run through STRUCTURE together and separated into two bar charts to highlight correspondence between varieties and morphological characters.

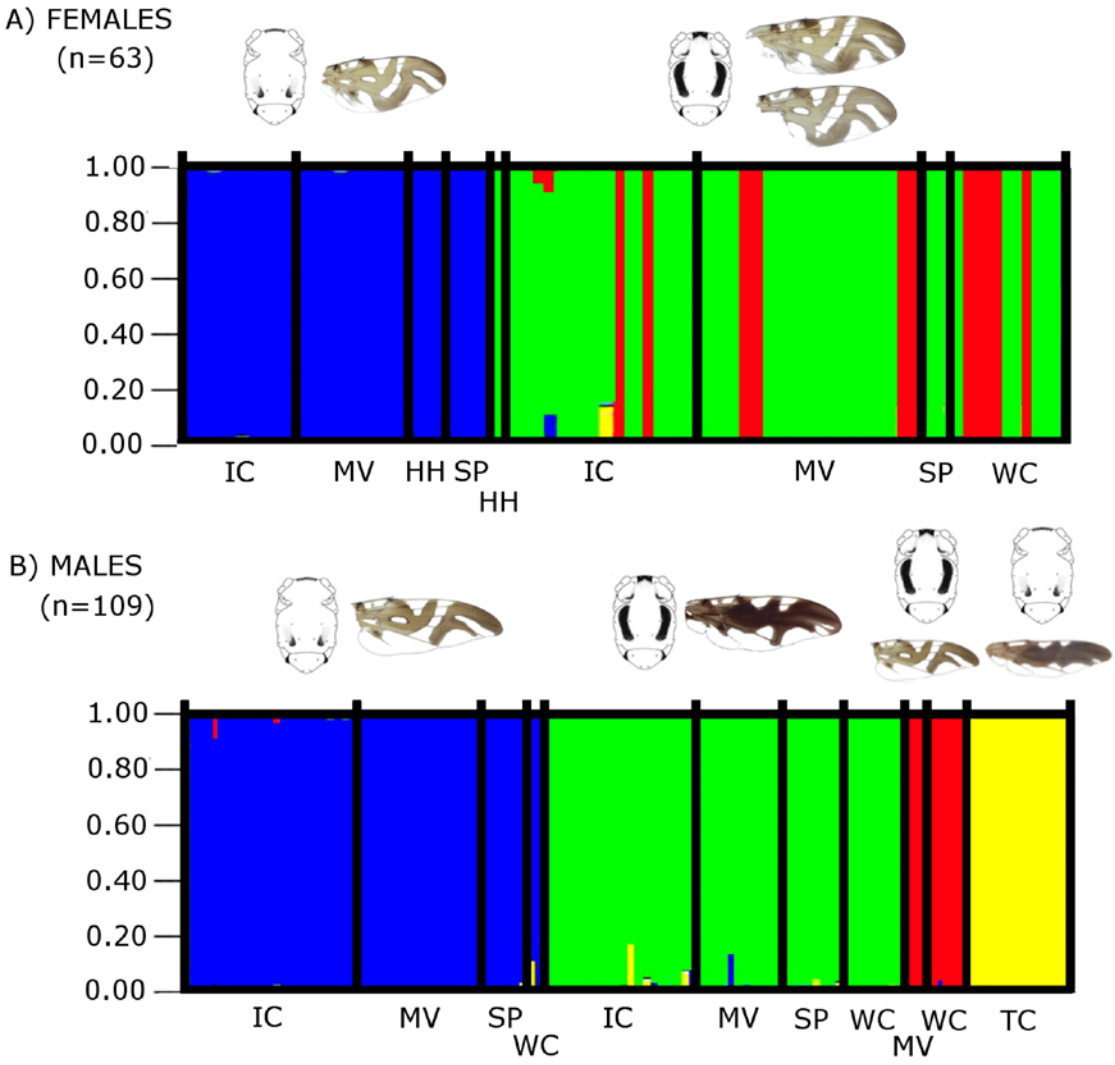

Table 2.1. Mean number of days until adult eclosion. Mean number of days (± SE) until adult eclosion for two varieties of *S. longipennis* across three years of pupal collections. Fall collections were overwintered in a 4˚C fridge for four or six months before moving to a warm room to break diapause. Spring collections were collected after the ground thawed and were moved immediately to the warm room. Different letters in the same row indicate significant differences between mean eclosion times as assessed by t-tests. Numbers in parentheses indicate sample sizes.

	Months in fridge	*S. longipennis* var. *vittigera*	*S. longipennis* var. *longitudinalis*
Fall Collections (lab overwintered)			
2012 (collected Fall 2011)	4	25.5 ± 1.9 days (13) a	58.9 ± 4.0 days (18) b
2013 (collected Fall 2012)	4	34.3 ± 3.2 days (15) a	48 ± 0.3 days (31) b
2014 (collected Fall 2013)	6	12.1 ± 0.4 days (23) a	26.9 ± 2.2 days (16) b
Spring collections (natural overwintered)			
2011 (collected 4/13-5/1)	N/A	11.4 ± 0.8 days (8) a	27.0 ± 1.8 days (24) b
2014 (collected 5/13-5/29)	N/A	6.4 ± 1.1 days (18) a	10.5 ± 0.7 days (15) b

Table 2.2. Calculated degree of temporal isolation. Degree of temporal isolation between *S. longipennis* var. *vittigera* and *S. longipennis* var. *longitudinalis* for four individual *Strauzia* emergence datasets and combined datasets across multiple years of collections. "Fall Collections" represents Fall 2012 and Fall 2014 combined. "Spring Collections" represents Spring 2013 and Spring 2014 combined. "All Collections" represents all emergence data collected from 2012-2014.

Collection	Degree of Temporal Isolation
Fall 2012	I=0.73
Spring 2013	I=0.88
Fall 2014	I=0.56
Spring 2014	I=0.26
Fall Collections	I=0.60
Spring Collections	I=0.20
All Pupal Collections	**I=0.30**

Table 2.3. Number of each *S. longipennis* variety captured. Numbers (and proportions of total) of each *S. longipennis* variety captured on *H. tuberosus* during each of five time periods. Numbers in each time period are totals from four years of collections (2011-2014). Goodness of fit statistics and P values reflect a null expectation of equal percentages during each time period.

Time period	*S. longipennis* var. *vittigera*	*S. longipennis* var. *longitudinalis*	*S. longipennis* var. *longipennis*	G	P
May 20 - June 3	51 (0.61)	19 (0.23)	13 (15.7%)	30.169	0.000
June 4 – June 18	73 (0.31)	110 (0.46)	56 (23.4%)	19.138	0.000
June 19 – July 3	13 (0.09)	65 (0.46)	62 (44.3%)	36.529	0.000
July 4 – July 18	3 (0.03)	39 (0.40)	55 (56.7%)	43.876	0.000
July 19 – August 3	2 (0.12)	4 (0.24)	11 (64.7%)	7.882	0.019

Table 2.4. Table of average mating frequency observed for *Strauzia* crosses. Average mating frequency ± SE observed for each *Strauzia* fly cross type. Numbers in parentheses indicate number of replicates that were set up for each cross. Mating frequencies between flies of the same variety or species are shown along the diagonal in bold text. Crosses were tested for deviation from expected mating frequencies, which were based on conspecific crosses of the male (M) and female (F) partners. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ^=significant with Bonferroni correction, NS=not significant. N/A indicates that the sample size was not large enough to make a comparison.

		Female Partner						
		S. arculata	*S. noctipennis*	*S. intermedia*	*S. perfecta*	*S. longipennis* var. *vittigera*	*S. longipennis* var. *longitudinalis*	*S. longipennis* var. *longipennis*
Male Partner	*S. arculata*	**0.58±0.04 (44)**	0±0 (4) M:***^; F:***^	0±0 (2) M:***^; F:***^	0.16±0.11 (9) M:**^; F:*	0.3±0.14 (4) M: NS; F:*	0.4±0.3 (2) M: NS; F:NS	0.12±0.07 (18) M:***^; F:***^
	S. noctipennis	0.05±0.03 (12) M:***^; F:***^	**0.38±0.08 (16)**	0	0.06±0.04 (7) M:***^; F:***^	0±0 (1) M: N/A; F: N/A	0.05±0.03 (12) M:***^; F:***^	0.08±0.03 (10) M:**^; F:***^
	S. intermedia	0±0 (4) M:***^; F:***^	0	**0.64±0.04 (28)**	0.3±0.18 (2) M:NS; F:NS	0.07±0.01 (3) M:**^; F:***^	0±0 (2) M:***^; F:***^	0.28±0.2 (5) M:NS; F:NS
	S. perfecta	0±0 (4) M:***^; F:***^	0±0 (5) M:***^; F:***^	0±0 (3) M:***^; F:***^	**0.48+±0.05 (38)**	0±0 (1) F: N/A; M: N/A	0.11±0.07 (9) M: ***^; F:***^	0.02±0.02 (12) M:***^; F:***^
	S. longipennis var. *vittigera*	0.29±0.1 (7) M:**^; F:*	0.6±0 (1) M: N/A; F: N/A	0±0 (5) M:***^; F:***^	0.15±0.06 (15) M:***^; F:***^	**0.71±0.05 (34)**	0.37±0.1 (20) M:**; F:**	0.26±0.05 (41) M:***^; F:***^
	S. longipennis var. *longitudinalis*	0.45±0.11 (11) M: NS; F:NS	0.12±0.08 (5) M:**^; F:*	0±0 (7) M:***^; F:***^	0.09±0.03 (47) M:***^; F:***^	0.21±0.07 (22) M:***^; F:***^	**0.66±0.03 (94)**	0.17±0.04 (69) M:***^; F:***^
	S. longipennis var. *longipennis*	0.04±0.04 (10) M:***^; F:***^	0.3±0.15 (8) M:NS; F:NS	0±0 (1) M: N/A; F: N/A	0.06±0.03 (29) M:***^; F:***^	0±0 (5) M:***^; F:***^	0.02±0.01 (21) M:***^; F:***^	**0.49±0.04 (97)**

Chapter III

HOST-PLANT PARTITIONING AMONG THREE SYMPATRIC VARIETIES OF THE SUNFLOWER MAGGOT FLY (*STRAUZIA LONGIPENNIS*)

Introduction

Many studies of speciation in insects focus on phytophagous (plant-feeding) insects that are specialists on a single or multiple host plant species (Funk 1998, Via 1999, Feder et al. 1994, Craig et al. 1993). Specialist insects are often the focus of speciation studies because changes in their host plant affiliations can lead to reproductive isolation between populations using different plants (Via 1999, Nosil et al. 2002). Three closely related varieties of the sunflower maggot fly, *Strauzia longipennis* (Wiedemann 1830), are in the process of diverging (Forbes et al. 2013, chapter II, this thesis). These three varieties (*S. longipennis* var. *vittigera*, *S. longipennis* var. *longitudinalis* and *S. longipennis* var. *longipennis*) are separated by multiple reproductive isolating barriers, and yet they continue to share a single host, *Helianthus tuberosus* L. (Hippee et al. 2016). Because host shifting has been cited as important in the early stages of divergence for so many other phytophagous insects (Nosil 2002, Via 1999), the coexistence of three *Strauzia* varieties on a single host is unusual, and raises the question: how is it that three closely related varieties can share the same host plant without facing competitive exclusion?

Competition is thought to be strong for phytophagous insects that share the same food resource, so selection should favor changes in resource use that reduce overlap between competing populations (Denno et al. 1995). Many phytophagous insects experience an increase in survivorship following a shift to a new environment, which decreases their competition for resources (Bolnick 2001, Rosenzweig 1978). Host-shifting may therefore be a consequence of selection that reduces competition for a shared resource between diverging insects. Alternatively, in some cases phytophagous insects have also been observed partitioning a single food resource into smaller microhabitats in response to competition (Shapiro and Carde 1970, Benson 1978). Changes in temporal and spatial use of host plants can also work to decrease the competition

between competing insect species (Denno et al. 1995). Changes in behavior that reduce competition can occur at any point in an insect's life cycle. For example, female mosquitoes searching for appropriate habitats for egg laying will actively select the most favorable habitat for her offspring (Minakawa et al. 2004). Additionally, ovipositing female mosquitoes will alter their egg laying behavior if they detect presence of conspecific larva in the oviposition medium that could be a source of competition for her offspring (Munga et al. 2006).

Current observations of *Strauzia longipennis* behavior indicate that all three varieties generally follow the same basic life cycle – the adult insects meet and mate on *H. tuberosus*, females lay eggs in the stem of the plant, larva mine the pith of the stem and pupate in the roots or surrounding soil. However, lineage specific differences in specific aspects of this life cycle may reduce competition and maintain the three varieties of *S. longipennis* on a single host. My primary hypothesis is that the three varieties of *S. longipennis* avoid competition by dividing the pith of the *H. tuberosus* plant spatially into three different heights, with each variety feeding at a different height. I will test this hypothesis by collecting *H. tuberosus* stems and determining the height of damage to pith on plants infested with each variety of *S. longipennis*.

I have also generated two hypotheses that address the potential mechanisms that may lead to host plant partitioning across the three varieties of *S. longipennis*. I will call the first of these the "partitioning by ovipositional choice" hypothesis (Figure 3.1). In this scenario, female *Strauzia* from each of the three varieties prefer plant nodes of different heights for oviposition, resulting in reduced competition between larvae feeding on plant pith. Here, *S. longipennis* var. *longipennis* (yellow in Figure 3.1) lays eggs at the highest position on the plant, *S. longipennis* var. *longitudinalis* (blue in Figure 3.1) oviposits in the middle of the stem, and *S. longipennis* var. *vittigera* (red in Figure 3.1) oviposits near the bottom of the stem. The partitioning by ovipositional choice hypothesis would be supported by observations of female flies of each variety preferentially ovipositing into different plant heights.

My second hypothesis I will call the "partitioning by allochrony" hypothesis. If the females of the three varieties do not select different locations on the plant stem for oviposition, their physical separation may occur naturally due to fly emergence time differences coupled with plant growth throughout the summer. Evidence of allochronic isolation has already been found to contribute to reproductive isolation among *S. longipennis* varieties (Stolzfus 1988, Axen et al. 2010, Forbes et al. 2013, chapter II, this thesis). *S. longipennis* var. *vittigera* emerges first in the summer, followed by *S. longipennis* var. *longitudinalis*. *S. longipennis* var. *longipennis* is the final variety to emerge each summer. As a plant grows over the course of the summer, new pith is added, which equates to an ongoing extension of available food for flies. This idea is incorporated into the partitioning by allochrony hypothesis (Figure 3.2). Here, all varieties oviposit into the growing tip / shoot apical meristem of the plant. The first variety to emerge and lay eggs (*S. longipennis* var. *vittigera* – red in Figure 3.2) oviposits into the youngest and shortest plants. Once the second variety (*S. longipennis* var. *longitudinalis* – blue in Figure 3.2) is ready to oviposit, the plant has grown, providing additional space above the *S. longipennis* var. *vittigera* oviposition site to place new eggs. Finally, the *S. longipennis* var. *longipennis* (yellow in Figure 3.2) females oviposit above the other two *S. longipennis* varieties because more stem is available at the top of the plant. This hypothesis would find support if we find that females from all three varieties favor the same location (at or near the shoot apical meristem) for oviposition.

Methods

Collections and Field Sites

From June-August of 2015, I collected a total of 318 adult *Strauzia longipennis* on *H. tuberosus* from field sites in the Iowa City area (Figure 3.3), including 100 *S. longipennis* var. *vittigera*, 118 *S. longipennis* var. *longitudinalis*, and 100 *S. longipennis* var. *longipennis*. I also collected 65 *S. arculata*. I collected all flies singly in plastic cups unless I caught flies *in copulo*, in which case they were recorded as a mating pair. I recorded host plant information and field site location for each *Strauzia* collected. I visually identified all male flies upon collection and identified

morphologically cryptic females following behavioral experiments by genotyping microsatellite loci (see methods in Chapter II, this thesis).

In the fall of 2014 and spring of 2015, I collected 186 *Strauzia* as pupae from *H. tuberosus* tubers and surrounding soils. I collected pupae by bisecting the stems and roots of host plant species. In addition, I sifted the soil surrounding host plants because *S. longipennis* var. *longipennis* tend to pupate in the soil. I stored pupae collected in the fall of 2014 individually in cups of moistened vermiculite and allowed them to overwinter in a refrigerator at approximately 4°C. After 6 months, I removed the cups from the refrigerator and allowed pupae to sit for one week at room temperature (approximately 18°C) before moving them into the fly incubator. I kept the pupae in the incubator with controlled environmental conditions (16:8 light:dark cycle; 25˚C) and check them daily for adult emergences. I placed pupae collected in the spring of 2015 directly into the fly incubator. Of the 186 pupae collected, 73 emerged as adults in the fly incubator. All of these emerged adults belonged to one of the three *S. longipennis* varieties. Flies reared from pupae were primarily *S. longipennis* var. *vittigera* (n = 37) and *S. longipennis* var. *longitudinalis* (n = 30). I collected fewer *S. longipennis* var. *longipennis* (n = 7) because most larvae of this variety pupate in the soil surrounding the host plant.

Plant Interaction and Oviposition Behavior

From April-July of 2015, I grew *H. tuberosus* - the known host of *S. longipennis* and *H. annuus* – a putative host of *S. longipennis* in the Biology Greenhouse at the University of Iowa. I also grew hosts plants of two other *Strauzia* species (*Rudbeckia laciniata* – host of *Strauzia intermedia* and *Helianthus grosseserratus* – host of *Strauzia arculata* and *Strauzia noctipennis*), and tested them as additional acceptable candidates for *S. longipennis* oviposition. The USDA National Plant Germplasm System provided all seeds from sources in the Midwest US. I germinated seeds in foam seed starting trays and transferred them to pots as seedlings. Plants were transferred to 10

gallon pots and remained in the Biology Greenhouse until they were at least 6 nodes tall. Plants were watered daily and fertilized weekly.

From June-August of 2015, I exposed 40 mated female *S. longipennis* flies to 107 plants grown in the greenhouse. I selected mated female *S. longipennis* varieties and *S. arculata* flies to participate in plant oviposition trials. I placed female flies in a mesh bag with a single plant for 1 hour daily. I rotated each female to plants of different species or to multiple plants of the same species each day as plants were available. No more than one female was exposed to any single plant and each female was rotated through her set of plants as many times as possible until the female died. Female behavior was observed and oviposition location based on node was recorded for each oviposition during the hour long observation period. Over the course of the summer, I exposed *S. longipennis* var. *vittigera* (n = 9) to 6 *H. tuberosus* plants and 4 *H. grosseserratus*. I exposed *S. longipennis* var. *longitudinalis* (n = 22) to 46 *H. tuberosus* plants, *7 H. grosseserratus*, 8 *H. annuus*, and 6 *R. laciniata*. I also exposed *S. longipennis* var. *longipennis* (n = 11) to 19 *H. tuberosus*, 4 *H. grosseserratus*, 5 *H. annuus*, and 2 *R. laciniata*. I counted plant nodes and measured plant height before each trial began. I recorded the location of oviposition for each female that oviposited on a plant during an observation trial. *S. longipennis* var. *vittigera* females (n = 3) oviposited on 3 *H. tuberosus* plants, *S. longipennis* var. *longitudinalis* females (n = 20) oviposited on 37 *H. tuberosus* plants, and *S. longipennis* var. *longipennis* females (n = 8) oviposited on 11 *H. tuberosus* plants. *S. arculata* females (n = 2) oviposited on 3 *H. grosseserratus* plants.

To compare oviposition location between all plants in the greenhouse, the measured oviposition locations needed to be standardized to a scale that allowed for direct comparison between individual flies and plants. I standardized all plant stems based on the number of nodes to a scale between 0 and 1. Plant apical meristems were considered point 0 for each plant and the base of

the stem was considered point 1. All oviposition location values were between 0 and 1 on this scale. I used the following equation to standardize plant stems:

$$X_{ST} = \frac{X_{NO}}{(N+1)}$$

where X_{ST} is the standardized node position, X_{NO} is the original node position and N is the total number of nodes on the plant with the apical meristem counted as node 0. Using this standardization, I compared oviposition location across *S. longipennis* varieties. Flies oviposited from 0 to 100 times on a plant during a trial period. Flies were only included in the analysis if they oviposited 5 or more times during a trial. I averaged the oviposition location for each fly on each plant using the average of the first five observed oviposition locations. I compared the oviposition locations of all three *S. longipennis* varieties using Mann-Whitney tests because data did not have a normal distribution.

Plant Dissections and Larval Travel Patterns

To determine where larvae of each variety begin feeding, I collected 76 additional *H. tuberosus* stems from 3 field sites in the Iowa City area (Figure 3.3) in the fall of 2015 and bisected each stem to find enclosed *Strauzia* larvae or pupae. I sifted the soil found around the plant stems to look for *S. longipennis* var. *longipennis* that may have pupated in the soil. Each *Strauzia* was stored in 95% ethanol. I destructively extracted DNA from larvae and pupae using the Qiagen Blood and Tissue Kit (Qiagen Sciences, Germantown, MD) DNA extraction protocol. I PCR-amplified three diagnostic microsatellite loci (ST34, ST42 and ST49) using fluorescently-labeled forward primers. I genotyped each sample on an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA) and scored raw reads using panels designed in the program GeneMarker (SoftGenetics LLC, State College, PA). All allele calls were checked visually following the automatic scoring and samples were sorted by variety following the recommendations from previous STRUCTURE analysis (Hippee et al. 2016). I collected 11 *S.*

longipennis var. *vittigera* larvae, 8 *S. longipennis* var. *longitudinalis* larvae, and 18 *S. longipennis* var. *longpennis* larvae from field collected stems. I also collected 8 *S. longipennis* var. *vittigera* pupae, 4 *S. longipennis* var. *longitudinalis* pupae, and 1 *S. longipennis* var. *longipennis* pupa from these field collected stems. To infer where oviposition occurred, I measured the highest point of damage in the pith of the stem. I also determined the length that each larva traveled in the stem by measuring the length of the damage trail in the pith of each stem, as well as the height that each *Strauzia* larva or pupa was found. I also noted damage from other insects. In some cases, it was impossible to determine which insect had caused the damage at a given location within the stem, so these samples were excluded from the analysis.

Results

Oviposition Behavior

All three *S. longipennis* varieties oviposited more frequently on *H. tuberosus* plants compared to other plants. The oviposition frequency, or average number of ovipositions per hour, for *S. longipennis* var. *longitudinalis* was significantly higher (t-test, $P<0.001$) for *H. tuberosus* plants (mean oviposition frequency = 12.7 ovipositions per hour; n = 46 trials) than for all other plants combined (mean oviposition frequency = 1.18 ovipositions per hour, n = 21 trials). The same pattern was present for *S. longipennis* var. *longipennis* females. The oviposition frequency for *S. longipennis* var. *longipennis* was significantly higher (t-test, $P<0.05$) for *H. tuberosus* plants (mean oviposition frequency = 2.96 ovipositions per hour; n = 19 trials) than for all other plants combined (mean oviposition frequency = 0.73 ovipositions per hour, n = 11 trials). There was no significant difference between *H. tuberosus* oviposition frequency and *H. grosseserratus* oviposition frequency for *S. longipennis* var. *vittigera*. Results are summarized in Table 3.1.

Two of the three *S. longipennis* varieties and *S. arculata* oviposited far more often on the top quarter of the plant stem (Figure 3.4). The median oviposition location was calculated for each trial using the standardized node locations based on the first 5 ovipositions. The median

oviposition location for *S. longpennis* var. *longitudinalis* (median node location = 0.23, n = 37) and *S. longipennis* var. *longipennis* (median node location = 0.14, n = 11) were not significantly different (Mann-Whitney test, U = 142), indicating that they oviposit in similar locations on plant stems. Both *S. longipennis* var. *longitudinalis* and *S. longipennis* var. *longipennis* oviposited significantly higher (Mann-Whitney test, U = 2 in both pairwise comparisons) on the plant stem than *S. longipennis* var. *vittigera* (median node location = 0.43, n = 3). The median oviposition location of all *S. longipennis* varieties combined (median node location = 0.22, n = 51) on *H. tuberosus* was not significantly different from the oviposition of *S. arculata* (median node location = 0.2, n = 3; Mann-Whitney test, U = 70) on *H. grosseserratus*.

Plant Dissections and Larval Travel Patterns

S. longipennis var. *vittigera* and *S. longipennis* var. *longitudinalis* larvae were generally found in the same relative location within the *H. tuberosus* stem when comparing the average location of each type after standardizing each stem (Table 3.2). *Vittigera* and *longitudinalis* larvae were found close to the bottom of the stem (*vittigera* mean height = 0.99, N = 11; *longitudinalis* mean height = 0.85, N = 8) when the top of the stem was given a value of 0 and the root was given a value of 1. *S. longipennis* var. *longipennis* larvae were found significantly closer to the top of the stem (*longipennis* mean height = 0.51, N = 18) than *S. longipennis* var. *vittigera* (t-test, $p<0.001$) and *S. longipennis* var. *longitudinalis* (t-test, $p<0.01$). The start height of larvae was similar across all three varieties with most pith damage beginning in the upper half of the plant stem (*vittigera* mean location=0.47, N=11; *longitudinalis* mean location = 0.25, N = 8; *longipennis* mean location = 0.32, N = 18). There was no significant difference between the start height of larval damage between any of the three *S. longipennis* varieties (pairwise t-tests, $p>0.05$ in all comparisons).

The relative length of the path of damage was significantly shorter for *S. longipennis* var. *longipennis* (average relative length = 0.26) than both *S. longipennis* var. *vittigera* (average

relative length = 0.52, t-test, $p<0.01$) and *S. longipennis* var. *longitudinalis* (average relative length = 0.59, t-test, $p<0.05$). All larval collection data is summarized in Table 3.2. There were no significant differences in any of the data collected for pupae found in plant stems (Data summarized in Table 3.3). This may be due to low sample sizes of *S. longipennis* var. *longipennis* because this variety is known to pupate in soil surrounding the plant, making plant damage associated with the variety difficult to identify.

Discussion

In general, observations of oviposition behavior supported the partitioning by allochrony hypothesis (Fig. 3.2). Female *S. longipennis* var. *longitudinalis* and *S. longipennis* var. *longipennis* both tended to oviposit in the topmost quarter of the plant stem, suggesting that they have a preference nodes close to the shoot apical meristem. Only *S. longipennis* var. *vittigera* females oviposited lower than the other varieties, but these flies were also represented by a very small sample size, which needs to be increased in the future. The overall result supports the prediction that females do not show a preference in oviposition location and simply oviposit into the growing tip, with allochrony in eclosion resulting in pith partitioning. *S. arculata* also oviposited near the top of *H. grosseserratus* plants, so this preference for growing tips may be shared across the genus.

While *S. longipennis* var. *vittigera* and *S. longipennis* var. *longitudinalis* larva were generally found in the same location in the stem, *S. longipennis* var. *longipennis* were found significantly higher in the stem than the other varieties. This pattern of larval location was also predicted by the partitioning by allochrony model. As plants grow taller and thicker over the course of the summer and if temporally isolated *S. longipennis* varieties oviposit exclusively near the growing tips, eggs from the latest-emerging variety (*S. longipennis* var. *longipennis*) would end up higher in the stem than those laid earlier in the season. The differences in *S. longipennis* var. *longipennis* larval location may be a result of a shorter amount of time to grow and pupate or less available

plant tissue for feeding. The larval location of each variety supports the idea that allochronic isolation and plant growth are both important factors driving the partitioning of larval food resources within a single plant stem among the diverging varieties of *S. longipennis*. The current data show that *S. longipennis* var. *vittigera* and *S. longipennis* var. *longitudinalis* larvae have overlapping stem locations, but additional collections and stem measurements are necessary because larval measurements may be biased because the timing of field stem collections may alter the location of larvae inside the plant stem.

Collections of larvae and pupae from *H. tuberosus* in 2015 supports the previous finding that all *S. longipennis* varieties share the same host plant species with no evidence of habitat isolation (Hippee et al. 2016). Females from all three *S. longipennis* varieties preferred to oviposit on *H. tuberosus* over other known *Strauzia* host plant species. All possible combinations of larvae and pupae from all three varieties were found sharing an individual host plant in 36.1% of stems collected in the field with a maximum of 6 pupae found in a single plant, indicating that inter-variety competition may be present between *Strauzia* varieties without the temporal division provided allochronic isolation. On some occasions, females were observed ovipositing in alternative host plants in the greenhouse, perhaps indicating that other barriers, such as reduced performance on alternative hosts or allochronic isolation are relevant in the maintenance of all three varieties on the same host. Oviposition in alternative hosts may also just reflect the nature of the experiment, which measured host acceptance and not host discrimination or host choice. For the moment, it is possible that the willingness of all *S. longipennis* variety females to oviposit on non-natal hosts illustrates a hierarchy of female preference instead of the single host preference which is a trait common across specialist insects (Thompson and Pellmyr 1991). The willingness of females to oviposit on different plants may be affected by the relative quality and abundance of potential hosts in a geographic region (Craig et al. 1989). This flexibility in *S. longipennis* may

explain previous observations of *S. longipennis* using *H. annuus* and *H. decapetalus* as hosts (Stolzfus 1988) where *H. tuberosus* is less common or absent.

We did not attempt to directly measure competition between *S. longipennis* varieties here, but this should be a future goal. Competition between phytophagous insect varieties is challenging to identify and varies greatly between species (Denno et al. 1995). Finding competing phytophagous insect species is limited by the difficulty in finding populations with densities high enough to experience intense competition (Jermy 1984). The three varieties of *S. longipennis* may be a suitable population for an analysis of competition because all three varieties are frequently found to share a single host plant in the field. In addition, it may be possible to evaluate the competition between varieties of *S. longipennis* by looking at egg and larval mortality rates between multiple varieties sharing the same host plant. Understanding the complex interactions between insects and their host plants requires a wide variety of scientific disciplines (Schoonhoven et al. 1998). Though this study addresses behavioral observations of insects interacting with their hosts, plant chemical defense mechanisms, insect host identification processes, and interactions between *S. longipennis* varieties need to be evaluated in future work to address the broader implications of insect and plant host interactions.

Figure 3.1. The partitioning by ovipositional choice model. This model shows oviposition and location of larvae for all three varieties of *S. longipennis*. The figure on the left shows the oviposition location of each variety as marked by the three arrows. The figure on the right shows the location that larva would be found for each variety after eggs have hatched. The red is *S. longipennis* var. *vittigera*, the blue is *S. longipennis* var. *longitudinalis* and the yellow is *S. longipennis* var. *longipennis*.

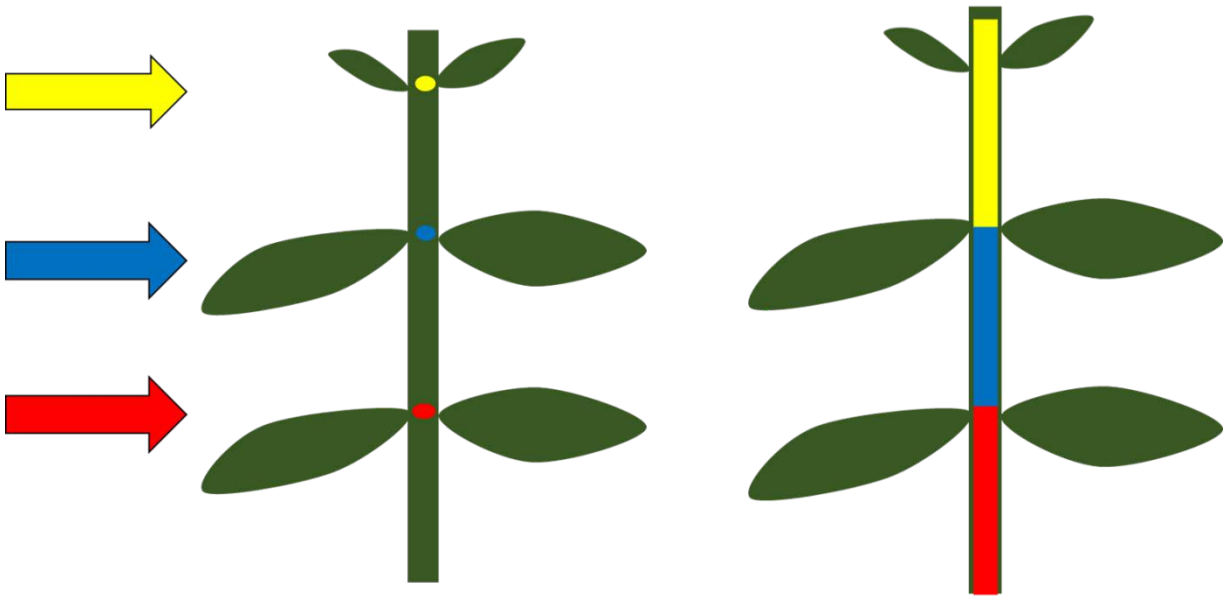

Figure 3.2. The partitioning by allochrony model. This model shows oviposition location and location of larvae for all three *S. longipennis* varieties. The three images on the left show the oviposition location for each variety as the plant grows as indicated by the colored arrows. The image on the right shows where larvae would be found if this model is correct. Red indicates *S. longpennis* var. *vittigera*, blue indicates *S. longipennis* var. *longitudinalis* and yellow indicates *S. longipennis* var. *longipennis*. The red arrow about the images on the left shows the direction of time as plants grow.

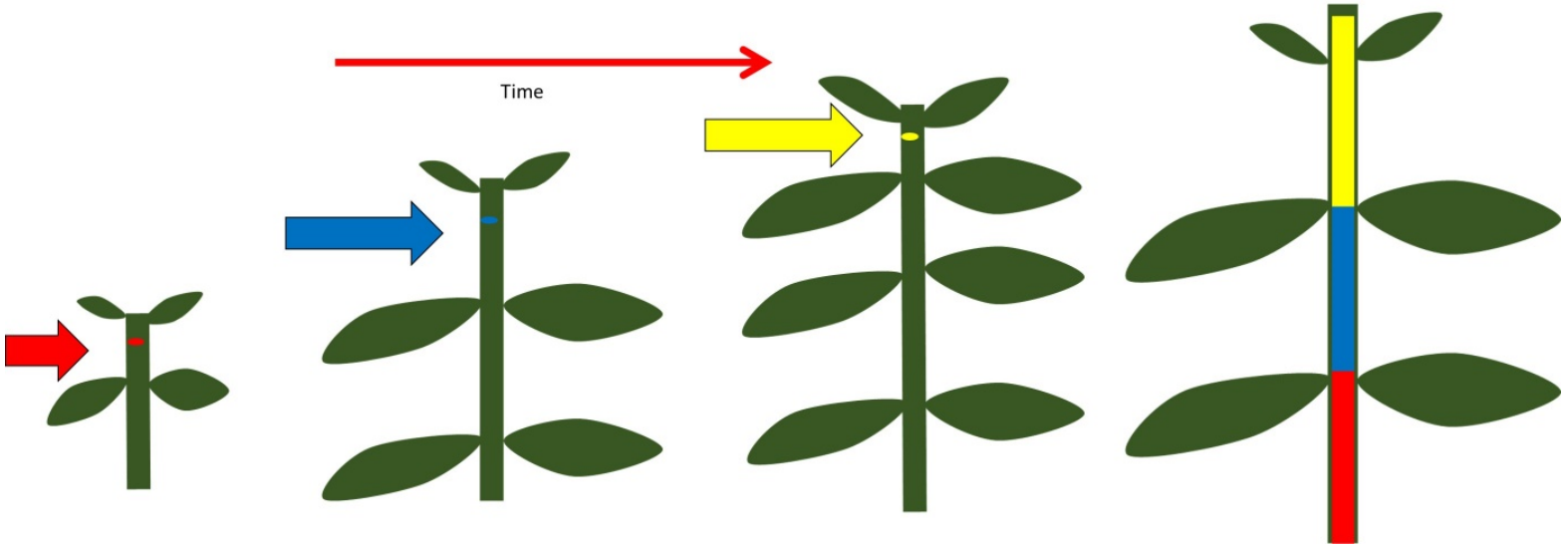

Figure 3.3. Map of collection sites used for 2015 collection season. F.W Kent Park (KP) is located in Oxford, IA and has a restored natural prairie with *H. grosseserratus*; Willow Creek Park (WC) is a park in Iowa City, IA with patches of *H. tuberosus*; Turkey Creek (TC) in Solon, IA is a reconstructed natural prairie with patches of *H. grosseserratus* and *H. tuberosus*; Scott Park (SP) in Iowa City, IA is a large residential park with stands of *H. tuberosus* and *A. trifida*; Hickory Hill Park (HH) is a large residential park in Iowa City, IA with patches of *H. tuberosus*; Waterworks Prairie Park (WW) is a recreational park area in Iowa City, IA with *H. tuberosus* near the parking lot.

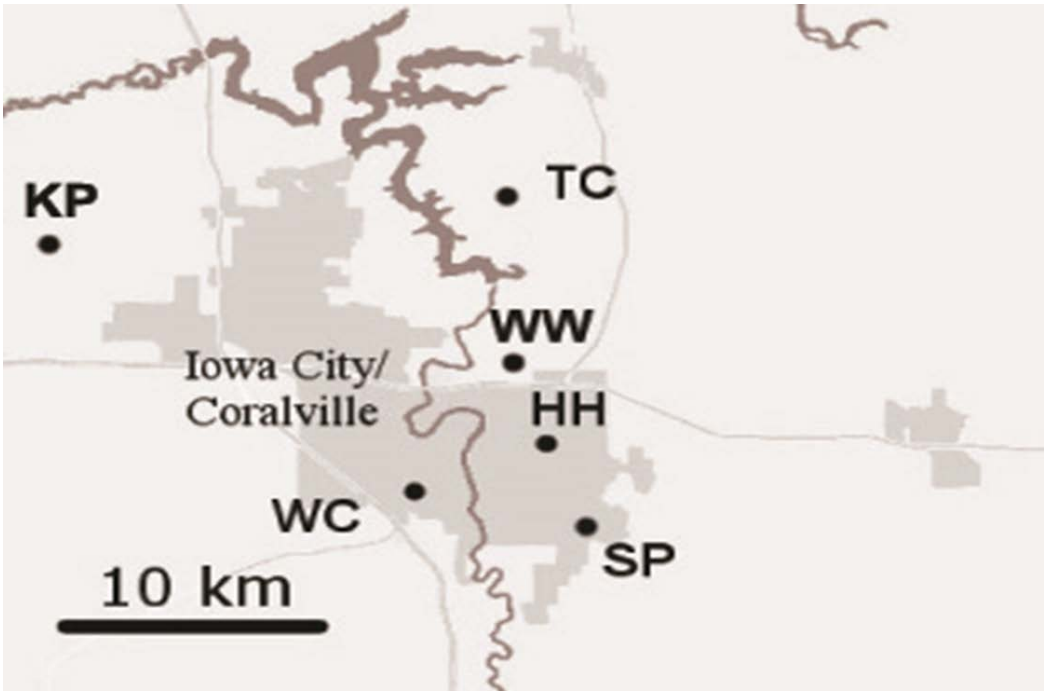

Figure 3.4. Box and whisker plot of average oviposition location. Plot shows average oviposition location of females ovipositing in *H. tuberosus* stems. A value of 0 represents the apical meristem and a value of 1 represents the bottom of the stem. Sample sizes are listed below each sample on the plot.

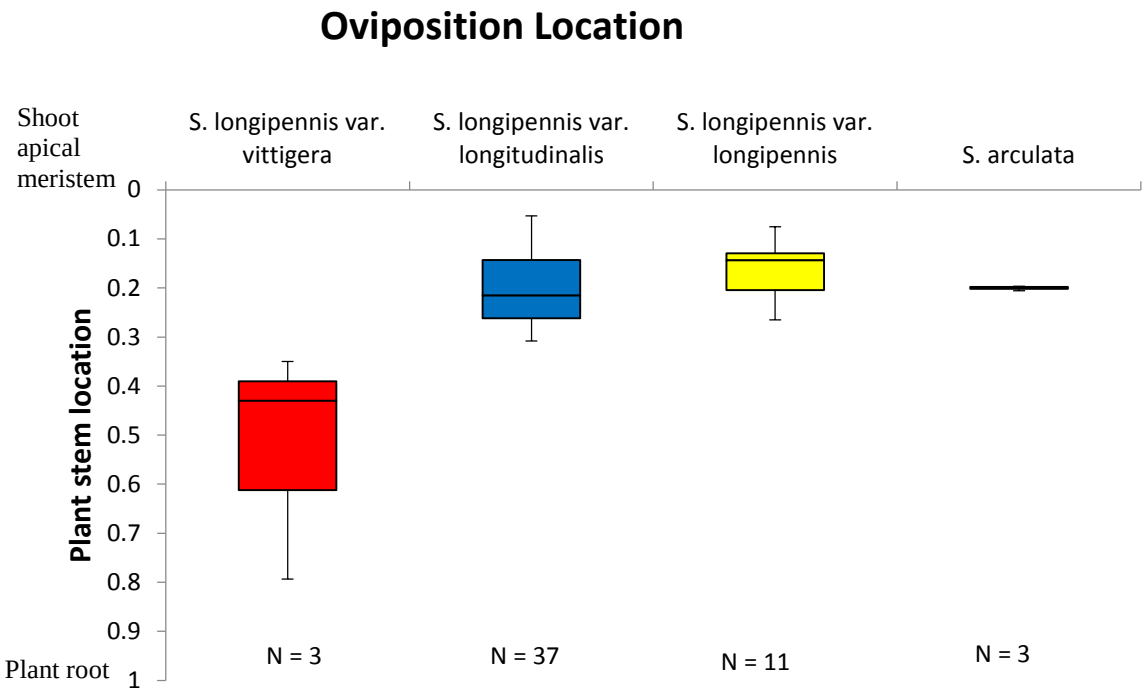

Table 3.1. Table showing the average oviposition frequency. This table show the average oviposition frequency (average number of ovipositions per hour) for each *S. longipennis* variety on *H. tuberosus* and all other *Strauzia* hosts used in this study. Standard error is included next to each average and sample size is included in parentheses. *S. longipennis* var. *vittigera* was not exposed to *H. annuus* or *R. laciniata*. An asterisk next to the *H. tuberosus* value indicates that the oviposition rate on *H. tuberosus* differs significantly from all other plant species for that *S. longipennis* variety.

Plant Species	*S. longipennis* Variety		
	S. longipennis var. *vittigera*	*S. longipennis* var. *longitudinalis*	*S. longipennis* var. *longipennis*
H. tuberosus	5.13±3.7 (6)	12.71±2.09 (46)*	2.96±0.79 (19)*
H. grosseserratus, *H. annuus* and R. *laciniata*	1.5±1.5 (4)	1.18±0.52 (21)	0.73±0.63 (11)

Table 3.2. Table summarizing results of larval collections. Averages and standard errors are listed for predicted oviposition height, height larva was found, and larval travel length from dissected *H. tuberosus* stems. Sample sizes are listed in parentheses. Averages with an "A" superscript differ significantly from varieties with the letter "B" in the same column.

S. longipennis variety	Oviposition Height	Height Found	Travel Length
S. longipennis var. *longipennis*	0.32±0.05 (18)	0.51±0.07 (18)^{B}	0.26±0.05 (18)^{B}
S. longipennis var. *longitudinalis*	0.25±0.07 (8)	0.85±0.08 (8)^{A}	0.59±0.1 (8)^{A}
S. longipennis var. *vittigera*	0.47±0.07 (11)	0.99±0.01 (11)^{A}	0.53±0.07 (11)^{A}

Table 3.3. Table summarizing results of pupal collections. Averages and standard errors are listed for predicted oviposition height, height pupae were found, and travel length from dissected *H. tuberosus* stems. Sample sizes are listed in parentheses. There are no significant differences between average values for each *S. longipennis* variety.

S. longipennis variety	Oviposition Height	Height Found	Travel Length
S. longipennis var. *longipennis*	0.12 (1)	0.42 (1)	0.3 (1)
S. longipennis var. *longitudinalis*	0.79±0.09 (4)	1.0±0 (4)	0.21±0.09 (4)
S. longipennis var. *vittigera*	0.69±0.11 (8)	0.98±0.02 (8)	0.33±0.11 (8)

REFERENCES

Axen HJ, JL Harrison, JR Gammons, IG McNish, LD Blythe, and MA Condon. 2010 Incipient speciation in *Strauzia longipennis* (Diptera: Tephritidae): two sympatric mitochondrial DNA lineages in Eastern Iowa. Annals of the Entomological Society of America. 103: 11-19.

Benson WW. Resource Partitioning in Passion Vine Butterflies. Evolution. 32(3): 493-518.

Berlocher SH and JL Feder. 2002. Sympatric Speciation in Phytophagous Insects: Moving Beyond Controversy?. Annual Reviews in Entomology. 47: 773-815.

Bolnick DI. Intraspecific competition favours niche width expansion in *Drosophila melanogaster*. Nature. 410: 463-466.

Borghuis A, J van Groenendael, O Madsen, and J Ouberg. 2009. Phylogenetic analyses of the leaf beetle genus *Galerucella*: Evidence for host switching at speciation? Molecular Phylogenetics and Evolution. 53: 361-367.

Caillaud MC and S Via. 2000. Specialized feeding behavior influences both ecological specialization and assortative mating in sympatric host races of pea aphids. American Naturalist. 156: 606-621.

Carvajal-Rodriguez A and E Rolan-Alvarez. 2006. JMATING: a software for the analysis of sexual selection and sexual isolation effects from mating frequency data. BMC Evolutionary Biology 6: 40.

Chou J and CA Mullin. 1993. Distribution and antifeedant associations of sesquiterpene lactones in cultivated sunflower (*Helianthus annuus* L.) on western corn rootworm (*Diabrotica virgifera vergifera* Leconte). Journal of Chemical Ecology. 19: 1439-1452.

Clusella-Trullas S, JH van Wyk, and JR Spotila. 2007. Thermal melanism in ectotherms. Journal of Thermal Biology. 32: 235–245.

Cocroft RB, RL Rodriguez, and RE Hung. 2010. Host shifts and signal divergence: mating signals covary with host use in a complex of specialized plant-feeding insects. Biological Journal of the Linnean Society. 99: 60-72.

Condon MA, SJ Scheffer, ML Lewis, and SM Swensen. 2008. Hidden neotropical diversity: greater than the sum of its parts. Science. 320: 928-931.

Condon MA, SJ Scheffer, ML Lewis, R Wharton, DC Adams, and AA Forbes. 2014. Lethal interactions between parasites and prey increase niche diversity in a tropical community. Science 343: 1240-1244.

Coyne JA and HA Orr. 1989. Patterns of Speciation in *Drosophila*. Evolution. 45(2): 362-381.

Coyne JA and HA Orr. 1997. "Patterns of Speciation in *Drosophila*" Revisited. Evolution. 51(1): 295-303.

Coyne JA and HA Orr. 2004. Speciation. Sunderland: Sinauer Associates, Inc.

Coyne JA, S Elwyn, E Rolan-Alvarez. 2005. Impact of experimental design on *Drosophila* sexual isolation studies: Direct effects and comparison to field hybridization data. Evolution. 59: 2588-2601.

Craig TP, JK Itami, WG Abrahamson, and JD Horner. 1993. Behavioral evidence for host-race formation in *Eurosta solidaginis*. Evolution. 47:1696-1710.

Craig TP, JK Itami, and PW Price. 1989. A Strong Relationship Between Oviposition Preference and Larval Performance in a Shoot-Galling Sawfly. Ecology. 70(6): 1691-1699.

Dambroski HR, C Linn Jr, SH Berlocher, AA Forbes W Roelofs, and JL Feder. 2005. The genetic basis for fruit odor discrimination in *Rhagoletis* flies and its significance for sympatric host shifts. Evolution. 59(9): 1953-1964.

Denno RF, MS McClure and JR Ott. 1995. Interspecific interactions in phytophagous insects: Competition Reexamined and Resurrected. Annu. Rev. Entomol. 40: 297-331.

Dres M and J Mallet. 2002. Host races in plant-feeding insects and their importance in ecological speciation. Philosophical Transactions of the Royal Society, Series B. 357:471-492.

Evanno G, S Regnaut, and J Goudet. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology. 14: 2611–2620.

Feder JL, TA Hunt, and GL Bush. 1993. The effects of climate, host plant phenology and host fidelity on the genetics of apple and hawthorn infesting races of *Rhagoletis pomonella*. Entomologia Experimentalis et Applicata. 69: 117-135.

Feder J, S. Opp, B Wlazlo, K Reynolds, W Go, and S Spisak. 1994. Host fidelity is an effective premating barrier between sympatric races. Proceedings of the National Academy of Sciences, USA. 91: 7990–7994.

Feder JL, SH Berlocher, JB Roethele, HR Dambroski, JJ Smith, WL Perry, V Gavrilovic, KE Filchak, J Rull, and M Aluja. 2003. Allopatric genetic origins for sympatric host-plant shifts and race formation in *Rhagoletis*. Proceedings of the National Academy of Sciences, USA. 100: 10314-10319.

Filchak KE, JB Roethele, and JL Feder. 2000. Natural selection and sympatric divergence in the apple maggot *Rhagoletis pomonella*. Nature. 407: 739-742.

Foote RH, FL Blanc, and AL Norrbom. 1993. Handbook of the fruit flies (Diptera: Tephritidae) of America north of Mexico. Ithaca: Cornell University Press.

Forbes AA, TH Powell, LL Stelinski, JJ Smith, and JL Feder. 2009. Sequential sympatric speciation across trophic levels. Science. 323: 776-779.

Forbes AA, PH Kelly, KA Middleton, and MA Condon. 2013. Genetically differentiated races and speciation-with-gene-flow in the sunflower maggot, *Strauzia longipennis*. Evolutionary Ecology. 27: 1017-1032.

Funk DJ. 1998. Isolating a role for natural selection in speciation: Host adaptation and sexual isolation in *Neochlamisus babbianae* leaf beetles. Evolution. 52: 1744-1759.

Funk DJ, KE Filchak, and JL Feder. 2002. Herbivorous insects: model systems for the comparative study of speciation ecology. Genetica 116: 251-267.

Funk DJ, P Nosil and WJ Etges. 2006. Ecological divergence exhibits consistently positive associations with reproductive isolation across disparate taxa. PNAS. 103(9): 3209-3213.

Hardy NB and SP Otto. 2014. Specialization and generalization in the diversification of phytophagous insects: tests of the musical chairs and oscillation hypotheses. Proceedings of the Royal Society, Series B. 281: 20132960.

Heiser CB, DM Smith, S Clevenger, and WC Martin. 1969. The North American sunflowers (*Helianthus*). Memiors of the Torrey Botanical Club. 22(3):1-218.

Hendry AP. 2001. Adaptive divergence and the evolution of reproductive isolation in the wild: an empirical demonstration using introduced sockeye salmon. Genetica. 112-113: 515-534.

Hippee AC, ME Elnes, JS Armenta, MA Condon, and AA Forbes. 2016. Divergence before the host shift? Prezygotic reproductive isolation among three varieties of a specialist fly on a single host plant. Ecological Entomology.

Hood GR, SP Egan, and JL Feder. 2012. Evidence for sexual isolation as a prezygotic barrier to gene flow between morphologically divergent species of *Rhagoletis* fruit flies. Ecological Entomology. 37: 521–528.

Hood GR, AA Forbes, THQ Powell, SP Egan, G Hamerlinck, JJ Smith, and JL Feder. 2015. Sequential divergence and the multiplicative origin of community diversity. PNAS. 112(44): E5980–E5989.

Jermy T. 1984. Evolution of insect/host plant relationships. American Naturalist. 609-630.

Joy JB and BJ Crespi. 2007. Adaptive radiation of gall-inducing insects within a single host-plant species. Evolution 61: 784-795.

Linn CE, HR Darnbroski, JL Feder, SH Berlocher, S Nojima, and WL Roelofs. 2004. Postzygotic isolating factor in sympatric speciation in *Rhagoletis* flies: reduced response of hybrids to parental host-fruit odors. Proceedings of the National Academy of Sciences, USA. 101: 17753–17758.

Lisowski E. 1979. Biochemical systematics of the genus *Strauzia* (Diptera: Tephritidae). M.S. thesis. University of Illinois, Urbana-Champaign.

Lisowski E. 1985. Taxonomy and Biology of *Strauzia* (Diptera: Tephritidae) in Illinois. PhD thesis. University of Illinois, Urbana-Champaign.

Loew H. 1873. Monographs of the Diptera of North America. Part III. Miscellaneous Collection of the Smithsonian Institution. 256: 1-351.

Martin EV. 1940. Effect of soil moisture on growth and transpiration in *Helianthus annuus*. Plant Physiology. 15(3): 449.

Matsubayashi KW, I Ohshima I, and P Nosil. 2010. Ecological speciation in phytophagous insects. Entomologia Experimentalis et Applicata. 134: 1-27.

Minakawa N, G Sonye, M Mogi and G Yan. 2004. Habitat characteristics of *Anopheles gambiae s.s.* larvae in a Kenyan highland. Medical and Veterinary Entomology. 13(3): 301-305.

Munga S, N Minakawa, G Zhou, O-OJ Barrack, AK Githeko, G Yan. 2006. Effects of Larval Competitors on Oviposition Site Selection of *Anopheles gambiae* Sensu Stricto. Journal of Medical Entomology. 43(2): 221-224.

Nosil, P. 2012. Ecological Speciation. Oxford: Oxford University Press.

Nosil P. 2004. Reproductive isolation caused by visual predation on migrants between divergent environments. Proc. R. Soc. Lond. B. 271: 1521-1528.

Nosil P and BJ Crespi. 2004. Does gene flow constrain trait divergence or vice-versa? A test using ecomorphology and sexual isolation in *Timema cristinae* walking-sticks. Evolution 58: 101-112.

Nosil P, BJ Crespi, and CP Sandoval. 2002. Host-plant adaptation drives the parallel evolution of reproductive isolation. Nature. 417: 440-443.

Nosil P, BJ Crespi, and CP Sandoval. 2003. Reproductive isolation driven by the combined effects of ecological adaptation and reinforcement. Proceedings of the Royal Society, Series B. 270: 1911-1918.

Nosil P, BJ Crespi, and CP Sandoval. 2004. Host-plant adaptation drives the parallel evolution of reproductive isolation. Nature. 417: 440-443.

Nosil P, BJ Crespi, Gries R, and Gries G. 2007. Natural selection and divergence in mate preference during speciation. Genetica. 129(3): 309-27.

Nosil P, Sandoval CP, and Crespi BJ. 2006. The evolution of host preference in allopatric vs. parapatric populations of Timema cristinae walking-sticks. Journal of Evolutionary Biology. 19: 929-942.

Powell THQ, AA Forbes, GR Hood, JL Feder. 2014. Ecological adaptation and reproductive isolation in sympatry: genetic and phenotypic evidence for native host races of *Rhagoletis pomonella*. Molecular Ecology. 23: 688-704.

Pritchard JK, M Stephens, and P Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics. 155: 945-959.

Rolan-Alvarez E and A Caballero. 2000. Estimating sexual selection and sexual isolation effects from mating frequencies. Evolution. 54: 30-36.

Rosenzweig ML. 1978. Competitive speciation. Biological Journal of the Linnean Society. 10(3): 275-289.

Sandoval CP. 1994. The effects of the relative geographic scales of gene flow and selection on morph frequencies in the walking-stick *Timema cristinae.* Evolution. 48(6): 1866-1879.

Schluter D. 2001. Ecology and the origin of species. Trends in Ecology & Evolution. 16(7): 372-380.

Schluter D. 2009. Evidence for ecological speciation and its alternative. Science 323: 737-741.

Schoonhoven LM, T Jermy and JJA van Loon. 1998. Insect-Plant Biology: From physiology to evolution. Cambridge: University Press.

Shapiro AM and RT Carde. 1970. Habitat Selection and Competition Among Sibling species of Satyrid Butterflies. Evolution. 24(1): 48-54.

Smith CD and RJ Prokopy. 1982. Mating behavior of *Rhagoletis mendax* (Diptera: Tephritidae) flies in nature. Annals of the Entomological Society of America. 75: 388-392.

Smith JJ and GL Bush. 1997. Phylogeny of the genus *Rhagoletis* (Diptera: Tephritidae) inferred from DNA sequences of mitochondrial cytochrome oxidase II. Molecular Phylogenetics and Evolution. 7: 33-43.

Steyskal GC. 1986. Taxonomy of the adults of the genus *Strauzia* Robineau-Desvoidy (Diptera, Tephritidae). Insecta Mundi. 1: 101-117.

Stireman III JO, JD Nason, and SB Heard. 2005. Host-associated genetic differentiation in phytophagous insects: general phenomenon or isolated exceptions? Evidence from a goldenrod insect community. Evolution. 59: 2573-2587.

Stoltzfus BW. 1988. The taxonomy and biology of *Strauzia* (Diptera: Tephritidae). Journal of the Iowa Academy of Science. 95: 117-126.

Thompson JN and O Pellmyr. 1991. Evolution of oviposition behavior and host preference in *Lepidoptera*. Annual Reviews of Entomology. 36: 65-89.

Via S. 1999. Reproductive isolation between sympatric races of pea aphids. I. Gene flow restriction and habitat choice. Evolution. 53:1446-1457.

Via S. 1991a. Specialized Host Plant Performance of Pea Aphid Clones is not Altered by Experience. Ecology. 72(4): 1420-1427.

Via S. 1991b. The Genetic Structure of Host Plant Adaptation in a Spatial Patchwork: Demographic Variability among Reciprocally Transplanted Pea Aphid Clones. Evolution. 45(4): 827-852.

Via S, AC Bouck, and S Skillman. 2000. Reproductive isolation between divergent races of Pea Aphids on two hosts. II. Selection against migrants and hybrids in the parental environments. Evolution. 54(5): 1626-1637.

Westdal PH and CF Barrett. 1960. Life-history and habits of the sunflower maggot, *Strauzia longipennis* (Wied.) (Diptera: Trypetidae), in Manitoba. Canadian Entomologist. 92: 481-488.

Wood, TK and SI Guttman. 1982. Ecological and behavioral basis for reproductive isolation in the sympatric *Enchenopa binotata* complex (Homoptera: Membracidae). Evolution. 36: 233–242.

Wood TK, and MC Keese. 1990. Host-plant-induced assortative mating in *Enchenopa* treehoppers. Evolution. 44: 619-628.

Zhang B, KA Segraves, H-J Xue, R-E Nie, W-Z Li, and X-K Yang. 2015. Adaptation to different host plant ages facilitates insect divergence without a host shift. Proceedings of the Royal Society, Series B. 282: 20151649.

APPENDIX

Figure A1. Map of collection sites for *Strauzia* flies used in this study. Willow Creek Park (WC) in Iowa City, IA is a residential park with many large patches of *H. tuberosus*; Hickory Hill Park (HH) in Iowa City, IA is a large city park with *H. tuberosus, H. grosseserratus* and *A. trifida*; Mount Vernon, IA (MV) has stands of *H. tuberosus, H. grosseserratus* and *A. trifida*; Indian Creek Nature Center (IC) in Cedar Rapids, IA has several stands of *H. tuberosus* and *A. trifida* in a managed prairie setting; Turkey Creek (TC) in Solon, IA is a reconstructed natural prairie with patches of *H. grosseserratus* and *H. tuberosus*; Scott Park (SP) in Iowa City, IA is a large residential park with stands of *H. tuberosus* and *A. trifida*; Prairie Meadows Drive (PM) is an unmanaged prairie site with *H. grosseserratus* and *A. trifida*. The University of Iowa's Lakeside Laboratory (LL) in Whapeton, IA has several large *H. grosseserratus* patches in managed natural prairie areas, and Cayler Prairie State Preserve (CY) in Spirit Lake, IA has two large patches of *H. grosseserratus* in one of Iowa's largest natural prairies. Wearin Prairie (WP) in Hastings, IA has *H. grosseserratus* interspersed with *R. laciniata* in another of Iowa's natural prairies and Hitchcock Nature Center (HC) in Honey Creek, IA, has one patch of *H. tuberosus* in an unmanaged trail area. Hoslett Study Area (HS) in Decorah, IA is a restored floodplain with large patches of *R. laciniata*. Cottonwood Road (CR) has patches of *H. tuberosus* and *A. trifida* in the ditches outside of St. Joseph, IL. Elephant Trunk Rock (ET), outside of Ithaca, WI is a park area with *H. tuberosus* and *H. grosseserratus*. Lake of the Woods (LW) is a park near Champaign, IL with *R. laciniata*.

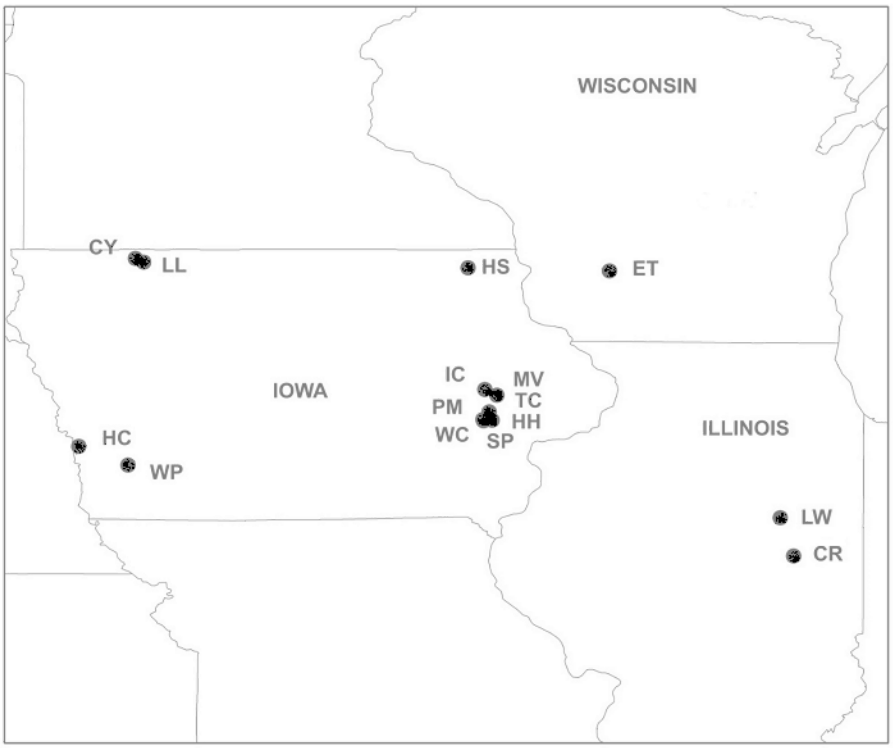

Table A1. Key to the names of each *Strauzia* variety discussed in this paper. Species names in this paper as compared to Lisowski (1979; 1985), Steyskal (186), Stoltzfus (1988), Axen et al. (2011) and Forbes et al. (2013). Note that "distinguishing features" only applies within *S. longipennis* and will not necessarily be useful in separating these flies from other species of North American *Strauzia*.

Distinguishing features	Lisowski (1979)	Lisowski (1985)	Steyskal (1986)	Stoltzfus (1988)*	Axen et al. (2011)	Forbes et al. (2013)	This paper
No notal stripes; Male wings posterior	Species F	*S. longipennis*		*S. longitudinalis**	*S. longipennis* var. *typica*	Cluster II	*S. longipennis* var. *longipennis*
Notal stripes; male wings coalesced	Species E	*S. longitudinalis*	*S. longipennis*	*S. longipennis**	*S. longipennis* var. *vittigera*	Cluster I	*S. longipennis* var. *longitudinalis*
Notal stripes; male wings "F" pattern	Species D	S. vittigera		*S. vittigera*		Cluster III	*S. longipennis* var. *vittigera*

*Descriptions of *S. longipennis* and *S. longitundinalis* in Stoltzfus (1988) run counter to all other descriptions.

Table A2. Microsatellite primers developed for *S. longipennis*. Table of microsatellite primers showing ranges of allele sizes for each variety (and for S. *noctipennis*). Three loci - ST34, ST42, and ST49 – proved diagnostic for easily discriminating between morphologically cryptic females of *S. longipennis* var. *vittigera* and *S. longipennis* var. *longitudinalis*. Primers for locus ST49 did not amplify for any *S. noctipennis* flies.

Locus name	Primers	Repeat type	Range of allele sizes *S. longipennis* var. *vittigera* (N =16)	Range of allele sizes *S. longipennis* var. *longitudinalis* (N = 72)	Range of allele sizes *S. longipennis* var. *longipennis* (N = 75)	Range of allele sizes *S. noctipennis* (N = 9 flies)
ST16	F: CAGCCATGTGCTTGGAGC R: AAGGTGAATGAAAGCAGCGG	AC	188-243	180-239	190-239	178-204
ST23	F: ACTGATAGCGCAAGCGACG R: ATGCAGTCGTGGGAAATGC	AC	312-341	312-341	317-344	323-328
ST24	F: AAATCCCTTAACTTGCAGAGGG R: GGGAAGAGTCAAGCAAGGAGC	AT	211-266	204-282	202-258	204-268
ST26	F: TGGTACAATCTCTGTCACTCTCCC R: TCAGAAATAGCTGAAAGGCTGC	TC	121-151	114-153	116-153	129-148
ST27	F: ACTGTAAAGGGAACGTGGCG R: AAATTTAATGCCAAGGCTCTCC	AC	436-480	424-495	430-493	460-480
ST32	F: TGCAAGTGCATGTGCAACC R: GCAACTACAGTCAGCAAATTCAGC	AC	191-204	187-226	191-221	198-200
ST34	F: GCAGTTTCTTGCCAACCACC R: AACCGGCGCAAATAACACC	TC	237-269	189-252	221-225	212-256
ST38	F: ACATCGTTTGTTTACACCACCC R: AGTCAAGGGCAACCGACG	AT	213-224	202-245	214-221	220
ST42	F: ACTACGATTCGAAAGCGTCC R: ATTCACGTGCACTCAATGG	AC	283-288	286-321	293-321	302
ST46	F: TTCGCTACATGCACAGTTGG R: GTTCATGCCTCATTGGCG	AT	281-283	272-300	279-300	278-323
ST49	F: CCAGGAAGCTCCTATGACTACG R: CACAATTAACAGTGAATACAGTGATGC	AT	297-311	307-334	300-343	N/A
ST50	F: TCGAAATAAACTATGAAGTTTGGTGG R: AAGCACGCCGGGTATTAGC	AT	141-145	127-155	135-148	145-148

Table A3. Field collections of adult *Strauzia* flies on *H. tuberosus.* Collections at 9 field sites from 2011-2015. Totals for each row and column are listed in bold. 2014 collections report male flies only, because females of *S. longipennis* var. *vittigera* and *S. longipennis* var. *longitudinalis* were not separated. Variation in the proportion of flies collected at each site reflects temporal differences in collection efforts (e.g., in 2011, we did not collect any *S. longipennis* var. *vittigera* flies at the IC site because these flies emerge early in the season (May) and we did not begin collections at that site until June).

	TOTAL FLIES	*S. longipennis* var. *vittigera*	*S. longipennis* var. *longitudinalis*	*S. longipennis* var. *longipennis*	Other *Strauzia*
H. tuberosus					
IC (2011)	**44**	0	15	29	0
IC (2012)	**0**	0	0	0	0
IC (2013)	**41**	17	20	4	0
IC (2014)	**146**	50	42	54	0
MV (2011)	**33**	1	11	21	0
MV (2012)	**20**	0	9	11	0
MV (2013)	**34**	3	17	14	0
MV (2014)	**39**	4	24	11	0
SP (2011)	**14**	0	9	5	0
SP (2013)	**32**	1	20	11	0
SP (2014)	**130**	63	42	25	0
SP (2015)	**180**	57	76	46	1
WC (2011)	**3**	0	1	2	0
WC (2012)	**44**	4	20	20	0
WC (2013)	**22**	3	17	1	1
WC (2014)	**21**	8	11	2	0
HC (2013)	**71**	47	4	20	0
HC (2014)	**227**	47	21	159	0
TC (2013)	**4**	2	2	0	0
TC (2014)	**59**	28	25	6	0
HH(2011)	**1**	0	0	1	0
HH(2012)	**3**	0	1	2	0
HH(2013)	**2**	1	0	0	1
HH(2014)	**13**	1	10	2	0
HH(2015)	**32**	21	9	2	0
ET (2013)	**127**	1	103	23	0
ET (2014)	**238**	48	150	39	1
CR(2013)	**58**	13	26	17	2
Total Flies	**1638**	**420**	**685**	**527**	**6**

Table A4. Field collections of adult *Strauzia* on *H. grosseserratus.* Collections are from 2012-2015 at 6 sites in Iowa. Totals for each row and column are listed in bold.

	TOTAL FLIES	*S. longipennis* var. *vittigera*	*S. longipennis* var. *longitudinalis*	*S. longipennis* var. *longipennis*	*S. noctipennis*	*S. arculata*	Other *Strauzia*
H. grosseserratus							
LL (2013)	**49**	0	0	0	47	2	0
LL (2014)	**24**	0	0	0	23	1	0
CY (2013)	**24**	0	0	0	5	19	0
CY (2014)	**24**	0	0	0	8	16	0
WP (2013)	**106**	0	0	0	77	29	0
WP (2014)	**32**	1	0	0	1	29	1
TC (2012)	**18**	0	0	0	10	8	0
TC(2014)	**1**	0	0	0	0	1	0
HH (2012)	**3**	0	0	0	0	3	0
KP (2015)	**74**	0	0	0	9	65	0
Total Flies	**355**	**1**	**0**	**0**	**180**	**173**	**1**

Table A5. Flies emerging from collections of *Strauzia longipennis* pupae. Collections are from *H. tuberosus* tubers (n=189) and soil (n=2) at 4 sites from 2011-2015. Only those that successfully eclosed in the lab were counted. Totals for each row and column are listed in bold. No other *Strauzia* species were reared from *H. tuberosus* plants.

	TOTAL FLIES	*S. longipennis* var. *vittigera*	*S. longipennis* var. *longitudinalis*	*S. longipennis* var. *longipennis*
IC (2011)	**20**	2	18	0
IC (2014)	**17**	12	5	0
MV (2011)	**1**	1	0	0
MV (2012)	**16**	5	11	0
MV (2013)	**11**	5	5	1
MV (2014)	**33**	11	20	2*
WC (2011)	**2**	2	0	0
WC (2012)	**22**	12	10	0
WC (2013)	**5**	5	0	0
WC (2014)	**14**	11	3	0
WC (2015)	**5**	4	1	0
SP(2015)	**43**	17	22	4
Total Flies	**189**	**87**	**95**	**7**

* These puparia collected from soil around *H. tuberosus.*

Table A6. Pupae and larvae collected from *H. tuberosus* stems and roots. *Strauzia* were collected from 3 sites in 2015. Insect varieties were determined using microsatellites. No other *Strauzia* species were found in *H. tuberosus* plants.

	TOTAL FLIES	*S. longipennis* var. *vittigera*	*S. longipennis* var. *longitudinalis*	*S. longipennis* var. *longipennis*
HH (2015)	**16**	8	4	4
SP (2015)	**36**	11	10	15
WW (2015)	**9**	3	5	1
Total Flies	**61**	**22**	**19**	**20**

Table A7. Table of count and frequency of each allele found at the ST34 locus. Table reports count and frequency of each allele found for *S. longipennis* var. *vittigera*, *S. longipennis* var. *longitudinalis*, *S. longipennis* var. *longipennis* and *S. noctipennis*.

	S. longipennis var. *vittigera*		*S. longipennis* var. *longitudinalis*		*S. longipennis* var. *longipennis*		*S. noctipennis*	
Allele	**Count**	**Frequency**	**Count**	**Frequency**	**Count**	**Frequency**	**Count**	**Frequency**
189	1	0	9	0.026087	0	0	0	0
190	0	0	1	0.0028986	0	0	0	0
191	1	0	5	0.0144928	0	0	0	0
192	0	0	0	0	0	0	0	0
193	1	0.00934579	28	0.0811594	0	0	0	0
194	0	0	0	0	0	0	0	0
195	9	0.08411215	60	0.173913	0	0	0	0
196	0	0	0	0	0	0	0	0
197	7	0.06542056	48	0.1391304	2	0.011695906	0	0
198	0	0	0	0	0	0	0	0
199	7	0.06542056	26	0.0753623	1	0.005847953	0	0
200	0	0	4	0.0115942	0	0	0	0
201	0	0	22	0.0637681	0	0	0	0
202	0	0	0	0	0	0	0	0
203	0	0	2	0.0057971	0	0	0	0
204	1	0.00934579	11	0.0318841	0	0	0	0
205	1	0.00934579	0	0	0	0	0	0
206	0	0	5	0.0144928	0	0	0	0
207	0	0	0	0	0	0	0	0
208	0	0	4	0.0115942	0	0	0	0
209	3	0.02803738	8	0.0231884	0	0	0	0
210	1	0.00934579	12	0.0347826	0	0	0	0
211	2	0.01869159	3	0.0086957	0	0	0	0
212	0	0	1	0.0028986	0	0	1	0.1
213	0	0	5	0.0144928	0	0	0	0
214	0	0	0	0	0	0	0	0
215	0	0	1	0.0028986	0	0	0	0
216	0	0	1	0.0028986	0	0	0	0
217	0	0	0	0	0	0	0	0
218	0	0	0	0	0	0	0	0
219	0	0	6	0.0173913	1	0.005847953	0	0
220	0	0	0	0	0	0	0	0
221	0	0	2	0.0057971	80	0.467836257	0	0
222	0	0	0	0	0	0	0	0
223	0	0	0	0	55	0.321637427	0	0
224	0	0	0	0	0	0	0	0

Table A7 - Continued

225	0	0	0	0	2	0.011695906	0	0
226	0	0	0	0	0	0	0	0
227	0	0	2	0.0057971	0	0	0	0
228	0	0	0	0	0	0	0	0
229	2	0.01869159	0	0	0	0	0	0
230	0	0	0	0	0	0	0	0
231	0	0	0	0	0	0	0	0
232	0	0	0	0	0	0	0	0
233	0	0	0	0	0	0	2	0.2
234	0	0	2	0.0057971	0	0	0	0
235	0	0	1	0.0028986	0	0	0	0
236	0	0	2	0.0057971	0	0	0	0
237	3	0.02803738	2	0.0057971	0	0	0	0
238	0	0	1	0.0028986	0	0	0	0
239	0	0	0	0	0	0	0	0
240	0	0	2	0.0057971	0	0	0	0
241	0	0	3	0.0086957	0	0	0	0
242	0	0	0	0	0	0	0	0
243	1	0.00934579	0	0	0	0	0	0
244	7	0.06542056	4	0.0115942	0	0	0	0
245	1	0.00934579	0	0	0	0	0	0
246	0	0	0	0	0	0	1	0.1
247	0	0	1	0.0028986	0	0	0	0
248	6	0.05607477	6	0.0173913	0	0	3	0.3
249	0	0	0	0	0	0	0	0
250	2	0.01869159	1	0.0028986	0	0	1	0.1
251	0	0	1	0.0028986	1	0.005847953	0	0
252	4	0.03738318	1	0.0028986	0	0	1	0.1
253	2	0.01869159	3	0.0086957	1	0.005847953	0	0
254	4	0.03738318	0	0	0	0	0	0
255	0	0	1	0.0028986	0	0	0	0
256	1	0.00934579	0	0	0	0	1	0.1
257	0	0	2	0.0057971	0	0	0	0
258	2	0.01869159	1	0.0028986	0	0	0	0
259	0	0	0	0	0	0	0	0
260	0	0	0	0	0	0	0	0
261	0	0	0	0	0	0	0	0
262	6	0.05607477	0	0	0	0	0	0
263	1	0.00934579	0	0	0	0	0	0
264	0	0	0	0	0	0	0	0

Table A7 - Continued

265	0	0	0	0	0	0	0	0
266	0	0	0	0	0	0	0	0
267	1	0.00934579	0	0	0	0	0	0
268	1	0.00934579	1	0.0028986	0	0	0	0
269	1	0.00934579	0	0	0	0	0	0
270	0	0	0	0	0	0	0	0
271	0	0	0	0	0	0	0	0
272	0	0	0	0	0	0	0	0
273	0	0	0	0	0	0	0	0
274	0	0	0	0	0	0	0	0
275	0	0	0	0	0	0	0	0
Total	**79**	**1**	**301**	**1**	**143**	**1**	**10**	**1**

Table A8. Table of count and frequency of each allele found at the ST42 locus. Table reports count and frequency of each allele found for *S. longipennis* var. *vittigera*, *S. longipennis* var. *longitudinalis*, *S. longipennis* var. *longipennis* and *S. noctipennis*.

	S. longipennis var. *vittigera*		*S. longipennis* var. *longitudinalis*		*S. longipennis* var. *longipennis*		*S. noctipennis*	
Allele	**Count**	**Frequency**	**Count**	**Frequency**	**Count**	**Frequency**	**Count**	**Frequency**
282	1	0.01	0	0.00	0	0	0	0.00
283	1	0.01	0	0.00	0	0	0	0.00
284	5	0.06	0	0.00	0	0	0	0.00
285	1	0.01	0	0.00	0	0	0	0.00
286	26	0.32	14	0.041543	1	0	0	0.00
287	0	0.00	0	0	0	0	0	0.00
288	2	0.02	0	0	0	0	0	0.00
289	0	0.00	0	0	0	0	0	0.00
290	0	0.00	0	0	0	0	0	0.00
291	0	0.00	0	0	0	0	0	0.00
292	0	0.00	0	0	0	0	0	0.00
293	2	0.02	2	0.0059347	1	0.00632911	0	0.00
294	0	0.00	0	0	0	0	0	0.00
295	0	0.00	0	0	0	0	0	0.00
296	0	0.00	2	0.0059347	0	0	0	0.00
297	0	0.00	1	0.0029674	0	0	0	0.00
298	0	0.00	0	0	0	0	0	0.00
299	0	0.00	7	0.0207715	0	0	0	0.00
300	0	0.00	2	0.0059347	3	0.01898734	0	0.00
301	1	0.01	13	0.0385757	0	0	0	0.00
302	0	0.00	3	0.0089021	1	0.00632911	16	1
303	6	0.07	23	0.0682493	0	0	0	0.00
304	10	0.12	8	0.0237389	25	0.15822785	0	0.00
305	5	0.06	31	0.0919881	0	0	0	0.00
306	0	0.00	0	0	9	0.05696203	0	0.00
307	0	0.00	18	0.0534125	1	0.00632911	0	0.00
308	2	0.02	14	0.041543	9	0.05696203	0	0.00
309	1	0.01	2	0.0059347	0	0	0	0.00
310	10	0.12	77	0.2284866	7	0.0443038	0	0.00
311	0	0.00	7	0.0207715	18	0.11392405	0	0.00
312	4	0.05	33	0.0979228	4	0.02531646	0	0.00
313	0	0.00	4	0.0118694	9	0.05696203	0	0.00
314	1	0.01	8	0.0237389	0	0	0	0.00
315	0	0.00	2	0.0059347	10	0.06329114	0	0.00
316	2	0.02	1	0.0029674	0	0	0	0.00
317	1	0.01	6	0.0178042	22	0.13924051	0	0.00

Table A8 - Continued

318	0	0.00	1	0.0029674	0	0	0	0.00
319	0	0.00	1	0.0029674	2	0.01265823	0	0.00
320	0	0.00	0	0	1	0.00632911	0	0.00
321	0	0.00	1	0.0029674	1	0.00632911	0	0.00
322	0	0.00	0	0	0	0	0	0.00
323	0	0.00	0	0	0	0	0	0.00
324	0	0.00	0	0	0	0	0	0.00
325	0	0.00	0	0	0	0	0	0.00
326	0	0.00	0	0	0	0	0	0.00
327	0	0.00	0	0	0	0	0	0.00
328	0	0.00	0	0	0	0	0	0.00
329	0	0.00	0	0	0	0	0	0.00
330	0	0.00	0	0	0	0	0	0.00
331	0	0.00	0	0	0	0	0	0.00
332	0	0.00	0	0	0	0	0	0.00
333	0	0.00	0	0	0	0	0	0.00
334	0	0.00	0	0	0	0	0	0.00
335	0	0.00	0	0	0	0	0	0.00
336	0	0.00	0	0	0	0	0	0.00
337	0	0.00	0	0	0	0	0	0.00
338	0	0.00	0	0	0	0	0	0.00
339	0	0.00	0	0	0	0	0	0.00
340	0	0.00	0	0	0	0	0	0.00
341	0	0.00	0	0	0	0	0	0.00
342	0	0.00	0	0	0	0	0	0.00
343	0	0.00	0	0	0	0	0	0.00
344	0	0.00	0	0	0	0	0	0.00
345	0	0.00	0	0	0	0	0	0.00
346	0	0.00	0	0	0	0	0	0.00
347	0	0.00	0	0	0	0	0	0.00
348	0	0.00	0	0	0	0	0	0.00
349	0	0.00	0	0	0	0	0	0.00
350	0	0.00	0	0	0	0	0	0.00
351	0	0.00	0	0	0	0	0	0.00
352	0	0.00	0	0	0	0	0	0.00
353	0	0.00	0	0	0	0	0	0.00
354	0	0.00	0	0	0	0	0	0.00
355	0	0.00	0	0	0	0	0	0.00
356	0	0.00	0	0	0	0	0	0.00
357	0	0.00	0	0	0	0	0	0.00

Table A8 - Continued

358	0	0.00	0	0	0	0	0	0.00
359	0	0.00	0	0	0	0	0	0.00
360	0	0.00	0	0	0	0	0	0.00
361	0	0.00	0	0	0	0	0	0.00
362	0	0.00	0	0	0	0	0	0.00
363	0	0.00	0	0	0	0	0	0.00
364	0	0.00	0	0	0	0	0	0.00
365	0	0.00	0	0	0	0	0	0.00
366	0	0.00	0	0	0	0	0	0.00
367	0	0.00	0	0	0	0	0	0.00
368	0	0.00	0	0	0	0	0	0.00
369	0	0.00	0	0	0	0	0	0.00
370	0	0.00	0	0	0	0	0	0.00
371	0	0.00	0	0	0	0	0	0.00
372	0	0.00	0	0	0	0	0	0.00
373	0	0.00	0	0	0	0	0	0.00
374	0	0.00	0	0	0	0	0	0.00
375	0	0.00	0	0	0	0	0	0.00
376	0	0.00	0	0	0	0	0	0.00
377	0	0.00	0	0	0	0	0	0.00
378	0	0.00	0	0	0	0	0	0.00
379	0	0.00	0	0	0	0	0	0.00
380	0	0.00	0	0	0	0	0	0.00
381	0	0.00	0	0	0	0	0	0.00
382	0	0.00	0	0	0	0	0	0.00
383	0	0.00	0	0	0	0	0	0.00
384	0	0.00	0	0	0	0	0	0.00
385	1	0.01	0	0	0	0	0	0.00
Total	**82**	**1**	**287**	**1**	**124**	**1**	**16**	**1**

Table A9. Table of count and frequency of each allele found at the ST49 locus. Table reports count and frequency of each allele found for *S. longipennis* var. *vittigera*, *S. longipennis* var. *longitudinalis*, *S. longipennis* var. *longipennis* and *S. noctipennis*.

	S. longipennis var. *vittigera*		*S. longipennis* var. *longitudinalis*		*S. longipennis* var. *longipennis*		*S. noctipennis*	
Allele	**Count**	**Frequency**	**Count**	**Frequency**	**Count**	**Frequency**	**Count**	**Frequency**
297	3	0.03658537	0	0	0	0	0	0
298	0	0	0	0	0	0	0	0
299	0	0	0	0	0	0	0	0
300	1	0.01219512	3	0.01140684	3	0.026086957	0	0
301	2	0.02439024	0	0	0	0	0	0
302	0	0	0	0	0	0	0	0
303	3	0.03658537	2	0.00760456	0	0	0	0
304	5	0.06097561	5	0.01901141	0	0	0	0
305	12	0.14634146	2	0.00760456	0	0	0	0
306	0	0	0	0	0	0	0	0
307	7	0.08536585	11	0.0418251	3	0.026086957	0	0
308	0	0	3	0.01140684	1	0.008695652	0	0
309	10	0.12195122	4	0.01520913	23	0.2	0	0
310	0	0	3	0.01140684	0	0	0	0
311	5	0.06097561	5	0.01901141	36	0.313043478	0	0
312	2	0.02439024	2	0.00760456	0	0	0	0
313	0	0	4	0.01520913	16	0.139130435	0	0
314	0	0	16	0.0608365	0	0	0	0
315	0	0	24	0.09125475	0	0	0	0
316	0	0	0	0	0	0	0	0
317	0	0	2	0.00760456	0	0	0	0
318	0	0	0	0	0	0	0	0
319	0	0	0	0	3	0.026086957	0	0
320	0	0	1	0.00380228	0	0	0	0
321	1	0.01219512	30	0.11406844	5	0.043478261	0	0
322	6	0.07317073	18	0.06844106	0	0	0	0
323	2	0.02439024	2	0.00760456	0	0	0	0
324	0	0	0	0	0	0	0	0
325	0	0	11	0.0418251	1	0.008695652	0	0
326	2	0.02439024	0	0	1	0.008695652	0	0
327	7	0.08536585	72	0.27376426	0	0	0	0
328	8	0.09756098	28	0.10646388	2	0.017391304	0	0
329	1	0.01219512	7	0.02661597	1	0.008695652	0	0
330	2	0.02439024	2	0.00760456	2	0.017391304	0	0
331	0	0	0	0	0	0	0	0

Table A9 - Continued

332	1	0.01219512	5	0.01901141	1	0.008695652	0	0
333	0	0	0	0	0	0	0	0
334	2	0.02439024	1	0.00380228	7	0.060869565	0	0
335	0	0	0	0	0	0	0	0
336	0	0	0	0	6	0.052173913	0	0
337	0	0	0	0	0	0	0	0
338	0	0	0	0	1	0.008695652	0	0
339	0	0	0	0	0	0	0	0
340	0	0	0	0	0	0	0	0
341	0	0	0	0	0	0	0	0
342	0	0	0	0	1	0.008695652	0	0
343	0	0	0	0	2	0.017391304	0	0
Total	**82**	**1**	**263**	**1**	**115**	**1**	**0**	**0**

Table A10. Table of calculated I_{PSI} values. Calculated I_{PSI} values of each possible pairwise combination of *Strauzia* involved in no choice mating trials. A and B are the two varieties being compared. AA, BB, AB and BA are the number of pairs observed mating on at least one occasion. N (AA), N (BB), N (AB) and N (BA) are the total number of pairings for each variety or species combination. Total N is the sum of all pairs being compared. Standard deviations are listed in parentheses.

Comparison		Homospecific Pairs				Heterospecific Pairs						
A	B	AA	N (AA)	BB	N (BB)	AB	N (AB)	BA	N (BA)	Total N	I_{PSI}(SD)	I_{PSI} P value
S. arculata	*S. noctipennis*	41	44	11	16	3	12	0	4	76	0.9043 (0.049)	0
S. intermedia	*S. perfecta*	27	28	32	38	0	3	1	2	71	0.9687(0.0302)	0
S. perfecta	*S. arculata*	32	38	41	44	2	9	0	4	95	0.9371(0.0385)	0
S. arculata	*S. intermedia*	41	44	27	28	0	4	0	2	78	1.0(0.0)	0
S. noctipennis	*S. perfecta*	11	16	32	38	0	5	2	7	66	0.9213(0.0505)	0
S. longipennis var. *longipennis*	*S. arculata*	70	97	41	44	6	18	1	10	169	0.8888(0.0394)	0
S. longipennis var. *longipennis*	*S. noctipennis*	70	97	11	16	4	10	3	8	131	0.8192(0.0751)	0
S. longipennis var. *longipennis*	*S. intermedia*	70	97	27	28	2	5	0	1	131	0.9619(0.0257)	0
S. longipennis var. *longipennis*	*S. perfecta*	70	97	32	38	1	12	5	29	176	0.8945(0.0409)	0
S. longipennis var. *vittigera*	*S. arculata*	31	34	41	44	3	4	6	7	89	0.7849(0.0679)	0
S. longipennis var. *vittigera*	*S. noctipennis*	31	34	11	16	0	1	1	1	52	0.9574(0.0404)	0
S. longipennis var. *vittigera*	*S. intermedia*	31	34	27	28	1	3	0	5	70	0.9682(0.0306)	0
S. longipennis var. *vittigera*	*S. perfecta*	31	34	32	38	0	1	5	15	88	0.8747(0.0477)	0
S. longipennis var. *longipennis*	*S. longipennis* var. *vittigera*	70	97	31	34	22	41	0	5	177	0.7384(0.0375)	0
S. longipennis var. *longitudinalis*	*S. arculata*	87	94	41	44	1	2	9	11	151	0.8673(0.0384)	0
S. longipennis var. *longitudinalis*	*S. noctipennis*	87	94	11	16	3	12	2	5	127	0.8915(0.0537)	0
S. longipennis var. *longitudinalis*	*S. intermedia*	87	94	27	28	0	2	0	7	131	1(0)	0
S. longipennis var. *longitudinalis*	*S. perfecta*	87	94	32	38	3	9	14	47	188	0.7648(0.0538)	0
S. longipennis var. *longitudinalis*	*S. longipennis* var. *longipennis*	87	94	70	97	2	21	26	69	281	0.7492(0.0394)	0
S. longipennis var. *longitudinalis*	*S. longipennis* var. *vittigera*	87	94	31	34	10	20	7	22	170	0.7379(0.0624)	0

Table A11. Identities and number of *Strauzia* pairs collected *in copulo*. *Strauzia* were collected on their respective host plants from 2011-2015. Numbers in bold are intra-species or intra-variety pairs. 112 pairs collected in 2014 were omitted because females of *S. longipennis* var. *vittigera* and *S. longipennis* var. *longitudinalis* cannot be distinguished morphologically.

		Female partner						
		S. arculata	*S. noctipennis*	*S. intermedia*	*S. perfecta*	*S. longipennis* var. *vittigera*	*S. longipennis* var. *longitudinalis*	*S. longipennis* var. *longipennis*
Male partner	*S. arculata*	**26**	0	0	0	0	0	0
	S. noctipennis	0	**8**	0	0	0	0	0
	S. intermedia	0	0	**27**	0	0	0	0
	S. perfecta	0	0	0	**22**	0	0	0
	S. longipennis var. *vittigera*	0	0	0	0	**29**	1	1
	S. longipennis var. *longitudinalis*	0	0	0	0	2	**10**	0
	S. longipennis var. *longipennis*	0	0	0	0	13	2	**26**