THE ECOLOGY OF INSECT MEDIATED TRANSMISSION OF THE FIRE BLIGHT PATHOGEN, *ERWINIA AMYLOVORA*, BY ORCHARD DWELLING DIPTERANS

A Dissertation
Presented to the Faculty of the Graduate School
of Cornell University
In Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy

by
Matthew T. Boucher
August 2020
Fire blight, caused by the bacterial pathogen *Erwinia amylovora*, is a devastating disease of pome fruit with worldwide distribution. The disease gets its name from the scorched appearance of diseased tissue and infects all tissue types of pomaceous fruit. Tissue in the early stages of infection exude a bacterial ooze containing the bacteria in a polysaccharide matrix, which acts as primary and secondary inoculum. Insects have been implicated in the transmission of *E. amylovora* since the pathogen was first discovered, with various mechanisms for insect mediated transmission proposed. The goal of this research was to further define the role of insects in transmission of *E. amylovora* by identifying its most important potential vectors in New York State and advancing our knowledge of the ecology of transmission by insects. In chapter 1, we describe field surveys used to identify key insect vectors over the course of a growing season, showing that while pollinating hymenopterans historically received attention as springtime disease disseminators, dipterans have an understudied and potentially outsized role in transmission throughout the entire season. Various families of Diptera were observed feeding on bacterial ooze and could shed bacteria for at least six days following an acquisition event from ooze. In chapter 2, we use lab bioassays to investigate biological factors affecting the ability of *Drosophila melanogaster* to acquire and transmit *E. amylovora*, showing that mating status, nutritional state, and sex had no effect on acquisition and transmission. The data presented in this chapter supports earlier hypothesis outlining Diptera as mechanical vectors. In chapter 3, we showed that *Delia platura*, an anthomyiid, can successfully initiate new infections in damaged apple saplings, and demonstrated that the bacterium has no deleterious effects on the flies. Based
on this data, we suggest that the relationship between *D. platura* and *E. amylovora* is at least commensal and possibly mutualistic, wherein the fly benefits by using ooze as a food source and the bacterium benefits by insect mediated transmission. In chapter 4, we showed that *E. amylovora* has minor behavioral effects on *D. platura*, but further research is required to understand how these changes in behavior impact disease transmission success. Collectively, this work provides a roadmap for the future study of insect mediated transmission of *E. amylovora*. The role of Diptera in the disease cycle is clear and has been largely ignored for more than 120 years. We argue for greater attention to the details of Diptera driven transmission, especially relating to insect behavior, as this space within the disease cycle has largely untapped potential to benefit management and disease outbreak models.
BIOGRAPHICAL SKETCH

Matthew was born in the small town of Southampton in Western Massachusetts in 1993 to Mary Belcamino. As a child, he desired to be a baseball player, falling in love with the thrill of the sport when his beloved Boston Red Sox made an ultimately devastating playoff run in 2003. Of course, dreams do not always come true, and by the time he aged out of Little League it was, in fact, clear that some modicum of hand eye coordination was lacking to attain such lofty goals. Matthew came to love the outdoors simply by having the privilege to have access to them, leading, at least as he tells it, to an interest in how the natural world functioned. He attended Hampshire Regional High School in Westhampton, Massachusetts, where a number of thoughtful mentors supported his budding interest in biology and encouraged him to pursue those interests in college.

At the University of Massachusetts, Amherst (pronounced AM-URST, not AM-HURST), he majored in biology and anthropology and conducted research under the guidance of John Burand and Joe Elkinton on entomopathogenic viruses of invasive insects, including winter moth, Operophtera brumata, and honeybees, Apis mellifera. He became a resident assistant in his sophomore year, engaging in the structural advantages afforded to him and reflecting on the depths of systematic oppression, especially relating to sustainability and the food system. His desire to pursue graduate studies relating to food and agriculture developed out of this reflection and he moved to Cornell University in 2015 to begin those pursuits with Greg Loeb.

Cornell AgriTech in Geneva, NY quickly became home for Matthew, and as he reflected on the last five years, he is eternally grateful for the friendships and mentors that have become a part of his life along the way. More can be done to make the world a better, safer place, and this notion underscores his research goals as he finally casts off the “student” label. He has the privilege of feeling hope in the dark days of the recent past, and will stand with those who have been standing for their entire life, and need rest.
To Mariam, with love

Equally, wholly, and always.
ACKNOWLEDGMENTS

Five years is a long time, and many people have moved in and out Cornell AgriTech during that time that have been essential to the completion of this dissertation. First, to my advisor Greg Loeb, thank you. Thank you for believing in me, and my independence. Thank you for your guidance on tough days and for listening to my rants about various injustices. Thank you for your willingness to grow with the times. Whether you know it or not, you have set an example for every PI around you to think more critically about how they support their students. I came to your lab incredibly young, a little angry, and though I do not like to admit it, a touch naïve. You gave me space for my instincts to mature and taught me a particular level of diplomatic finesse that I certainly lacked. I am still pretty much always fired up about something. It is my stasis, but I am now better equipped to use my fire and my privilege to push for change that the academy needs, and that is in no small part because of your mentorship.

To Steve Hesler, our research support specialist, I may have never gotten that Red Sox hat on your head, but that will not change the depths of my appreciation for all you have done for me. I do not really have a father figure in my life, yet you filled that role time and again in ways that I truly did not appreciate until I sat back and reflected on it all. A singular conversation stands out to me, shortly after I got engaged, at the lab bench on the fifth floor processing our ten millionth Drosophila sample. We talked about what marriage means, to you, to me, to the world. We talked about the commitment to the work of marriage, and the joy of traveling from place to place with someone important to you. I’m not sure that you’ll remember it, because those types of conversations are normal to you, but their impact on me is unquantifiable.

To Kayli Harling, Rowan Collins, and Sam Willden, this work would not have gotten done without you. Thank you for the energy you brought to the team each day, you lifted the spirits of everyone around you, and kept us going through work and life related hardships. Thank you for every talk about justice, about truth, about what it means to be a good person. You are all change makers, and you will make change in your own way. You are powerful and I believe in you, always.
To the Kerik and the entire Cox lab, thank you for welcoming me into your lab, this project would truly not have gotten off the ground without your guidance. Thank you for your energy and lightheartedness, which always got me through tough times and manuscript rejections. I am excited to watch your lab grow, as the camaraderie you build keeps people energized and excited about their work.

To the folks in Geneva, Karen, Gabe, Molly, Holly, Kyle, Abby, Brian, Wendy, Ping, and all the rest: Geneva is a welcoming place because of you. I shed a tear (or a dozen) on the day I was awarded the Villani Award a few years ago. Receiving that award is truly the most honored I have ever been. This dissertation is a commitment to, and a reaffirmation of, the values embodied by Dr. Villani through its content and through the responsibility I carry with me to the work of building community in research spaces. To Karen Wentworth specifically, I promise that The Trough will open one day, and I will always have a booth reserved for you. The Trough is the future of food, thank you for believing in my dream.

To Mason Clark and Mike Wolfin, you have both served as confidants and mentors to me throughout my time here. Mason, your ability to be vulnerable in a world full of men who are poorly in touch with their feelings is inspiring, and your search for truth and meaningfulness in all aspects of life is second to none. Mike, you pushed me, always, to be better and to keep thinking and questioning. Your endless curiosity and willingness to listen brought this dissertation forward at times when I thought it was stuck, and your advocacy for bringing new students to Geneva has started a tradition that has built a vibrant group that only seems to be getting bigger.

To my Geneva crew, Anna, Zach, Lori, Bryan, Tony, Jane, and Erica. You, to me, are family. I love you all, your heart, your compassion, your goodness. To your core you are all good and just and truthful. It is hard to find people like you, and for all of our lives to converge in a small town in upstate New York is special. I will carry our memories and our friendships wherever I go, and I will see you all soon, and often.

To Talya, John, Natalie, and Silas, I would not have chosen another group to experience
this with. I like to think that we looked after each other in our toughest times, and if that is not how it always played out, I felt safe knowing that I had you turn to. Thank you each, you were there at momentous moments in my life, and sharing that happiness with you was itself a purveyor of joy.

To Max, Jeff, Hudson, Adam, Reid, Molly, and Eric, one of my many chosen families, thank you. Thank you for allowing me to step out of my world and relax, to take time just being with you all. As I reflect on the years and years of friendship between us, I see a lot change. We’re different from who we were nearly a decade ago when we took our first New Hampshire trip, and we’re different from who were five years ago when I hugged you all goodbye on the balcony of that giant house after the Franconia Loop Trail. I was filled with fear that day, that maybe this was the last time we would all be together, but of course that was never going to happen. We have grown together, in weird ways, mostly good. Some of us are married and some us will be soon, and the next five years will likely lend itself to more change and more happiness. Closing this chapter of my life has brought these thoughts to the forefront, that wherever I go I’m taking you with me, hell or high water, and I know the same is true for you. Celebrating our moments together is what I look forward to the most, and there will always be time for that, each more enduring than the last.

To my brother Mike, and my sisters Miranda and Madison, I really do not know what to say to you weirdos. I am so proud of you, so incredibly proud. Your work ethic and pursuit of passion is fun to watch, and I consider myself lucky to have grown with you. Mike, from our one-person wiffle ball teams in the backyard to your exit seminar, you have cut a path that I followed, and I am not sure I could have made here without first seeing you do it all. Miranda, I have never seen anyone more singularly focused on your goals than you. You have a drive that I do not think anyone else can muster. Wherever you go, you will be great, because that is what you have done so far. Madison, you are the master of controlled chaos, a whirlwind of personality, and an inspiration. Follow your heart, wherever it takes you, it has gotten you this far. Take chances, big ones, and never forget that there is always, always, always time to change
course.

Mom, I did it. I do not really know what else to say. Thank you. For everything. It was not always easy, but when I needed you, you had my back. You were often the first one to tell me to take a break, or to remind me what my goals were. You kept me going at times when I felt stuck, often in small ways, as simple reminders to take care of myself. Sometimes you just need your mom to tell you that you will be fine, and I am privileged to know that I will get that when I vent my frustrations. We have come a long way. I’m all grown up, and I am thankful that you have been there for it.

To the Talebs and the Bigelows, thank you for bringing me into your family. Your goodness is tangible and welcoming and safe. I am excited to build a life with you all in it, and your advice, encouragement, and pride are rejuvenating. Haytham, thank you for your bits and pieces of advice, and nurturing spirit. I do not really know how to capture your energy in words, but your kindness and your depth of belief in Mariam and I is steadying, always.

Finally, Mariam. We did this. Together. Reflecting on the last five years, from the stages of uncertainty, to the parallel conversations we were having with the people around us, to the sureness and togetherness that we have committed to, I see change. I see the fight that you fight every day, and every day, I am proud of you. I am proud of the power of your compassion, and the lengths at which you will go to protect your people. You are tireless in the face of injustice and true to your values like no other. Your exploration and reflection on truth and justice is your driving force, and I’ll be here, always, to remind of you that. When you speak, people listen. The room quiets down because you have something to say. You speak not from power, but from belief, from hope, which you dare to feel on a daily basis, even when the world chooses not to. I see it in you every day, and it is the honor of my life to witness it. It is with love that we will build a world together, wherever, together. Follow your arrow, and I’ll be there too.
TABLE OF CONTENTS

BIOGRAPHICAL SKETCH...........................................................................................................v
ACKNOWLEDGEMENTS...........................................................................................................vii
LIST OF FIGURES.....................................................................................................................xii
LIST OF TABLES......................................................................................................................xiv
PREFACE......................................................................................................................................xv

CHAPTER 1: Field evaluation of interactions between insects and *Erwinia amylovora* in a New York apple orchard...................................................................................................................1

CHAPTER 2: Effects of exposure time and biological state on acquisition and accumulation of *Erwinia amylovora* by *Drosophila melanogaster*...........................................................................................................37

CHAPTER 3: Interactions between *Delia platura* and *Erwinia amylovora* associated with insect mediated transmission of shoot blight.........................................................................................69

CHAPTER 4: The effect of *Erwinia amylovora* infection in apple saplings and fruit on the behavior of *Delia platura*..................................................................................................................116
LIST OF FIGURES

Figure 1.1: Population of Delia spp. captured on all yellow sticky cards in 2016 and 2017……32
Figure 1.2: Percentage of E. amylovora ooze droplets by color……………………………………33
Figure 1.3: Percentage of E. amylovora ooze droplets by location………………………………..34
Figure 1.4: Persistence of E. amylovora over 7 days in 10 dipteran samples collected after
    feeding on ooze........................................................................................................35
Figure 1.5: Mean log10 CFU/mL + 95% CI E. amylovora in control and insect visited blossoms
    by sampling date......................................................................................................36
Figure 2.1: Effect of exposure time on overall percentage of flies positive for E.
    amylovora..................................................................................................................65
Figure 2.2: E. amylovora log10 CFU/Insect values at 3, 6, 12, and 24 hours of exposure……….66
Figure 2.3: E. amylovora log10 CFU/Insect values for food deprived and satiated D.
    melanogaster.............................................................................................................67
Figure 2.4: E. amylovora log10 CFU/Insect values for mated and unmated D.
    melanogaster.............................................................................................................68
Figure 3.1: Percentage of Gala apple saplings positive for E. amylovora when exposed to
    pathogen by 35 D. platura..........................................................................................103
Figure 3.2: Mean disease severity in Gala apple saplings.....................................................104
Figure 3.3: Median initial dose in Log10 CFU/mL between flies that acquired E. amylovora from
    ooze and from filter disks.........................................................................................105
Figure 3.4: Predicated probability that a fly will shed E. amylovora over time…………………..106
Figure 3.5: Mean E. amylovora shed by D. platura in Log10 CFU/mL separated by strain…….107
Figure 3.6: Predicted probability that a fly will be contaminated with E. amylovora on external or
    internal surface depending on acquisition method.....................................................108
Figure 3.7: Predicted probability that a fly will be contaminated with E. amylovora when
    subjected to different acquisition methods..............................................................109
Figure 3.8: Predicted probability that a fly will be internally or externally contaminated with E.
Figure 3.9: Mean *E. amylovora* load on internal and external surfaces of *D. platura* in Log_{10} CFU/mL by acquisition method

Figure 3.10: Estimated marginal mean of total *E. amylovora* colony forming units on external or internal surface of *D. platura* over time

Figure 3.11: Mean internal *E. amylovora* load in *D. platura* by strain over time

Figure 3.12: Estimated marginal mean showing predicted change in internal load over time by strain

Figure 3.13: Probability of *D. platura* survival over time when fed one of two *E. amylovora* strains or a sucrose control

Figure 4.1: Diagram of fruit preference arena

Figure 4.2: Diagram of Y-tube set up

Figure 4.3: Mean total *D. platura* counts on apple fruit in three choice assays

Figure 4.4: Mean total *D. platura* counts on apple saplings in three choice assays

Figure 4.5: Frequency of *D. platura* choice in apple sapling Y-tube assays

Figure 4.6: Frequency of *D. platura* choice in apple fruit Y-tube assays
LIST OF TABLES

Table 1.1: Breakdown of pollinating hymenopterans collected in 2016 and 2017 by genus……26
Table 1.2: Monthly breakdown of positive insects sampled from yellow sticky cards……………27
Table 1.3: PCR results for pollinator and yellow sticky card surveys in 2016 and 2017………28
Table 1.4: Summary of feeding behaviors for Dipterans observed on ooze in June 2018………29
Table 1.5: Summary of blossom visitation and behavior by insects during bloom 2018…………30
Table 1.6: Summary of positive blossoms visited by insects and collected as controls………31
Erwinia amylovora is a bacterial pathogen of rosaceous plants that causes fire blight, an economically devastating disease that impacts apple and pear producers across the world (Van der Zwet et al. 2012). *E. amylovora* can infect any wound or natural opening that is present on a plant and diseased tissues are characterized by their scorched appearance, the presence of ooze droplets on succulent green tissue, and shepherd’s crooking of dying young shoots (van der Zwet and Keil 1979). The disease cycle begins in the spring, when bacteria that overwintered at the margins of cankers in previously infected trees begins to ooze (Norelli et al. 2003). Ooze is a mix of *E. amylovora* encased in a bacterially produced exopolysaccharide (EPS) matrix that serves as inoculum for new infections (Oh and Beer 2005, Slack et al. 2017). Ooze can be windblown and splashed by rain into natural openings, or distributed by insects (van der Zwet et al. 2012b). In the spring, bacteria that has been deposited on the stigma of a blossom can be washed into the hypanthium, where the bacteria multiplies and initiates blossom blight (Thomson 1986). From there, the bacteria can spread systematically and infect all plant tissues (Norelli et al. 2003), migrating via the parenchyma and building to such high levels that the plant epidermis ruptures, allowing ooze to escape and for further reintroduction of the bacterium within and between trees (Slack et al. 2017). *E. amylovora* continues to migrate within the plant throughout the season, forming cankers in late summer through fall, which serve as an overwintering site for *E. amylovora* (Norelli et al. 2003). *E. amylovora* has been hypothesized to be introduced from external sources as well, including from infected, rosaceous plants in forests surrounding orchards (Emmett and Baker 1971).

Insects have been associated with *E. amylovora* since before the scientific community began research on the disease in the middle of the 19th century (Arthur 1886). The first reports of
fire blight were published in 1793 and 1817 (Denning 1793, Coxe 1817), and observers thought an ambrosia beetle (*Xyleborus dispar* Fabricious) was the cause of the malady (Arthur 1886). It was not until the late 1870s and early 1880s that a microbiological agent was scientifically identified (Arthur 1886, van der Zwet et al. 2012a), and researchers disagreed over whether the agent was bacterial or fungal (Arthur 1886). Koch’s postulates were successfully completed on the microbe in 1885, definitively supporting the bacterial theory (Arthur 1885). In 1884, insects were implicated as vectors in the fire blight disease cycle when tarnished plant bug (*Lygus lineolaris* Linnaeus) was recorded in association with bacterial cankers (van der Zwet et al. 2012a), suggesting that insects were not causal agents of the disease, but maintained a distinctive role its movement. From here the role of insects in the spread of *E. amylovora* was studied and debated frequently.

In 1901, Waite hypothesized that pollinators transmitted *E. amylovora* during bloom, showing that healthy blossoms on a diseased tree that were protected from pollinators would not become infected, while those accessible to pollinators did (van der Zwet et al. 2012a). Waite also recovered *E. amylovora* from the mouthparts and legs of *Apis mellifera* Linnaeus, and a fire blight outbreak in 1914 was attributed to high populations of honeybees in the orchard (Reed 1914, Gossard and Walton 1922). This early research created a consensus that pollinators were the primary insect vectors of fire blight (van der Zwet et al. 2012b). Rosen (1930) noted that Waite’s early work created an assumption that bees visited oozing cankers in the spring and acquired *E. amylovora* there, but there was no evidence to support this hypothesis (Hildebrand 1936, Parker 1936). This lack of evidence led to suggestions that *E. amylovora* could overwinter in honeybee hives, and that new infections could be initiated by bees from this inoculum source in the spring (Rosen 1930). This theory explained why disease outbreaks occurred when oozing
holdover cankers were rare and despite maintenance of the most up to date management protocols (Rosen 1930). In support of this hypothesis, Gossard and Walton (1922) showed that cultured bacteria mixed with pure honey and inserted into honeycomb survived between 72 and 100 hours, and inoculation tests on shoots with this mixture yielded high rates of positive infections. Additionally, artificial inoculations with pollen collected from honeybee corbiculae did not yield definitively positive tests while artificial inoculations using honeybee mouthparts did (Gossard and Walton 1922). Other experiments recovered bacteria from honeycomb up to 55 days from initial deposition, which led to suggestions that honeycomb provided protection for the bacteria (Thomas 1930).

The hive as initial *E. amylovora* inoculum source was hotly contested, especially by those involved in the honeybee industry (van der Zwet et al. 2012b). Some argued that the conditions necessary for honeybee dissemination were simply uncommon, and thus the economic benefit of a well pollinated orchard outweighed the economic detriment of fire blight related losses (Root 1923). Others reasoned that orchardists who used the same hives year after year often experienced only sporadic fire blight outbreaks, rendering the hive as an overwintering site infeasible (Pierstorff and Lamb 1933). Gossard and Walton (1922) ceded that varying environmental factors made definitive judgements on the role of bees in transmission difficult, but posited that *E. amylovora* was not present in the hive at the beginning of the season (Gossard and Walton 1922). Pierstorff and Lamb (1934) showed that the bacterium could not be recovered from hive material twenty-four hours after artificial inoculation. They added that *E. amylovora* could only be recovered from the heads of bees up to two days after artificial inoculation. The authors argued that these data were more relevant because they were conducted under field conditions, while previous bee and hive recovery experiments were done in the laboratory.
Pierstorff and Lamb (1934) supported a blossom-to-blossom theory of pollinator driven *E. amylovora* transmission with data showing that non-inoculated bees visiting inoculated blossoms transmitted the bacteria to healthy blossoms, but bees traveling from an inoculated hive did not transmit bacteria to healthy blossoms (Pierstorff and Lamb 1934, Thomas and Ark 1934, Parker 1936). This new theory was supported by similar data elsewhere (Ark and Thomas 1936, Hildebrand 1936, Keitt 1941), and it was suggested that bees are overall inefficient transmitters of *E. amylovora* despite their capability to do so (Parker 1936, Keitt 1941). Later research confirmed that bees are not responsible for initial blossom blight infections in the spring, and that bees are a potential threat after *E. amylovora* was delivered to blossoms by an external mechanism such as rain (van der Zwet et al. 2012b).

While the pollinator as blossom blight vector debate raged, efforts to define the role of hemipterans in the spread of shoot blight took a less combative tone (van der Zwet et al. 2012b). After earlier anecdotal observations (van der Zwet et al. 2012a), *Lygus lineolaris* was shown to transmit *E. amylovora* and was considered especially harmful to nursery stock (Stewart 1913). Stewart (1913) hypothesized 1) that the feeding wounds of *L. lineolaris* doubled as entry courts for *E. amylovora*; 2) that the insect acquired the bacteria by walking across oozing cankers; and 3) that bacteria were mechanically transmitted to feeding wounds on succulent shoot tissue (Stewart 1913). Similar experiments implicated additional hemipterans, such as potato leafhopper (*Empoasca fabae* Harris) and various aphid species (Burrill 1915, Stewart and Leonard 1915, 1916, Emmett and Baker 1971, Pfeiffer et al. 1999), and researchers suggested
that most hemipterans would be capable of transmitting *E. amylovora* through the hypothesized mechanism because of their shared behavior and feeding strategy (Stewart and Leonard 1916, Parker 1936). Studies showed that using insecticides to reduce hemipteran populations in the orchard reduced disease incidence, offering indirect evidence in support of this theory (Merrill 1915, Gossard and Walton 1922, Parker 1936, Pfeiffer et al. 1999). A corollary to this work posited that varying environmental conditions across time and space made the relative importance of any particular species difficult to determine at any one time (Burrill 1915, Stewart and Leonard 1916, Pfeiffer et al. 1999).

Some argued that hemipterans were not as important to *E. amylovora* transmission as mainstream thought suggested (van der Zwet et al. 2012b). Miller (1929) found low hemipteran populations but high rates of *E. amylovora* infection in the spring and cited infections that occurred in insect exclusion experiments in the summer as evidence that the role of hemipterans in secondary spread of shoot blight was overstated. Multiple studies found no change in fire blight incidence when controlling for hemipterans with insecticides (Miller 1929, Pfeiffer et al. 1999, van der Zwet et al. 2012b). Later research demonstrated that aphids could acquire the bacteria from an infected source but were unable to transfer high enough quantities to initiate an infection (Plurad et al. 1965, 1967, Clarke et al. 1992). Plurad et a (1967) noted that it took roughly over one hundred individual bacterial cells to initiate a new infection, but most aphids egested less than fifty. This study did not investigate if external contamination of the aphid was an important factor in transmission and a later study demonstrated this possibility (Hildebrand et al. 2000). Stahl (1977) hypothesized that *Lygus* spp. were not involved in transmission of shoot blight and played only a minor role in the transmission of fruit blight. This study argued that mechanical damage created the majority of entry courts for *E. amylovora* (Stahl and Luepschen
1977). Overall, no final consensus on the hemipteran question was ever reached (Parker 1936, Pfeiffer et al. 1999), and the discussion faded into the background of fire blight research.

A role for dipterans in the spread of fire blight was theorized in early studies, and this role received more attention recently. Stewart and Leonard (1915) implicated flies with anecdotal evidence, stating that these insects were often observed feeding on bacterial ooze and that this behavior likely led to mechanical transmission. In 1916, the same authors showed that various fly species were unable to transmit the bacteria and reasoned that flies do not cause the necessary damage to initiate an infection (Stewart and Leonard 1916). They and others hypothesized that flies could transmit the bacteria to wounds created by weather events or other insects (Stewart and Leonard 1916, Stahl and Luepschen 1977), but this mechanism was rendered unlikely due to environmental and behavioral factors (Stewart and Leonard 1916, Hildebrand et al. 2000). This transmission mechanism was revisited in recent research (Slack et al. 2017, Boucher et al. 2019), with one study arguing that it is more reasonable pathway than previously believed (Boucher et al. 2019).

Thomas and Ark (1934) revisited the role of flies in behavioral experiments, showing that flies strongly preferred oozing bark chips to molasses and hypothesizing that the fermenting odors of the oozing cankers were attractive to the flies (Thomas and Ark 1934). Parker (1936) catalogued flies feeding on ooze regularly and conducted experiments specifically demonstrating that dipterans could inoculate blossoms from oozing cankers under controlled conditions (Parker 1936). Later research showed that the Mediterranean fruit fly (Ceratitis capitata Wiedemann) could transmit *E. amylovora* to fruits and shoots (Ordax et al. 2015). Additional research added that several fly species could transmit *E. amylovora* to their offspring and that the bacteria survived as long as eight days in the guts and feces of some flies (Ark and Thomas 1936, Ordax
et al. 2015). Emmett and Baker (1971) proposed that large, contaminated fly populations could be disseminated by storms and strong winds, leading to movement of flies from orchards to wild, susceptible hosts and vice versa. Exchanges of contaminated flies between managed and unmanaged environments could factor into fire blight outbreaks (Emmett and Baker 1971).

While research has winnowed the possible culprits and mechanisms over the years, the ecological underpinnings of insect mediated *E. amylovora* transmission are relatively understudied. This dissertation explores various aspects of plant-insect-microbe interactions pertinent to the ecology of insect mediated transmission of *E. amylovora*. In chapter 1, we surveyed insects in an experimental orchard that was heavily infested with *E. amylovora*. We sampled pollinators in the spring and hemipterans and dipterans from petal fall (mid to late May) through August and tested them for *E. amylovora*. We also observed insect visitation to blossoms and ooze droplets to better understand how insects interact with infected or potentially infected tissue. We found that pollinators did not test positive for *E. amylovora*, and that their role in the disease cycle is likely limited in New York. We demonstrate an understated role for dipterans, as flies are the most regular visitors to blossoms in the spring and the most common order of insect observed feeding on ooze. We also show that flies that fed on ooze could retain and shed viable *E. amylovora* for at least 5 days.

In chapter 2, we evaluated how biotic and abiotic factors affect acquisition of *E. amylovora* by *D. melanogaster*. Sex, nutritional state, and mating status do not affect the likelihood of acquisition by *D. melanogaster* and higher exposure time to bacterial ooze increased the likelihood of acquisition but did not affect the bacterial load acquired. Flies that had been food deprived had higher *E. amylovora* counts than satiated flies, suggesting that bacterial ooze can be an important food source for foraging flies. We also demonstrate that *D. melanogaster*
can transmit viable *E. amylovora* to a surface, supporting the hypothesized mechanical transmission mechanism.

In chapter 3, we studied *E. amylovora* transmission dynamic using *Delia platura* as a model. *D. platura* was identified in chapter 1 as a highly abundant insect in the orchard that visited ooze frequently, feeding on ooze droplets for extended time. In this chapter, we show that *D. platura* that carry *E. amylovora* can initiate infections in mechanically damaged apple saplings and that acquisition from ooze leads to higher rates of infections compared to acquisition from epiphytic sources. We also show that the bacteria can survive internally and externally on the fly for at least 5 days and that *E. amylovora* has no negative effect on *D. platura* survival. We demonstrated no differences in the ability of *D. platura* to transmit *E. amylovora* in multiple strains. This work underscores the importance of flies in the transmission process, especially when there are high densities of flies in an orchard. This chapter also exemplifies that insects that are not considered pests of apple because they do not cause direct damage to the plant can be an indirect pest by facilitating the spread of *E. amylovora*.

In chapter 4, we studied the effect of *E. amylovora* infection on the behavior of *D. platura* to understand whether disease symptoms could enhance transmission by flies. We found that *D. platura* preferred infected fruit when given a choice between diseased fruit and healthy fruit and that choice was not odor mediated. Flies did not exhibit preference for diseased shoots, indicating that disease associated changes to shoots do not drastically impact the likelihood of transmission by *D. platura*. We underscore that *E. amylovora* has low vector specificity, meaning that many insects can conceivably transmit the pathogen, and the challenges of studying insect behavior in relation to low vector specificity.

Collectively, this dissertation outlines an outsize role for orchard dwelling dipterans in
the transmission of *E. amylovora* relative to the attention received in the literature. Fly communities are likely to vary based on geography and management, and transmission by flies is likely to be impacted by the life histories of the flies present in any given orchard. More attention on the ecology of Diptera mediated transmission is necessary, and our work provides a roadmap for future research on this transmission mechanism.
REFERENCES


Burrill, A. C. 1915. Insect control important in checking fire blight. Phytopathology. 5: 343–347.


Coxe, W. 1817. A view of the cultivation of fruit trees, and the management of orchards and cider. Published by M. Carey and son. Nov. 1, Philadelphia.


CHAPTER 1

FIELD EVALUATION OF INTERACTIONS BETWEEN INSECTS AND ERWINIA
AMYLOVORA IN A NEW YORK APPLE ORCHARD

Matthew Boucher,¹* Rowan Collins,¹ Kayli Harling,¹ Gabrielle Brind’Amour,¹ Stephen Hesler,¹
Karen Wentworth,¹ Kerik Cox,² and Greg Loeb¹

¹Department of Entomology, Cornell AgriTech, New York State Agricultural Experiment
Station, Cornell University, Geneva, New York, 14456 USA

²Section of Plant Pathology & Plant Microbe Biology, School of Integrated Plant Sciences,
Cornell AgriTech, New York State Agricultural Experiment Station, Geneva, New York, 14456
USA

*Corresponding author: M. T. Boucher; Email: mtb245@cornell.edu
Department of Entomology, Cornell AgriTech, College of Agriculture and Life Sciences,
Cornell University, 15 Castle Creek Drive, Geneva, NY 14456

A version of this manuscript has been submitted to Plant Disease
Abstract
The role of insects in dissemination of *Erwinia amylovora* has been studied for over 100 years. Pollinating bees do not feed on bacterial ooze but are suggested to transmit between flowers. It has been suggested that various hemipteran species walk on bacterial ooze and subsequently shed acquired bacteria into their own feeding wounds. Dipterans have been observed readily feeding on ooze, but their importance has been understudied. The goal of this study was to advance understanding of the ecology of insect mediated transmission of *E. amylovora* through field collections and observations conducted in a research apple orchard with actively oozing fire blight symptoms. We found that field-collected pollinating bees did not test positive for the bacterium, suggesting that their role in blossom blight dissemination may be overstated. Flies were prominent flower visitors, underscoring the need for further research into their role in bloom time bacterial dissemination. Flies were observed feeding on ooze droplets in the late spring and early summer and the insects retained bacteria for at least seven days. Flies shed transmissible amounts of *E. amylovora* for the duration of the experiment. The role of hemipterans was not clarified in this study, but it is possible that their role is connected to fly mediated disease transmission. Collectively, this research outlines the ecological role of different insects in disease transmission and underscores the underappreciated potential importance of flies in transmission of *E. amylovora*, providing a roadmap towards a better understanding of the complex dynamics at play.

Introduction
Fire blight is a bacterial disease of pomaceous fruit, primarily apple and pear, caused by the bacterium *Erwinia amylovora* (Van der Zwet et al. 2012). The bacterium can infect any plant tissue as long as there is an available entry court and symptoms include wilting of succulent
shoots, necrosis of blossoms and fruit, development of cankers on woody tissue, and the presence of bacterial ooze (Oh and Beer 2005). Bacterial ooze is a mixture of \textit{E. amylovora} encased in exopolysaccharides that builds to high enough levels underneath the plant surface that the pressure ruptures the epidermis and the ooze exudes from the injury (Slack et al. 2017). Ooze serves as a primary inoculum in the spring when it emerges from the margins of overwintering cankers (van der Zwet et al. 2012), and as a secondary inoculum source that infects succulent shoots throughout the growing season until succulent tissue lignifies (Slack et al. 2017). One of the primary mechanisms through which \textit{E. amylovora} is disseminated from ooze is insect activity (van der Zwet et al. 2012).

High populations of \textit{E. amylovora} in ooze are hypothesized to account for the inefficiencies associated with mechanical transmission by Dipterans (Slack et al. 2017). After failing to demonstrate transmission from various fly species, early researchers suggested that flies were incapable of generating wounds that act as entry courts for \textit{E. amylovora}, but that it was possible for contaminated flies to deliver bacteria to wounds created by piercing/sucking insects such as \textit{Lygus lineolaris} (Stewart and Leonard 1916). However, the authors argued that this mechanism was unlikely because \textit{E. amylovora} would perish due to desiccation before having the chance to colonize the wound, and posited that the most important role for flies in the disease cycle was in mechanically transporting \textit{E. amylovora} from ooze to blossoms (Stewart and Leonard 1916). A later study reported that flies exposed to cankers and then bagged to blossoms resulted in roughly 2-4% symptomatic blossoms compared to no symptomatic blossoms in unexposed controls (Parker 1936). Another survey showed that flies were the most common insect sampled in a diseased orchard, and 56% of flies were positive for \textit{E. amylovora} with loads ranging from $1 \times 10^1$ to $1 \times 10^5$ cells/insect (Miller 1972). Other studies reported similar
CFU ranges for flies under laboratory settings (Ordax et al. 2015, Boucher et al. 2019). The Mediterranean fruit fly, *Ceratitis capitata*, was shown to carry viable *E. amylovora* for as long as 28 days externally and 8 days internally, which is the first time survival of *E. amylovora* in the gut of an insect was reported (Ordax et al. 2015).

Pollinating insects such as honeybees have also been implicated in the spread of fire blight (van der Zwet et al. 2012). Honeybees do not visit oozing cankers (Parker 1936), but have primarily been shown to transmit *E. amylovora* from blossom to blossom (Pierstorff and Lamb 1934, Parker 1936). Honeybees caged with a tree containing 19 artificially inoculated blossoms resulted in roughly 67% of originally healthy blossoms becoming symptomatic, while honeybees caged to control trees without inoculated blossoms resulted in no symptomatic blossoms (Pierstorff and Lamb 1934). Another experiment demonstrated that bees transmitted *E. amylovora* to 52% of healthy blossoms when confined with one diseased dwarf tree and two healthy dwarf trees (Keitt 1941). The same study argued that nectar concentration limited honeybee transmission efficiency because healthy flowers that had medium and high nectar concentrations did not develop fire blight symptoms when visited by contaminated bees (Keitt 1941).

Hemipterans have received attention as potential vectors but their role in transmission varies (van der Zwet et al. 2012). Stewart (1913) caged tarnished plant bugs, *L. lineolaris*, to healthy pear shoots smeared with cultured *E. amylovora*, resulting in roughly 70% of shoots exhibiting fire blight symptoms. This led the author to hypothesize 1) that feeding wounds doubled as entry courts for *E. amylovora*; 2) that insects acquired bacteria by walking across oozing cankers; and 3) that bacteria were mechanically transmitted to feeding wounds on succulent shoot tissue (Stewart 1913). Later work showed that contaminated *L. lineolaris* could
initiate infections in an average of 24% of pear fruit they were exposed to (Stahl and Luepschen 1977). Studies on potato leafhopper (PLH), *Empoasca fabae*, suggested that insecticides to control for the pest significantly reduced fire blight incidence (Pfeiffer et al. 1999, Leahy et al. 2006), leading to the hypothesis that leafhoppers facilitate translocation of epiphytic *E. amylovora* into the plant (Leahy et al. 2006), but no follow up research was published. Molecular studies on transmission of *E. amylovora* by *Aphis pomi* showed that 0% of 200 aphids exposed to *E. amylovora* could initiate an infection in apple shoots, likely because they could not egest enough bacteria to overcome plant defenses (Plurad et al. 1967). Overall, the most commonly hypothesized role for hemipterans in the disease cycle is that their feeding injuries can act as entry courts for *E. amylovora* (Stewart 1913, Plurad et al. 1967, Leahy et al. 2006).

Despite foundational knowledge implicating insects in the spread of *E. amylovora*, the ecological underpinnings of insect mediated *E. amylovora* transmission remain relatively understudied. Only one field survey has been conducted (Hildebrand et al. 2000), and the interactions between implicated insects and diseased plants have not been described well enough to determine when and if transmission by insects will occur. The goal of this study was to examine interactions between commonly implicated mechanical vectors of *E. amylovora* and the bacterium to better understand the relative importance of each potential vector. To this end, the objectives of this study are to 1) evaluate how often three common insect groups (pollinators, dipterans, and hemipterans) test positive for *E. amylovora* across the growing season in a field relevant setting; to 2) observe and quantify interactions between dipterans and *E. amylovora* ooze in the field; and to 3) quantify the frequency of interaction between blossoms containing *E. amylovora* and insects visiting flowers.
Methods

Field site. All surveys and experiments were conducted in an experimental research orchard at Cornell AgriTech in Geneva, NY (42°52’23.2”N, 77°01’40.1”W). The orchard was planted in 2012 in an open field surrounded by research plots that varied in crops produced from year to year and included grains, beets, beans, and cabbage. No other fire blight susceptible crop was grown nearby. The orchard consisted of four blocks of fifty trees each (*Malus domestica* cv. Idared), with each tree spaced roughly three meters apart. Each tree was assigned a unique identification number based on its block, row, and column. The orchard was experimentally inoculated with *Erwinia amylovora* strain ea273 (ATCC 49946) several years before surveys were conducted (Sebaihia et al. 2010).

In 2015, prior to this study, the incidence of blossom and shoot blight in the survey orchard was extremely high and active fire blight was present throughout the study. On June 3, 2015 the incidence of blighted blossom clusters ranged from 2.5-89%, and the incidence of blighted shoots ranged from 23-88% across all trees. By September 15<sup>th</sup> the incidence of shoot blight was > 60% in the entire plot with an apparent 72% tree mortality. By 2016 the incidence of blighted blossom clusters ranged from 0.0-31%, and the incidence of blighted shoots ranged from 3-22% on living trees. Only a few additional trees seemed to recover from the infection in 2016. In 2017, the incidence of blighted blossom clusters ranged from 0.0-20%, and the incidence of blighted shoots ranged from 0-57% on living trees. By end of the 2017 season, more than 80% of the trees were killed by fire blight. In 2018, the incidence of blighted blossom clusters ranged from 0.0-31.3%, and the incidence of blighted shoots ranged from 0.0-47% on living trees. The remaining trees continued to decline by Sept 2018. In both 2017 and 2018, living trees produced a crop, but underdeveloped fruit and blighted shoots were commonly observed to ooze with cells of *Erwinia amylovora*. 
2016 – 2017 pollinator survey. Starting in late April we monitored flower development daily until king flowers began to bloom, which occurred in the first two weeks of May. From then to the end of petal fall, 2-3 observers each walked individual orchard blocks for thirty minutes to an hour and captured hymenopteran pollinators that landed on blossoms in 50mL conical tubes. In 2016 and 2017, we captured hymenopterans visiting flowers and in 2018 we included dipterans in our sampling. Pollinators were put on ice and returned to the lab to be tested for *E. amylavora*.

In 2016-2017, bees were stored at -20°C until processing, at which point they were removed and quickly identified to genus or species under a dissecting microscope. Insects were then transferred into a tube containing 500-1000µl of autoclaved distilled water depending on insect size and either hand homogenized with a plastic pestle (2016) or mechanically homogenized with a mixer mill (model MM400; Retsch®, Haan, Germany) for two minutes at 25 Hz (2017). In 2016, samples were pooled by combining 10µl of ten individual samples from the same species into one Eppendorf tube, and pooled samples were subject to DNA extraction with DнAzol® (catalog no. 10503027; ThermoFisher, Waltham, MA) using the manufacturer’s protocol (Broadley et al. 2017). In 2016, if a pool tested positive, then the ten individuals comprising the pool were tested individually to acquire a definitive count of positive samples.

In 2017, DNA extractions were conducted on individual samples using a lysis buffer (50mM KCl, 10mM Tris-HCl (pH 8.3), 2.5mM MgCl₂, 0.45% Triton x 100, 0.45% Tween 20, 0.01% gelatin, and 60µg/mL Proteinase K) (Li et al. 2014). DNA extraction was done following Li et al. (2014), in which a 1:1 ratio of lysis buffer and homogenized sample was added to a 0.2mL PCR tube, centrifuged for 30 seconds at 10,000 RPM, then stored at -80°C for one hour. After incubation, samples were incubated in a thermocycler at 60°C for one hour, then 95°C to
deactivate the Proteinase K and stored at -20°C until use in PCR assays (Li et al. 2014). 2016 and 2017 DNA extraction protocols were compared side by side to ensure results were similar before switching to the new method.

In both 2016 and 2017, samples were tested for the presence of the pEA29 plasmid using Polymerase Chain Reaction (PCR) (Tancos et al. 2016). PCR assays were conducted using EmeraldAmp Max PCR Master Mix (catalog no. RR320; Takara Bio USA, Inc., Mountain View, CA) and primers AJ75/76 (McManus and Jones 1995). Individual reactions contained 12.5µl of master mix, 9µl of dH2O, 0.5µl each of forward and reverse primers, and 2.5µl DNA template. Thermal cycler (catalog no. 170-9703; Bio-Rad Laboratories, Inc., Hercules, CA) conditions matched those used in Tancos et al. (2016) and PCR products were run on a 1% agarose gel in 1X Tris-acetate-EDTA buffer and stained with SYBR™ Safe DNA Gel Stain (Catalog no. S33102; ThermoFisher, Waltham, MA) at 90 V for 45 minutes. Gels were imaged with a KODAK Gel Logic 200 System (KODAK, Rochester, NY). All samples that tested positive using AJ75/76 primers were re-tested using primers for the rpsL gene as a check on original results (Russo et al. 2008).

**Sticky card survey.** Yellow sticky card surveys were conducted for 20 weeks in 2016 and 18 weeks in 2017 starting in the first week of May in each year and lasting until mid to late August. Each week, we used the RANDOM function in Microsoft Excel to select five trees from each orchard block and deployed two 7.62x12.7 cm yellow sticky cards (Olson Products, Inc., Medina, OH) in each tree for a total of forty cards per week. One card was deployed at knee level around the trunk and the other at shoulder or eye level in the canopy. Cards were removed weekly, placed into clear plastic sandwich bags, and returned to the lab where insects were
identified for sampling.

Insects were identified to genus or species on cards when possible, based on morphology. In 2016, we sampled hemipterans in high abundance (*Empoasca fabae* Harris, *Macrosteles quadrilineatus* Forbes, *Paraphlepsius* spp., and *Philaeus spumarius* Linnaeus) and in 2017 we added dipterans in high abundance (*Delia* spp., and miscellaneous) based on their prevalence in 2016, observations of various dipterans feeding on ooze while collecting samples in 2016, and feedback from growers. Hemipterans that did not test positive in 2016 were not tested in 2017 and abundances of *P. spumarius* were too low to sample in 2017. Insects were removed from sticky cards by first cutting away the plastic surrounding the insect, then adding a small drop of Goo Gone® liquid degreaser (Weiman Products, Gurnee, IL) to the sample (Cieniewicz et al. 2018). After 10-30 seconds, insects were removed from the card without damaging them and stored at -20°C until PCR. Samples were tested as described above.

**Ooze location and color survey.** On three occasions in June 2018, the location and color of *E. amylovora* ooze droplets detected in the research orchard were recorded. We counted 100 droplets on each occasion, and 20-25 droplets were haphazardly sampled per tree. Separate sets of trees were used in each of the three samplings to ensure no droplet was double counted. Ooze droplet color was recorded as white, red, orange, or yellow (Slack et al. 2017). Location was recorded as shoot, leaf, fruit, or woody tissue.

**Fly feeding and persistence assay.** On days between 60°F (15.56°C) and 85°C (29.44°C) from June 1 to June 21, 2018, observers located oozing trees and waited for dipterans to land in a block heavily infested with *E. amylovora*. This temperature range was chosen because it is the
range at which common flies in the orchard are most active (Miller and McClanahan 1960). Upon landing, insects would approach or encounter ooze droplets, and were timed for the duration of feeding on the droplet. Feeding initiated when the proboscis extended to the surface of the droplet and ended when the proboscis retracted. When feeding terminated, we attempted to capture the fly in a 15mL conical tube. Location and color of droplets that were fed on were recorded. Color and location of droplets visited by flies were compared to observed droplet color and location distributions in R using a chi-square goodness of fit test (R Core Team 2019).

Captured flies were returned to the lab, identified as either *Delia* spp. or miscellaneous, and used to evaluate persistence of *E. amylovora* on/in the insect. Flies were transferred to a new 15mL conical tube with a 1cm x 1cm square of paper towel soaked in 5% sucrose, and every day for seven days flies were transferred to a new, identical tube. The paper towel was removed from the old tube, and 1mL of autoclaved, distilled water was added to the tube. Tubes were vigorously shaken by hand to wash the tube and the mixture was serially and plated on Crosse-Goodman media, a selective media on which *E. amylovora* colonies exhibit a characteristic cratering pattern (Crosse and Goodman 1973). Plates were sealed with Parafilm® and incubated at 28°C for roughly 60 hours. After incubation, positive samples were identified and *E. amylovora* cells were counted under 30X magnification. The detection threshold of this assay was 200 CFU/mL. Positive tests were spot checked by growing bacteria overnight in LB broth (catalog no. 12795-027; Invitrogen, Carlsbad, CA) at 28°C, and then 500µl of resulting suspension was inoculated into immature apple fruits (cv. Gala). Immature fruit were incubated at 28°C and >95% RH for 7-14 days until ooze began to exude from the fruit surface. Dilutions were plated on Crosse-Goodman media and incubated and evaluated as described above (Crosse and Goodman 1973).
**Blossom visitation survey.** On 6 days spanning the start of bloom (May 17, 2018) to the end of bloom (May 25, 2018), observation teams sampled flowers visited by insects for *E. amylovora* in the research orchard. One observation constituted the first three flowers visited by a single insect (recorded as honeybee, small bee, medium bee, large bee, or fly). Each time an insect landed on a flower, it was timed for the duration of its visit; if it approached the blossom from the top (landing on anthers) or side (landing on petals); and if it fed on nectar (whether or not its proboscis extended into the floral cup). Each visited flower was then collected into a 2mL tube and stored on ice. Teams collected five observations per tree and sampled one to two trees per sampling day. For each observed tree, ten control blossoms were arbitrarily collected prior to insect observations to compare the overall prevalence of infected flowers to the prevalence of infected flowers visited by insects.

Samples were returned to the lab and processed the same day they were collected. Flowers were tested individually by removing extraneous tissue and chopping up the flower with a small pair of scissors. Scissors were sterilized for 30 seconds in 95% ethanol before using on a new flower. Shredded flowers were pulverized in a 1.5mL Eppendorf tube by hand using a plastic pestle, and soaked in 1mL autoclaved, distilled water for one hour. Samples were serially diluted, plated on Crosse-Goodman media (Crosse and Goodman 1973), and incubated and evaluated as described above. The detection threshold of this assay was 200 CFU/mL. Differences in CFU counts between insect visited flowers and control flowers were analyzed in R using a generalized linear mixed-effects model with the glmmTMB library and compared using the emmeans library (Brooks et al. 2017, R Core Team 2019, Lenth 2020). A negative binomial distribution was used due to overdispersion of the counts, and flower source (insect visited or control) was a fixed effect while block and tree were included as random effects.
In a separate survey, hymenopterans and dipterans that visited open flowers were captured using methods described in 2016 – 2017 pollinator survey, returned to the lab and quickly cataloged as a honeybee, a large bee, a medium bee, a small bee, or a fly. Insects were kept on ice and then added individually to a vial containing 1000 µl of autoclaved, distilled water and hand homogenized using a plastic pestle. Homogenate was serially diluted and dilutions were plated on Crosse-Goodman media and incubated and evaluated as described above (Crosse and Goodman 1973). The detection threshold of this assay was 200 CFU/mL.

**Results**

**2016 – 2017 pollinator survey.** No pollinating hymenopterans tested positive in 2016 or 2017 out of a total of 749 individual pollinators captured representing a diversity of blossom visitors (Table 1.1).

**Sticky card survey.** Of the 66 positive samples from both years, 45 (68%) were collected in June and July (Table 1.2). In 2017, *Delia* spp. accounted for 15 out of 24 positive samples (63%). *E. amylovora* was detected in two hemipteran species, one hemipteran genus, and one dipteran genus (Table 1.3). Meadow spittlebug, *Philaenus spumarius*, tested positive in 2016, but no samples of this insect were collected in 2017. *Delia* spp. constituted a mix of *Delia platura* and *Delia florilega* samples, which were the predominant dipteran captured in the field in both 2016 and 2017 (Figure 1.1). In 2016, the population of *Delia* spp. on yellow sticky cards was relatively stable across the growing season. Numbers captured were higher in 2017 compared to 2016, peaking in June and July before declining to numbers comparable to 2016. In 2017, *Delia* spp. tested positive for *E. amylovora* less than 1% of the time and no other dipteran tested positive (Table 1.3).
**Ooze location and color survey.** The distribution of ooze droplet colors observed in the field and the distribution of ooze droplet colors visited by flies was determined (Figure 1.2). Orange and yellow droplets were the most prevalent in the orchard and the most often visited by flies. There were no significant differences in the distribution between observed droplet color and color visited by flies ($\chi^2 = 2.5898, p = 0.4503$). The distribution of ooze droplet location on the tree and the location of ooze droplets visited by flies was also determined (Figure 1.3). Ooze droplets were found on green succulent shoots most often (75%) and flies visited droplets on shoots roughly 67% of the time. There was a significant difference between observed droplet location and location of ooze visited by flies ($\chi^2 = 55.129, p = 0.0005$), indicating that flies visited ooze more frequently on fruit and less frequently on wood then would be expected based on the observed distribution of ooze.

**Fly feeding and persistence assay.** A total of 30 observations of flies feeding on ooze were recorded (Table 1.4). *Delia* spp. accounted for roughly 58% of flies observed, with individuals from the Muscidae and Sarcophagidae families making up the remaining 42%. Feeding times on ooze ranged widely, from short bouts of 2 seconds to bouts exceeding 6 minutes. The mean feeding time was close to 3 minutes for *Delia* spp. and about 2 minutes for other dipterans. Of the 30 dipterans recorded, 16 were captured and tested for *E. amylovora* daily for 7 days. Ten of these flies were initially positive for *E. amylovora* (Table 1.4). Insects were positive for *E. amylovora* for 1-7 days, and the population of *E. amylovora* in initially positive flies declined from a mean of roughly $1 \times 10^7$ CFU/Insect on day 1 to roughly $1 \times 10^3$ CFU/Insect by day 7 (Figure 1.4). Of the 10 samples, only 1 remained positive for 7 days, 2 were positive for 6, 4, and 3 days, and 3 were positive for only 1 day.
**Blossom visitation survey.** Out of a total of 20 *Apis mellifera*, 60 wild bees, and 41 flies collected from 38 different trees, 0% tested positive for *E. amylovora* (Table 1.5). Out of these 121 insect samples, 30 were collected from trees known to be positive based on positive flowers collected. Of 21 trees from which flowers were sampled, 4 trees (19%) were positive for *E. amylovora* by petal fall, but only 1 tree had infected blossoms in the first 4 days of sampling. Dipterans were the most frequent flower visitors, constituting 54% (90/167) of all visitors observed and roughly 67% (8/12) of the visits to positive flowers (Table 1.5). Overall, 11 out of 12 positive flowers visited by insects were collected from one tree, and 27 out of 36 positive control flowers were collected from that same tree (Table 1.6). Insect visitation in the first 2 days of sampling accounted for 39% of total visits compared to 41% of control samples. Insect visitation in the middle 2 days of sampling accounted for 48% of total visits compared to 41% of control samples. Insect visitation in the last 2 days of sampling accounted for 12% of total visits compared to 18% of control samples. Bacterial populations of human sampled flowers were significantly higher than bacterial populations in insect visited flowers (t-ratio = -2.259, \( p = 0.03 \), Figure 1.5).

Honeybees and large bees spent the least amount of time interacting with each flower visited, while medium bees, small bees, and flies spent nearly 20 seconds longer on average interacting with flowers (Table 1.5). Bees landed on flower anthers (approach from top) greater than 50% of the time, while flies were more likely to land on the petals (approach from side) (Table 1.5). Additional visitation and behavioral data are summarized in Table 1.5.
Discussion

Our research shows that hymenopteran pollinators did not test positive for *E. amylovora* during bloom and that dipterans visited blossoms more frequently than bees, casting doubt on the role of pollinating bees in the spread of *E. amylovora* (Johnson et al. 1993). Flies captured on blossoms did not test positive for *E. amylovora* and blossom blight was rare in 2018. We also show that dipterans and hemipterans test positive at low rates across the growing season and close to 70% of positive insects were collected in June and July in 2016 and 2017. Hemipterans that tested positive in 2016 tested positive at lower rates in 2017 and dipterans dominated the population of positive insects in 2017. Further, we show that flies are readily observed feeding on ooze droplets in the field after bloom and that these insects will occasionally feed on ooze uninterrupted for greater than six minutes. Ooze droplets were located on shoots in 75% of observations, and 67% of the droplets visited by flies were on shoots. Furthermore, fly visits to ooze were more frequent when ooze was located on fruit and less frequent when located on wood relative to the observed distribution of ooze in the field. Flies retained infectious levels of bacteria for as long seven days after feeding on ooze. These data should be interpreted with caution, as we only conducted experiments at one location and in one cultivar, but the fact that general patterns held over multiple study years leads us to believe that the trends we observed will be consistent in New York.

The absence of pollinating hymenopterans positive for *E. amylovora* indicates that these insects may not play a significant role in transmission of blossom blight in New York. Pollinators are thought to transmit bacteria from blossom to blossom, and that mechanism is plausible based on earlier research (Pierstorff and Lamb 1934), but we believe that the factors that need to align for such transmission to occur are uncommon at this ecological scale. The sporadic nature of blossom blight likely plays a role here (Steiner 1990), as only one tree
contained positive blossoms for the first four days of sampling, and 75% of all positive control blossoms and 92% of all positive insect visited blossoms were collected from this tree in 2018. While interpretation is difficult with so few trees with infected blossoms at the time of sampling, we suggest that the spatial distribution of diseased blossoms will affect the likelihood of pollinator mediated transmission. The significantly higher bacterial count in control samples is likely due to more positive control blossoms being collected later in bloom (19/35) than positive insect visited blossoms (1/12) as bacterial populations on flowers are generally higher later in bloom. (Thomson 1986), though a larger sample of positive, insect visited blossoms is necessary to confirm this hypothesis. It is also possible that pollinators can detect and avoid highly diseased blossoms (Cellini et al. 2019), but additional research is necessary to understand how *E. amylovora* infected flowers affect pollinator behavior.

Moreover, pollinating hymenopterans may not be behaviorally equipped to efficiently facilitate new *E. amylovora* infections during bloom (Thomson 1986). We show that the bacterial population in blossoms increases over time, but insect visitation decreases as bacterial populations hit their peak. This misalignment of insect visitation and bacterial population reduces the potential for interaction between a bee and a positive blossom, which reduces the potential for an insect to acquire and transmit the bacteria in orchards with a uniform blooming period. It is also possible that bees do not interact with individual blossoms long enough to acquire and transmit *E. amylovora* (Thomson 1986), as the average blossom visitor interacted with flowers for only 22 seconds. However, few bees encountered positive blossoms, so we cannot confidently define the role of this behavior at this time.

Another key observation of our study is that flies are the primary insect visitors to blossoms. The observed prevalence of flies on blossoms is in accordance with recent attention paid to flies
as underappreciated pollinators (Raguso 2020). In a meta-analysis of farmland pollination, dipterans accounted for two thirds of total pollinator abundance (Orford et al. 2015), underscoring significant interactions between flies and blossoms in agroecosystems. Previous researchers observed several families of diptera feeding on overwintering cankers and suggested that these flies are the primary disseminators of *E. amylovora* from cankers to blossoms (Parker 1936). Considering the recent attention paid to flies as pollinators, we argue that new efforts should be made to understand the canker-to-blossom transmission mechanism and the broader role of flies in the dissemination of blossom blight (Slack et al. 2017). In our study, flies fed on nectar during 75% of visits and spent an average of 30 seconds interacting with a single blossom, but none tested positive for *E. amylovora*. We believe that this result stems from the rarity and patchiness of blossom blight in the orchard. The role of flies in the spread of blossom blight requires further study, and will likely depend on the fly species, life history, and population size of the insect, and how these factors interact with the distribution and availability of active overwintering cankers across ecological scales (Orford et al. 2015, Raguso 2020).

Our data indicate that flies continue to be problematic in the months following bloom. The overall counts of positive samples were low in 2016 and 2017, and we believe we are underreporting the actual prevalence of positive insects in the field based on our 2018 observations. Our 2018 survey of bacterial persistence in live flies showed a mean retention of 4 days, indicating that 7-day intervals between sticky card collections may have been too long. It is also possible that sample desiccation on the yellow sticky cards led to degradation of *E. amylovora* in those samples, but this has not been a problem in related pathosystems (Bextine et al. 2005). Despite the low counts, some patterns were apparent. The rate of positive samples in June and July relative to the rest of the growing season suggests this two-month window is when
the potential for insect transmission in New York is at its highest. This window coincides with
the highest prevalence of ooze in diseased orchards (Slack et al. 2017), making inoculum readily
available to insects during this time. The population of Delia spp., the most prevalent fly in our
orchard, peaked during these months, indicating that these flies will be most prevalent at the
same time ooze is. Additionally, visitation to ooze by flies during this time was observed
frequently. The high level of bacteria acquired and shed over time suggests that flies can transmit
infectious levels of bacteria to plant surfaces over several days. This result demonstrates that
flies are able to deliver bacteria to plant surfaces, after which E. amylovora could
opportunistically colonize any wound or natural opening (Stewart and Leonard 1916, Stahl and
exceeding what is required for infection supports the hypothesis that acquisition of high bacterial
titers by flies is necessary to account for: 1) time passage between acquisition and transmission;
and 2) for insect interactions with non-host plants (Slack et al. 2017).

Further observations of flies feeding on ooze define some behaviors associated with
acquisition. We disprove a previous suggestion that ooze droplet colors differentially attract flies
(Slack et al. 2017), finding no differences between the observed distribution of droplet colors in
the field and the distribution of ooze droplet colors visited by flies. We did identify a difference
in observed droplet location in the field compared to droplet location visited by flies. While close
to 70% of flies visited ooze where it is most often located on shoots, flies also visited oozing
fruit more frequently than the observed distribution of ooze on fruit. This may be mediated by
unknown qualities associated with ooze or possibly by the odor profile of diseased fruit. Previous
research hypothesized that fermenting odors brought on by E. amylovora infection may be
attractive to flies (Thomas and Ark 1934). Understanding how insects locate diseased tissue is a
key aspect of the transmission mechanism that remains understudied. The fact that flies were most often observed on shoots suggests that this is where acquisition and transmission driven by flies is most likely to occur.

The role of hemipterans in the spread of *E. amylovora* remains a question. While field samples were occasionally positive in both survey years, we never observed hemipterans feeding on ooze and we do not know if positive individuals can successfully transmit bacteria. The count of positive hemipterans is likely underestimated for reasons described above and additional research is necessary to determine the transmission capacity of common hemipterans in apple orchards. Early researchers proposed that the feeding wounds created by hemipterans act as entry courts for bacteria that is delivered to plant surfaces by flies (Stewart and Leonard 1916, Stahl and Luepschen 1977). We propose that hemipteran feeding could release surface tension created by subdermal bacterial growth and EPS production (Slack et al. 2017), allowing ooze to leak out of the resulting feeding wound. A similar phenomenon occurs in healthy trees damaged by hemipterans (Downes and Dahlem 1987). Moreover, flies have been observed feeding on the sap exuding from the hemipteran feeding wounds (Stewart and Leonard 1916, Downes and Dahlem 1987), and Downes and Dahlem (1987) argued that honeydew was a source of concentrated sugar for flies prior to the evolution of flower nectar. If dipterans actively seek honeydew, then they might be predisposed to encounter hemipteran feeding wounds, which could then act as entry courts or inoculum sources.

Collectively, this study provides a window into the ecological roles of different insect guilds involved in the transmission of *E. amylovora* in New York. Pollinating bees likely have a smaller role in transmission of blossom blight than previously thought, while flies that visit blossoms likely have an understudied role relative to the attention they have received. Dipterans are readily
observed feeding on ooze in the early summer, and their potential to disseminate bacteria from this source was demonstrated. The role of hemipterans in the disease cycle is still unclear, and interactions between hemipterans, dipterans, and *E. amylovora* should be studied in more depth. Dipterans, especially those in the Muscidae and Anthomyiidae families, preliminarily appear to play a significant role in facilitating transmission of shoot blight, and they should be a continued focus of research.

**Acknowledgements**

We thank George Sundin of Michigan State University for helpful discussions leading to the development of this study. We thank Erika Mudrak of the Cornell Statistical Consulting Unit for helpful comments on data analysis. This research was funded by NSF-GRFP grant #DGE-1650441; Northeast SARE Graduate Student Grant grant #GNE16-115-29994; New York State Department of Agriculture and Markets Apple Research Development Program grant #C200849; Federal Capacity Funds #2016-17-199, the Grace Griswold Endowment, and the Arthur Boller Research Fund.
REFERENCES


Sebaihia, M., A. M. Bocsanczy, B. S. Biehl, M. A. Quail, N. T. Perna, J. D. Glasner, G. A.


24
**Table 1.1:** Breakdown of pollinating hymenopterans collected in 2016 and 2017 by genus

<table>
<thead>
<tr>
<th>Genus</th>
<th>2016</th>
<th>2017</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apis</td>
<td>62</td>
<td>66</td>
</tr>
<tr>
<td>Bombus</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Xylocopa</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>Andrena</td>
<td>98</td>
<td>164</td>
</tr>
<tr>
<td>Lasioglossum</td>
<td>241</td>
<td>47</td>
</tr>
<tr>
<td>Halictus</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>Osmia</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Augochlorella</td>
<td>1</td>
<td>19</td>
</tr>
<tr>
<td>Augochlora</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 1.2: Monthly breakdown of positive insects sampled from yellow sticky cards

<table>
<thead>
<tr>
<th></th>
<th>May</th>
<th>June</th>
<th>July</th>
<th>August</th>
<th>September</th>
</tr>
</thead>
<tbody>
<tr>
<td>2016</td>
<td>0</td>
<td>8</td>
<td>18</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>2017</td>
<td>4</td>
<td>17</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 1.3: PCR results for pollinator and yellow sticky card surveys in 2016 and 2017

<table>
<thead>
<tr>
<th>Order</th>
<th>Insect/Group</th>
<th>2016</th>
<th>2017</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Samples collected</td>
<td>Positive samples</td>
</tr>
<tr>
<td>Hymenoptera</td>
<td><em>Apis mellifera</em></td>
<td>62 0 0%</td>
<td>66 0 0%</td>
</tr>
<tr>
<td></td>
<td>Other Hymenoptera</td>
<td>354 0 0%</td>
<td>249 0 0%</td>
</tr>
<tr>
<td>Diptera</td>
<td><em>Delia</em> spp.</td>
<td>534 -a -a 1.91%</td>
<td>1645 15 0.91%</td>
</tr>
<tr>
<td></td>
<td>Other Diptera</td>
<td>123 -a -a</td>
<td>189 0 0%</td>
</tr>
<tr>
<td>Hemiptera</td>
<td><em>Macrosteles quadrilineatus</em></td>
<td>95 0 0%</td>
<td>0 0 0%</td>
</tr>
<tr>
<td></td>
<td><em>Philaenus spumarius</em></td>
<td>153 2 1.31%</td>
<td>0 0 0%</td>
</tr>
<tr>
<td></td>
<td><em>Lygus lineolaris</em></td>
<td>66 0 0%</td>
<td>60 0 0%</td>
</tr>
<tr>
<td></td>
<td><em>Empoasca fabae</em></td>
<td>896 13 1.45%</td>
<td>1531 8 0.52%</td>
</tr>
<tr>
<td></td>
<td><em>Paraphlepsius</em> spp.</td>
<td>393 27 6.87%</td>
<td>237 1 0.42%</td>
</tr>
</tbody>
</table>

*a* Diptera populations were monitored in both 2016 and 2017 but only tested for *E. amylovora* in 2017.
Table 1.4: Summary of feeding behaviors for Dipterans observed on ooze in June 2018

<table>
<thead>
<tr>
<th>Diptera observed</th>
<th>Range of feeding times (minutes)</th>
<th>Mean feeding time (minutes)</th>
<th>Individuals captured</th>
<th>Individuals positive for <em>E. amylovora</em></th>
<th>Percent positive</th>
<th>Mean number of days positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delia spp.</td>
<td>0:12 – 5:09</td>
<td>2:55</td>
<td>7</td>
<td>4</td>
<td>57%</td>
<td>3.0</td>
</tr>
<tr>
<td>Misc. Diptera</td>
<td>0:02 – 6:34</td>
<td>2:03</td>
<td>9</td>
<td>6</td>
<td>67%</td>
<td>4.5</td>
</tr>
<tr>
<td>Total</td>
<td>0:02 – 6:34</td>
<td>2:22</td>
<td>16</td>
<td>10</td>
<td>63%</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>Number of insects</td>
<td>Number of blossoms visited</td>
<td>Mean blossoms visited (max. 3)</td>
<td>Number of positive blossoms visited</td>
<td>Percentage of positive blossoms visited</td>
<td>Percentage of individuals that fed on nectar</td>
</tr>
<tr>
<td>----------------</td>
<td>-------------------</td>
<td>----------------------------</td>
<td>--------------------------------</td>
<td>--------------------------------------</td>
<td>------------------------------------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td><em>Apis mellifera</em></td>
<td>5</td>
<td>12</td>
<td>2.4</td>
<td>0</td>
<td>0%</td>
<td>20%</td>
</tr>
<tr>
<td>Small bee</td>
<td>40</td>
<td>64</td>
<td>1.6</td>
<td>1</td>
<td>1.6%</td>
<td>60%</td>
</tr>
<tr>
<td>Medium bee</td>
<td>22</td>
<td>42</td>
<td>1.9</td>
<td>3</td>
<td>7.1%</td>
<td>60%</td>
</tr>
<tr>
<td>Large bee</td>
<td>10</td>
<td>15</td>
<td>1.5</td>
<td>0</td>
<td>0%</td>
<td>79%</td>
</tr>
<tr>
<td>Diptera</td>
<td>90</td>
<td>128</td>
<td>1.4</td>
<td>8</td>
<td>6.3%</td>
<td>75%</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>167</strong></td>
<td><strong>261</strong></td>
<td><strong>1.6</strong></td>
<td><strong>12</strong></td>
<td><strong>4.6%</strong></td>
<td><strong>69%</strong></td>
</tr>
</tbody>
</table>
Table 1.6: Summary of positive blossoms visited by insects and collected as controls

<table>
<thead>
<tr>
<th></th>
<th>Total blossoms visited/sampled</th>
<th>Percent of positive blossoms on day 1 – 2</th>
<th>Percent of positive blossoms on day 3 – 4</th>
<th>Percent of positive blossoms on day 5 – 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insect visits</td>
<td>261</td>
<td>0.97% (1/103)</td>
<td>7.94% (10/126)</td>
<td>3.13% (1/32)</td>
</tr>
<tr>
<td>Control samples</td>
<td>315</td>
<td>0.78% (1/128)</td>
<td>12.40% (16/129)</td>
<td>32.76% (19/58)</td>
</tr>
</tbody>
</table>
Figure 1.1: Population of *Delia* spp. captured on all yellow sticky cards in 2016 (grey solid line) and 2017 (block dashed line) in an experimental research orchard inoculated with *E. amylovora* in Geneva, NY.
Figure 1.2: Percentage of *E. amylovora* ooze droplets by color in an experimental orchard (A) and visited by flies (B). There were no significant differences between observed frequency of droplet colors and frequency of droplet colors visited by flies ($\chi^2 = 2.5898, p = 0.4503$).
Figure 1.3: Percentage of *E. amylovora* ooze droplets by location in an experimental orchard (A) and visited by flies (B). There was a significant difference between the frequency of droplet at each location and the frequency of droplet location visited by flies ($\chi^2 = 55.129, p = 0.0005$).
Figure 1.4: Persistence of *E. amylovora* over 7 days in 10 dipteran samples collected after feeding on ooze in the field. Data is presented on logarithmic scale. Decline in mean *E. amylovora* CFU/Insect roughly approximated exponential decay.
Figure 1.5: Mean log$_{10}$ CFU/mL + 95% CI *E. amylovora* in control (black bars) and insect visited (grey bars) blossoms by sampling date. Figure only shows mean log$_{10}$ CFU/mL for positive samples (insect visited: n = 12; control: n = 35), and all sampling dates were combined due to low n. *, $p < 0.05$. 
CHAPTER 2

EFFECTS OF EXPOSURE TIME AND BIOLOGICAL STATE ON ACQUISITION AND ACCUMULATION OF *ERWINIA AMYLOVORA* BY *DROSOPHILA MELANOGASTER*

Matthew Boucher,¹* Rowan Collins,² Kerik Cox,³ and Greg Loeb¹

¹Department of Entomology, Cornell AgriTech, New York State Agricultural Experiment Station, Cornell University, Geneva, New York, 14456 USA

²Department of Environmental Studies, Mount Holyoke College, Hadley, Massachusetts, USA

³Section of Plant Pathology & Plant Microbe Biology, School of Integrated Plant Sciences, Cornell AgriTech, New York State Agricultural Experiment Station, Geneva, New York, 14456 USA

*Corresponding author: M. T. Boucher; Email: mtb245@cornell.edu
Department of Entomology, Cornell AgriTech, College of Agriculture and Life Sciences, Cornell University, 15 Castle Creek Drive, Geneva, NY 14456

Abstract

Fire blight, caused by the bacterium *Erwinia amylovora*, is a disease devastating to the production of rosaceous crops, primarily apple and pear, with worldwide distribution. Fire blight begins in the spring when primary inoculum is produced as ooze, which consists of plant sap, *E. amylovora*, and exopolysaccharides. Ooze is believed to be transferred to healthy tissues by wind, rain, and insects. However, the mechanisms by which insects locate and transmit ooze are largely undocumented. The goals of this study were to investigate the biological factors affecting acquisition of *E. amylovora* from ooze by a model dipteran, *Drosophila melanogaster*, and to determine if flies were able to mechanically transfer this bacterium after acquisition. We found that the percentage of positive flies increased as exposure time increased, but nutritional state, mating status, and sex did not significantly alter the number of positive individuals. Bacterial abundance was highly variable at all exposure times, suggesting that other biological factors play a role in acquisition. Nutritional state had a significant effect on *E. amylovora* abundance, and food deprived flies had higher *E. amylovora* counts than satiated flies. We also demonstrate that *D. melanogaster* transmits *E. amylovora* to a selective media surface, and hypothesize that the same is possible for plant surfaces, where bacteria could persist until an opportunity to colonize the host arises. Collectively, these data suggest a more significant role for flies than previously thought in transmission of fire blight and contribute to a shift in our understanding of the *E. amylovora* disease cycle.

Importance

A recent hypothesis proposed that dissemination of *Erwinia amylovora* from ooze by flies to native Rosaceous trees was likely key to the life cycle of the bacterium during its evolution. Our study validates an important component of this hypothesis by showing that flies
are capable of acquiring and transmitting this bacterium from ooze under various biotic conditions. Understanding how Dipterans interact with ooze advances our current knowledge of its epidemiological function and provides strong evidence supporting an underappreciated role of flies in the disease cycle. These findings may be especially important as it pertains to shoot blight, as this stage of the disease is poorly understood and may involve a significant amount of insect activity. Broadly, this study underscores a need to consider the depth, breadth, and origin of interactions between flies and *E. amylovora* to better understand its epidemiology.

**Introduction**

*Erwinia amylovora* (Enterobacteriaceae) is a gram-negative bacterium that causes fire blight, a necrotic disease of pome fruits and other rosaceous plants (Oh and Beer 2005). This bacterium is hypothesized to have originated in the northeastern USA on wild rosaceous hosts such as crabapple, and likely shifted to rosaceous crop plants including apple and pear when they were introduced to North America by European colonizers (van der Zwet and Keil 1979). Presently, *E. amylovora* has spread to at least 47 countries, becoming a global risk to pome fruit production (Van der Zwet et al. 2012). In 2000, apple producers in Michigan lost nearly 3 million bushels, which had a lasting financial impact that cost growers roughly $42 million over five years (Longstroth 2001). A 1997 fire blight outbreak in Australia cost growers nearly $15 million and estimates suggest that future outbreaks could cost upwards of $195 million across five years (Rodoni et al. 2006). Moreover, the annual cost of disease management to pome fruit growers in the United States is estimated at $100 million USD. These costs include sanitation of any diseased plant material along with applications of copper and antibiotics prior to and at bloom to reduce inoculum levels (Norelli et al. 2003).

Adding to these economic stressors is a dramatic shift in the structure of modern apple
orchards. Growers are shifting to high-density plantings using dwarf trees on highly susceptible rootstocks, and newer cultivars are more susceptible to *E. amylovora* than older ones (Norelli et al. 2003). These high-density plantings produce a greater yield of higher quality fruit, but are more expensive to establish, leading to greater economic losses due to fire blight than in the past (Norelli et al. 2003). Compounding these costs is a realized risk of antibiotic resistance across the United States (Forster et al. 2015). Streptomycin has been used as part of *E. amylovora* control programs for over 50 years, but the continued presence and emergence of resistant *E. amylovora* strains and lack of cost effective alternatives necessitates a more integrated approach to disease management (Forster et al. 2015, Tancos et al. 2016).

Management programs require a more thorough understanding of the *Erwinia amylovora* disease cycle than is currently available (Smits et al. 2017). The disease cycle begins in spring, when overwintering cankers from previous seasons exude bacterial ooze, which is primarily a mixture of *E. amylovora* and bacterial exopolysaccharide (Van der Zwet et al. 2012). This ooze serves as the primary inoculum source in the spring, and is transmitted to plant surfaces by wind, rain, and insects (van der Zwet et al. 2012). Once internalized, *E. amylovora* systematically migrates through the plant via the parenchyma and can form biofilms in the xylem (Koczan et al. 2009, Slack et al. 2017). The bacteria accumulates in such high titers in the parenchyma of young green shoots that it ruptures the epidermis, generating new ooze that serves as secondary inoculum (Slack et al. 2017). As the growing season ends, cankers form on woody tissue, where *E. amylovora* overwinters and oozes the following spring (van der Zwet et al. 2012).

The primary component of *Erwinia amylovora* ooze in all strains and hosts is the exopolysaccharide (EPS) amylovoran, which is also a key pathogenicity factor for the bacterium (Bennett and Billing 1980, Nimtz et al. 1996, Koczan et al. 2009). Amylovoran aides in
preventing recognition of bacterial cells by host plant defenses as well as nutrient binding (Bellemann et al. 1994). Amylovoran-mutant *E. amylovora* do not produce ooze or other disease symptoms, and *E. amylovora* does not proliferate in apple tissue without functional amylovoran genes (Zhao et al. 2009). The polysaccharide matrix in ooze constitutes roughly 80% of a droplet, with the other 20% being *E. amylovora* cells (Keil and Van der Zwet 1972). Structurally, amylovoran is made up of several carbohydrates, with the primary ones being galactose and glucose (Nimtz et al. 1996).

The role of insects as vectors of primary and secondary inoculum has been anecdotally recognized since the early 1900s (Burrill 1915, Stewart and Leonard 1916, Ark and Thomas 1936), but controversy remains over which insects are chiefly responsible for transmission and how these insects transmit the bacteria (Ordax et al. 2015). For example, honeybees (*Apis mellifera* Linnaeus) are hypothesized to transmit *E. amylovora* from flower to flower (Johnson et al. 1993), but do not feed on or approach fire blight ooze, thus making them unlikely disseminators of primary inoculum (Hildebrand 1936). By comparison, flies (Diptera) approach and feed on fire blight ooze, acquiring *E. amylovora* and becoming capable disseminators in the process (Ark and Thomas 1936, Ordax et al. 2015, Slack et al. 2017). Dipterans are known vectors of numerous related bacterial phytopathogens, including potato black leg and other soft rots (Basset 2000, Grenier et al. 2006, Rossmann et al. 2018), and can act as alternative hosts for these bacteria, which aids in retention and transmission of these diseases to plant hosts (Nadarasah and Stavrinides 2011). Historically, the relationship between numerous fly species and fire blight incidence has been studied (Stewart and Leonard 1916, Ark and Thomas 1936, Ordax et al. 2015), but the role of flies was downplayed at the time because researchers suggested that the transmission mechanism was unrealistic (Stewart and Leonard 1916). Stewart
(1916) found that when flies were caged on an apple sapling that was smeared with cultured *E. amylovora*, no disease occurred. When the experiment was repeated with hemipterans that damage sapling tissue, disease did occur (Stewart and Leonard 1916). Stewart then proposed that it was possible that flies could transmit *E. amylovora* to the surface of a sapling, after which the bacteria could colonize an injury created by a hemipteran, but indicated that transmission via this mechanism was likely over estimated, citing adverse environmental factors (Stewart and Leonard 1916). However, this study failed to investigate that mechanism and did not evaluate acquisition of *E. amylovora* by flies. Further, the testing method is questionable, as cultured bacteria smeared on the plant surface does not account for the effects ooze may have on the behavior of flies or the quantity of viable bacteria available to flies in the field. Slack et al (2017) reinvigorated interest in this transmission mechanism and broadened the scope of its importance by proposing that it may have been the primary mode of transmission in *E. amylovora*’s original evolutionary environment. Prior to the introduction of rosaceous fruit crops to the Northeast USA, *E. amylovora* was a pathogen of native rosaceous plants, which were less likely to be adjacent to each other as is the case in an orchard (Slack et al. 2017). Flies that harbor the bacteria on or in their bodies could traverse the distance between pathogen hosts, where the bacteria would be left behind on a plant not protected by a management regime and thus more likely to be damaged in some way. In this proposed mechanism, ooze plays a central role as the main site of *E. amylovora* acquisition by flies (Slack et al. 2017).

While the relationship between *Erwinia amylovora* and potential dipteran transmission facilitators has been explored recently (Ordax et al. 2015, Slack et al. 2017), neither study focused on how the biological state of a fly affected acquisition of *E. amylovora*. For instance, it is not known if flies use ooze as a nutrient source. If flies consume *E. amylovora* ooze for
nutrition, then it is reasonable to predict that factors affecting general nutrient consumption in flies should apply to their consumption of fire blight ooze. This behavior could have broad implications for the quantities of *E. amylovora* an individual fly acquires from ooze and thus how likely it is to transmit the disease to new hosts. The goal of this study was two-fold: 1) to experimentally establish fly acquisition and transmission of *E. amylovora* from ooze; and 2) to evaluate which biological factors contribute to acquisition. We conducted this experiment using the common Dipteran laboratory model *Drosophila melanogaster*, which was used in similar phytopathological studies and has been implicated as a facilitator of fire blight transmission (Ark and Thomas 1936, Grenier et al. 2006). Based on several studies that investigate the involvement of various Dipterans in this system (Stewart and Leonard 1916, Ark and Thomas 1936, Ordax et al. 2015, Slack et al. 2017), we assume that flies are broadly capable of acquiring *E. amylovora* from ooze. Thus, in this instance, we used *D. melanogaster* as a general model for how flies interact with ooze during acquisition and to show that flies can transmit the bacteria after interacting with ooze. In a factorial design, we tested the effect of mating status, nutritional state, and exposure time on the acquisition and abundance of *E. amylovora* on individual *D. melanogaster*. Fly sex for each individual was recorded after treatment and included in the analysis as an additional factor. In a separate experiment, flies were exposed to ooze and then allowed to walk on selective media to demonstrate transmission.

**Methods**

*Drosophila melanogaster colony and *E. amylovora* cultures.* *Drosophila melanogaster* colony was started from individuals collected from the Rochester, NY area in 2013 and obtained from Dr. John Jaenike at the University of Rochester. Flies were maintained on formula 4-24® instant Drosophila medium (herein referred to as ‘blue diet,’ catalog no. 173210, Carolina
Biological Supply Co.) in an environmental chamber (model I-30BL: Percival Scientific, Inc.; Perry, IA, USA) at 24°C with 13 hours of daylight at 50% relative humidity. Flies were transferred to new diet bottles once weekly.

*Erwinia amylovora* strain ea273 (ATCC 49946), a well characterized virulent strain isolated from apple (Malus × Domestic cv. Rhode Island Greening) in New York, was maintained on Crosse-Goodman (CG) media and used in all experiments (Crosse and Goodman 1973). CG media is a selective media on which *E. amylovora* presents with a distinct cratering. This cratering allows us to differentiate *E. amylovora* from other bacteria that may be present in/on the insect (Crosse and Goodman 1973). We chose this strain because it is common in New York State and highly virulent on apple (Bocsanczy et al. 2008, Sebaihia et al. 2010). Overall, *Erwinia amylovora* strain diversity is low, requiring CRISPR spacer array analysis to properly characterize, so use of a single strain in this experiment was appropriate (Tancos and Cox 2016). Cultures were transferred weekly; a sterile inoculation loop was dipped into *E. amylovora* colonies on CG media and grown overnight in LB broth (catalog no. 12795-027, Invitrogen) at 28°C in a shake incubator. One microliter of LB grown *E. amylovora* was plated on CG plates and incubated upside down at 28°C with high humidity for 48 hours, then stored at 4°C until used.

**CAFE (Capillary Feeding) Assay.** All experiments were conducted in a CAFE arena (Ja et al. 2007). The CAFE arena is made up of two chambers; an outer chamber (50mL conical tube) with a water-soaked paper towel placed in the bottom; and an inner chamber (plastic vial) secured to the inner rim of the conical tube cap with a large hole drilled in the bottom underlaid with mesh. A hole is drilled in the center of the conical tube cap, with a truncated 200µl pipette tip inserted into the hole. The pipette tip secured a 5µl capillary tube (catalog no. 53432-706,
VWR) with a food source for the flies. The outer chamber provides humidity for the fly to prevent desiccation. Only one fly is tested per CAFE arena.

**Acquisition study design.** This study was conducted using a factorial design with 4-day old flies evaluating the effect of mating status (mated or unmated), exposure time (3, 6, 12, 24 hours), and nutritional state (food deprived or satiated) on whether *D. melanogaster* 1) acquired *E. amylovora* from ooze and 2) how much *E. amylovora* an individual acquires. Fly sex was determined after each replicate was tested and included in the analysis as a factor. There were 16 possible combinations of factors and 29-30 flies were tested per factorial treatment (e.g. 30 mated, food deprived flies individually exposed to ooze for 3 hours), for a total of 478 replicates. We tested an equal number of controls for each factorial combination, ensuring that control replicates were run on the same day as treatment replicates. These flies were provided sucrose rather than ooze to ensure no cross contamination occurred during experimentation or processing. If controls tested positive for *E. amylovora*, that day’s replicates were discarded, and a new set was tested. Only 2 control replicates tested positive over the course of the experiment, occurring on one day, so contamination was not generally an issue. Experiments were conducted in an environmental chamber with the same conditions as described above.

For unmated *D. melanogaster*, pupated individuals close to eclosion (red eye spots and black wing spots visible through pupal case) were removed from diet bottles with a paintbrush and placed into individual vials containing blue diet and plugged with cotton. After eclosion, flies were allowed to feed on blue diet for 24 hours before being transferred into a CAFE arena for a 24-hour acclimation period. For mated flies, emerged adults were transferred out of diet bottles daily into new, fly free diet bottles and given 24 hours to feed and mate (Hudak and Gromko 1989). After mating, flies were transferred into individual CAFE arenas. Both mated
and unmated flies were provided 5% sucrose via capillary tube during habituation. After
habituation, flies were either deprived of food or allowed continued access to sucrose for another
24 hours. Food deprived flies were provided a capillary tube with water to prevent desiccation.
On the fourth day, flies were subjected to treatment. On treatment day, flies were provided with
either 5µl ooze solution or 5µl 5% sucrose solution via capillary tube and exposed to the food
source for 3, 6, 12, or 24 hours. After exposure, flies were transferred to 1.5mL Eppendorf tubes
and put on ice for 10 minutes to anesthetize them, after which individuals were sexed. Flies were
then homogenized with a sterile pestle in 100µl of dH2O, and 1µl of homogenate was plated on
CG plates. Plates were sealed with Parafilm® were incubated upside down at 28°C with high
humidity for about 48 hours, after which *E. amylovora* cells were counted under a light
microscope. To ensure identification accuracy, positive plates were spot checked throughout the
experiment by inoculating immature apple fruit with colonies identified as *E. amylovora.*
Immature fruit were inoculated as described below.

**Transmission study design.** This study was conducted on 4-day old, mixed sex and
mixed mating status *D. melanogaster.* Flies were isolated from colony 24 hours after eclosion
and placed into CAFE arenas, where they were provided 5% sucrose via capillary tube for 2
days. At the end of the third day post eclosion, flies were provided a capillary tube containing *E.
amylovora* ooze prepared as described below for roughly 15 hours. After exposure to ooze, flies
were removed from CAFE arenas and placed on individual petri dishes containing CG media.
Petri dishes were covered, and flies were allowed to walk around on the media surface for a two-
hour transmission period. Next, flies were removed and discarded, and a cell spreader was used
to distribute any bacteria on the plate. Plates were sealed with Parafilm® and incubated upside
down at 28°C with high humidity for about 48 hours, after which each plate was visually
assessed for the presence of *E. amylovora* cells under a light microscope.

On each of three testing dates, we tested 30 individual *D. melanogaster* (n=88). On the second testing day, 2 individuals died during the two-hour transmission period, so those replicates were discarded. To ensure no contamination occurred, 10 *D. melanogaster* controls were tested on each date in the manner described above (n=30). These flies were provided sucrose instead of ooze for the final exposure period before testing and 0% of controls tested positive. To confirm that the bacteria identified on positive plates was *E. amylovora*, five positive plates were randomly selected from each testing day, and *E. amylovora* from those plates was used to inoculate immature apple fruit (n=15). Colonies were selected from each plate, grown in separate aliquots of LB broth, and then 500µl of broth was pipetted onto an immature fruit half on the flesh side. Fruit halves were placed flesh side down on a piece of filter paper and sealed in a plastic cup before being incubated at 28°C in a growth chamber with high humidity (>90% RH) for 4-7 days until ooze droplets formed on the surface of fruit. All inoculated fruit developed ooze droplets, confirming our visual assessment.

**Preparation of *E. amylovora* ooze for assay.** Ooze was harvested from lab inoculated immature apples (cultivar ‘Gala’) collected from fire blight free research blocks in May in Geneva, NY using a method adapted from Goodman et al (Goodman et al. 1974). Immature fruits were surface sterilized by washing in 10% bleach solution for 10 minutes, then dried and punctured 4-8 times with a dental pick. Two hundred microliters of *E. amylovora* grown overnight in LB broth was deposited into each wound, then wounds were “sealed” with *E. amylovora* colonies grown on CG media by scraping colonies over wound surface with a dental pick. Immature fruit were placed in plastic cups on top of water-saturated filter paper with the lid sealed to the cup with parafilm. Fruit were kept at 28°C in a growth chamber with high humidity
(>90% RH) for 4-7 days until ooze droplets formed on the surface of fruit.

Capillary tubes were loaded with ooze solution by drawing 5µl solution into a pipette and placing the tip of the pipette to the end of the capillary tube. Capillary action then drew the solution into the tube. Loading the capillary tube in this manner ensured that *E. amylovora* did not contaminate any surface that the insect walked on, meaning that any bacteria the insect would acquire would be done so via ingestion. Undiluted ooze droplets were too viscous to be drawn into a 5µl capillary tube for the assay, so 3-5 droplets were collected from the fruit surface and homogenized in 200µl dH2O prior to loading into tubes. On every testing day, an aliquot of the ooze solution used was serially diluted and plated on CG media to determine the initial concentration of *E. amylovora*, this value was included as a random effect in statistical analyses.

**Statistics.** Infection status data (whether the insect acquired *E. amylovora* or not) were analyzed in R using a generalized linear mixed effect model with a binomial distribution (R Core Team 2019). Exposure time, sex, mating status, and nutritional state were fixed effects while ooze solution CFU and testing date were random effects. A likelihood ratio test using a chi-square distribution was performed to assess model significance. Each fly constituted an individual replicate in this analysis. The model was fit using lme4 package and analyzed using the car package (Bates et al. 2015, Fox and Weisberg 2019). All fixed effects were analyzed for interactions and model was simplified to remove insignificant terms.

*Erwinia amylovora* abundance was analyzed using a linear mixed effect model with a normal distribution (R Core Team 2019). CFU/insect data were log transformed and resulting distribution approached normality. Data were then analyzed in the manner described above. All figures were generated in R using the ggplot2 package (Wickham 2016).
**Results**

Exposure time affects the percentage of flies that acquire *E. amylovora* but not the abundance of bacteria for individual flies. To evaluate the likelihood that *D. melanogaster* acquires *E. amylovora* from ooze, individual flies were exposed to ooze in a CAFE (CApillary FEeding, described below) arena for 3, 6, 12, and 24 hours within a factorial experiment that also included mating status and nutritional state (Ja et al. 2007). Fly sex was part of this analysis but was not evaluated factorially. Following exposure, flies were homogenized in water and immediately plated on Crosse-Goodman media. Colony forming units (CFU) were determined after 48 hours of incubation at 28°C (Crosse and Goodman 1973). The minimum detection threshold was 100 CFU/insect and the maximum threshold was 100,000 CFU/insect. Exposure time was analyzed for its effect and potential interactions with other variables. There were no significant interactions between exposure time and other variables.

Exposure time had a significant positive effect on acquisition of *E. amylovora* by *D. melanogaster* such that increasing exposure time led to an increased percentage of positive individuals ($\chi^2 = 24.08, p < 0.001$) (Figure 2.1). 12.6% of *D. melanogaster* tested positive after 3 hours of exposure to ooze, 33.3% of individuals tested positive for fire blight after 6 hours, 56.3% of individuals tested positive for fire blight after 12 hours and 56.7% of *D. melanogaster* tested positive after 24 hours. We did not evaluate acquisition at enough exposure times to determine if the similar percentage of positive flies at 12 and 24 hours represented saturation.

Exposure time had a significant negative effect on the abundance of *E. amylovora* in individual *D. melanogaster* indicating that a longer exposure time led to decreased CFU/insect levels ($\chi^2 = 3.92, p = 0.048$) (Figure 2.2). Mean *E. amylovora* abundances were similar at 3 and 6 hours (13,227 CFU/insect and 13,145 CFU/insect respectively), before increasing to 20,421 CFU/insect at 12 hours and then decreasing to 8,853 CFU/insect at 24 hours (Figure 2.2).
However, CFU/insect varied considerably within each exposure time, and that variation was consistent across all exposure times (Figure 2.2). At 3 hours of exposure, cell counts ranged from 100-72,300 CFU/insect, and cell counts at 6, 12, and 24 hours of exposure ranged from 100-100,000 CFU/insect.

**Food deprived D. melanogaster accumulate more E. amylovora than satiated D. melanogaster, but nutritional state does not affect the percentage of positive individuals.** To evaluate the effect of food deprivation on *E. amylovora* acquisition within the factorial experiment, diet was either withheld from or provided to individual *D. melanogaster* for 24 hours before being exposed to *E. amylovora* in a CAFE arena (Ja et al. 2007).

While 42.5% of food deprived flies and 37% of satiated flies tested positive for *E. amylovora* across all exposure times, this difference was not significant ($\chi^2 = 1.00, p = 0.32$). There were no significant interactions between nutritional state and the other variables tested. There was a significant positive effect of food deprivation on *E. amylovora* abundance for individual *D. melanogaster* ($\chi^2 = 5.36, p = 0.02$) (Figure 2.3). Mean CFU/insect values differed two-fold between food deprived and satiated flies, measuring 17,885 CFU and 9,888 CFU respectively.

**Unmated flies accumulate marginally more E. amylovora than mated flies, but mating status does not affect the percentage of positive individuals.** To evaluate the effect of mating status on acquisition of *E. amylovora* within the factorial experiment, individual *D. melanogaster* were isolated as pupae and either maintained in isolation or allowed to mate until exposure to *E. amylovora* ooze in a CAFE arena (Ja et al. 2007).

There were no significant differences in the percentage of infected individuals between mated and unmated flies with 38.24% of mated flies and 41.25% of unmated flies testing
positive for *E. amylovora* across all exposure times ($\chi^2 = 0.45, p = 0.50$). There were no significant interactions between mating status and the other variables tested. There was a marginally significant positive effect of mating status on *E. amylovora* abundance for individual flies such that unmated flies acquired slightly higher titers than mated flies ($\chi^2 = 3.68, p = 0.055$) (Figure 2.4). The mean CFU/insect of unmated flies (18,378 CFU/insect) was nearly double the mean of mated flies (9,615 CFU/insect).

**Sex of *D. melanogaster* had no bearing on either abundance of *E. amylovora* in individuals or percentage of positive individuals.** Sex was determined after each replicate was completed and analyzed for its effect and potential interactions with other variables. Fly sex was not a statistically significant predictor of either *E. amylovora* abundance or percentage of positive individuals and had no significant interaction with exposure, mating status, or nutritional state. Overall, 39% of females and 40% of males tested positive for *E. amylovora* ($\chi^2 = 1.63, p = 0.20$). The mean abundance for female flies was 16,230 CFU/insect and the mean for male flies was 16,314 CFU/insect ($\chi^2 = 0.04, p = 0.84$).

**Drosophila melanogaster can transmit *E. amylovora* to a surface after acquiring bacteria from ooze.** To test if *D. melanogaster* can transmit *E. amylovora*, flies were exposed to ooze in the CAFE arena for 24 hours, then allowed to walk on the surface of individual petri dishes containing selective media. After two days of incubation, plates were checked for presence or absence of *E. amylovora*.

Overall, 58% of *D. melanogaster* (n=88) transmitted *E. amylovora* to the selective media surface. Replicates were run in 3 batches of 30 individuals across 3 weeks, and batches tested positive at 63% (n=30), 46% (n=28), and 63% (n=30).
Discussion

*Erwinia amylovora* proliferates to such high numbers in the parenchyma of infected plants that it ruptures the plant epidermis and oozes from within, forming large ooze droplets on the surface of green shoots, woody tissue, and immature fruit (Slack et al. 2017). Ooze has been hypothesized as a source of bacteria that dipterous insects could acquire and transmit, and may also be an appealing food source for flies due to its sugary constitution (Koczan et al. 2009, Slack et al. 2017). Dipteran contact chemoreceptors on the tarsi react to various types of carbohydrates and initiate feeding behavior regardless of the fly’s nutritional state (Dethier et al. 1956), so a carbohydrate rich resource such as ooze represents a likely source of stimulation and feeding. A fly that feeds on ooze could potentially harbor the bacteria in its crop or its alimentary canal, and the bacteria could be regurgitated or defecated onto susceptible plant surfaces. Several pathogenic bacteria in the family Enterobacteriaceae are harbored by flies internally and contaminate plant surfaces via this mechanism (Nayduch et al. 2018). The results from this study establish that *D. melanogaster* can acquire *E. amylovora* from ooze in high enough titers to be infectious to healthy hosts, as previous reports indicate that *E. amylovora* requires as few as 35 cells to generate a new infection (Crosse 1972). We demonstrated that food deprivation and lack of mating increased bacterial abundances of individual *D. melanogaster*, while fly sex had no effect on *E. amylovora* abundances. Additionally, we show that the longer an insect is exposed to *E. amylovora* ooze, the more likely it is to acquire the bacteria. Finally, we established that acquisition of *E. amylovora* from ooze can lead to transmission, as *D. melanogaster* that fed on ooze transmitted bacteria to a petri dish containing selective media.

It is important to note that the bacterial abundances reported in this study are likely conservative estimates of acquisition, as we focused solely on evaluating the ingestion of ooze. In the field, a fly would be able to access the ooze droplet more intimately by walking or
roosting on it. This would increase the contact rate between insect and droplet, leading to the acquisition of the bacteria on the external surface of the fly, thus increasing the overall bacterial titer. Furthermore, we had to dilute ooze droplets to reduce droplet viscosity, as droplets were too thick to be drawn into the capillary tube by themselves or would harden in the capillary tube if concentrations were too high, thus disallowing ingestion by the fly. This had the effect of reducing bacterial titers to the $10^6$ CFU/µl range, which falls within the range of what is found in the field, but abundances in the field often exceed $10^7$ CFU/µl and into the $10^8$ CFU/µl range (Slack et al. 2017). Nonetheless, D. melanogaster acquired high, transmissible amounts of bacteria even with the reduced titers required to conduct this experiment.

Despite statistical significance, we believe that exposure time has no biologically relevant effect on E. amylovora abundance for D. melanogaster. The significant negative effect is due to the drop in mean E. amylovora abundance at 24 hours. While we do not know the reason for this sharp decline, it is possible that it was a result of our experimental methodology. The ooze solution in the capillary tube was viscous and could harden over time, reducing access to the inoculum in the tube. Biologically, feeding durations on ooze could have been reduced at higher exposure time as fly taste receptors adapted to the concentration of sugar in solution (Dethier et al. 1956), reducing overall ingestion. Alternatively, the wide range of E. amylovora abundances at each time point could indicate that the biological effect of exposure time on bacterial abundance is relatively weak, and that other biotic and abiotic factors are more important (Thomson et al. 2017). Similar abundances across exposure times have been reported in previous studies on related Enterobacteriaceae (Thomson et al. 2017). Abundances of Escherichia coli did not change in the gut of house flies over time (Musca domestica Linnaeus), which was hypothesized to occur due to the immobilization/lysis of bacteria in the food bolus in the fly gut,
combined with replacement of dead cells via continued ingestion of bacteria (Thomson et al. 2017). In nature, a fly residing in a diseased orchard could have a range of exposure to *E. amylovora* ooze depending on the severity of the infection and the time of year. Being that exposure time has a strong positive affect on the percentage of positive flies, and at best a weak negative effect on the abundance of *E. amylovora* in the fly, short or intermittent interactions with ooze could yield flies that acquired transmissible amounts of bacteria. This suggests that flies could be key factors in the disease cycle of *E. amylovora* in orchards with both low and high levels of infection.

The strong effect of food deprivation on *E. amylovora* abundance for *D. melanogaster* was expected, as a food-deprived fly is more likely to engage in food searching behavior compared to a satiated fly (Dethier 1976). Stretching of the gut due to high food levels in satiated flies creates a negative feedback loop that inhibits proboscis extension and sucking behavior, thus reducing ingestion (Gelperin and Dethier 1967, McKellar 2016). This supports the idea that *E. amylovora* ooze could be used as a nutrient source for flies, but further studies are required to advance this hypothesis. This result also underscores the importance of an insect’s biological state when evaluating its capacity to acquire *E. amylovora* or related pathogens. The biological state of an insect will impact its behavior, which in turn would likely govern its interactions with *E. amylovora* ooze. A similar conclusion could be drawn from the mating status data in this study, where unmated flies acquired marginally more *E. amylovora* from ooze than did mated flies. While further study is required, this trend could be due to motivation on the part of mated flies to find an oviposition site rather than a food source compared to unmated flies. For example, one study found that odor preferences in female *Drosophila suzukii* Matsumara shift from fermentation odors to fruit odors as egg load increased. This shift is hypothesized to occur
as female priorities shift from finding mates/feeding sources to laying eggs (Wong et al. 2018). If a similar behavioral shift occurs in *Drosophila melanogaster*, then we would predict lower feeding rates on ooze for mated female flies and thus lower *E. amylovora* abundances in those flies.

The fact that fly sex had no effect on *E. amylovora* acquisition by *D. melanogaster* was surprising, given that sex is a determinant of bacterial acquisition in other systems. In the case of *E. coli*, female house flies (*M. domestica*) acquire higher titers than male house flies. This is likely due to behavioral differences between the two sexes, as females require a protein rich meal after each egg laying event and are thus more likely to seek out bacteria rich manure than males (Dethier 1961, Thomson et al. 2017). *Drosophila melanogaster* feed and oviposit on fermenting fruit, where both protein and carbohydrate sources are available (Becher et al. 2012), so sex differences in *E. amylovora* acquisition due to sex based behavioral differences in *D. melanogaster* may be less pronounced or nonexistent compared to other fly species. It is also possible that our experimental design dilutes any sex effects, as the only source of nutrition in the arena was ooze. A more targeted study on sex effects may be required to properly elucidate any differences.

These varying effects of biological state on acquisition illustrate the complexity of dissemination of *E. amylovora* by flies. It is likely that Diptera are generally capable of acquiring this bacterium, as *D. melanogaster*, *M. domestica*, *Lucilia sericata* Meigen, and *Ceratitis capitata* Wiedemann have all been shown to acquire *E. amylovora* (Ark and Thomas 1936, Ordax et al. 2015). In our own research, we have observed *Delia* spp. (Anthomyiidae) visiting and feeding on ooze in diseased orchards. Nutritional state, mating status, sex, and exposure time may have different effects on different fly species, and these differences are likely to vary based
on the life history and behavior of the individual species (Rossmann et al. 2018). Even if the effect of the above factors does not vary drastically, there could simply be between-species differences in *E. amylovora* acquisition as was shown between *M. domestica* and *Phormia regina* Meigen with related Enterobacteriaceae (Pace et al. 2017). The role of a fly’s biological state on acquisition of ooze is likely variable, and that role should be considered when detailing the capacity of different fly species in acquisition and transmission of *E. amylovora*.

Dipterans have been anecdotally implicated in the *E. amylovora* disease cycle as far back as 1914 (Jones 1914), but have received only sporadic attention in the literature since. Stewart and Leonard (1916) proposed the mechanism through which flies could transmit fire blight, suggesting that flies are not capable of directly initiating new infections, but can mechanically transmit *E. amylovora* to a healthy surface where the bacteria could colonize a natural opening or an injury created by another insect (Stewart and Leonard 1916). Despite claims by Stewart and Leonard that this mechanism was unlikely, additional research demonstrated that *E. amylovora* could be recovered from the frass of three fly species, including *D. melanogaster*, for up to six days post exposure (Ark and Thomas 1936). Others built on this hypothesis by suggesting that high densities of flies could be effective *E. amylovora* disseminators from wild hosts such as hawthorn (*Crataegus* spp.), as some flies can harbor the pathogen for up to nine days, providing ample time for dissemination (Emmett and Baker 1971). Collectively, these studies broadly supported a role for Dipterans in the *E. amylovora* disease cycle but did not evaluate conditions that could affect this process, which was an original criticism of its validity. Our study examines biological conditions of *D. melanogaster* that affect acquisition, showing that various biological conditions affect the number of new infections a single fly could initiate, but those effects do not interrupt the acquisition process. Previous studies failed to account for possible effects of
bacterial encasement in a polysaccharide matrix, having provided flies *E. amylovora* from
cultured bacteria rather than ooze. Our study is the most biologically relevant to date,
exemplifying that flies can acquire and transmit *E. amylovora* from this naturally occurring
source in an orchard environment.

This study supports two of the major tenants of the hypothesized mechanism originally
proposed by Stewart and Leonard (1916); 1) that flies acquire high enough titers of *E. amylovora*
from ooze to theoretically be infectious to numerous hosts, and 2) that flies can deposit *E.
amylovora* on a surface after acquisition from ooze. To this end, we dispute Stewart and
Leonard’s original discreditation of this hypothesis and support the proposal that interactions
with insects, primarily flies, drive the movement of this pathogen (Slack et al. 2017). In
furtherance of this proposal, multiple studies have demonstrated that *E. amylovora* can persist for
several days on or in a fly, and that persistence can be genetically mediated (Ark and Thomas
1936, Emmett and Baker 1971, Zhao et al. 2009, Ordax et al. 2015). The *E. amylovora* genome
contains three type three secretion systems (T3SS), two of which are not required for virulence in
plants and are hypothesized to be involved in *E. amylovora*-insect interactions (Zhao et al. 2009).
Type three secretion systems are needle like structures used by bacteria to deliver effector
proteins to host cells and are prevalent in both plant and animal bacterial pathogens (Galan and
Collmer 1999). The two non-phytopathogenic T3SS in *E. amylovora* are believed to have been
acquired via horizontal gene transfer and are closely related to T3SS found in animal pathogens
and endosymbionts (Zhao et al. 2009). We hypothesize that these T3SS play a role in extended
persistence of *E. amylovora* in flies, increasing their dissemination potential. A similar
phenomenon occurs in *Pantoea stewartii*, the bacterium that causes Stewart’s wilt of corn
(Correa et al. 2012). This close relative of *E. amylovora* is vectored by flea beetles and contains
two type three secretion systems, one of which is not involved in plant infection. When the non-
pathogenic T3SS was knocked out, the bacterium could not persist in the flea beetle gut (Correa
et al. 2012). If type three secretion systems in *E. amylovora* enhance persistence in flies, then
that would suggest a more intimate relationship between flies and this bacterium than previously
thought. Enhanced persistence of *E. amylovora* in flies magnified by high densities of flies at
given times of the year could make an outbreak particularly destructive and difficult to eradicate
in commercial orchards. Future studies should focus on the unknowns defined above to better
understand the threat that flies pose during a fire blight outbreak and the depth of interactions
between Dipterans, *E. amylovora*, and plant hosts.

**Acknowledgements**

We are grateful to the Cornell Statistical Consulting Unit for aiding in model fitting and
simplification as well as Mei-Wah Choi and the Jaenike lab for providing *E. amylovora* and *D.
melanogaster*, respectively. This research was supported by the NSF-GRFP (grant #DGE-
1650441), the Northeast SARE Graduate Student Grant (grant #GNE16-115-29994), New York
State Department of Agriculture and Markets Apple Research and Development Program (grant
#C200849), Federal Capacity Funds (grant #2016-17-199), the Grace Griswold Endowment, and
the Arthur Boller Research Fund.
REFERENCES


Burrill, A. C. 1915. Insect control important in checking fire blight. Phytopathology. 5: 343–347.


Compact Fruit Tree. 34: 16–19.


R Core Team. 2019. R: A Language and Environment for Statistical Computing.

Rodoni, B. C., P. R. Merriman, S. J. McKirdy, and G. Wittwer. 2006. Costs associated with


Figure 2.1: Marginal effect of exposure time on overall percentage of flies positive for *Erwinia amylovora* (black line with ± 95% CI) as predicted by a generalized mixed effect model with exposure as a fixed effect and date tested as a random effect with a binomial distribution. This line shows the predicted probability of infection as exposure time increases based on the model ($\chi^2 = 24.08, p < 0.001$). Blue dots represent raw overall percent of positive flies at each exposure time (3, 6, 12, and 24 hours).
Figure 2.2: *Erwinia amylovora* log$_{10}$ CFU/Insect values at 3, 6, 12, and 24 hours of exposure.

Red dot and error bars represent mean log$_{10}$ CFU/Insect ± 95% CI. The data are presented as discrete here to better visualize the variability at each time point, but were analyzed as a continuous variable. There was a negative effect of exposure time on abundance ($\chi^2 = 3.92, p = 0.048$). Black dots represent raw log transformed colony forming units (CFU) of individual *Drosophila melanogaster*.
**Figure 2.3:** *Erwinia amylovora* $\log_{10}$ CFU/Insect values for food deprived and satiated *Drosophila melanogaster*. Red dot and error bars represent mean $\log_{10}$ CFU/Insect ± 95% CI.

Food deprived flies had significantly higher bacterial abundances than satiated flies ($\chi^2 = 5.36, p = 0.02$). Black dots represent log transformed colony forming units (CFU) of individual *Drosophila melanogaster*. “*”: $p < 0.05$
Figure 2.4: *Erwinia amylovora* log_{10} CFU/Insect values for mated and unmated *Drosophila melanogaster*. Red dot and error bars represent mean log_{10} CFU/Insect ± 95% CI. Unmated flies had marginally higher bacterial abundances than mated flies ($\chi^2 = 3.68, p = 0.055$). Black dots represent log transformed colony forming units (CFU) of individual *Drosophila melanogaster*. “.”: $p = 0.055$
CHAPTER 3

INTERACTIONS BETWEEN *DELIA PLATURA* AND *ERWINIA AMYLOVORA*

ASSOCIATED WITH INSECT MEDIATED TRANSMISSION OF SHOOT BLIGHT

Matthew Boucher,¹* Rowan Collins,¹ Kayli Harling,¹ Gabrielle Brind’Amour,¹ Kerik Cox,² and Greg Loeb¹

¹Department of Entomology, Cornell AgriTech, New York State Agricultural Experiment Station, Cornell University, Geneva, New York, 14456 USA

³Section of Plant Pathology & Plant Microbe Biology, School of Integrated Plant Sciences, Cornell AgriTech, New York State Agricultural Experiment Station, Geneva, New York, 14456 USA

*Corresponding author: M. T. Boucher; Email: mtb245@cornell.edu

A version of this manuscript has been submitted to *Plant Disease*
Abstract

*Erwinia amylovora* is a bacterial pathogen of Rosaceous plants that can be devastating to commercial apple and pear production worldwide. Exopolysaccharide (EPS) production is essential for pathogenicity, aiding in biofilm production, and plant defense protection. EPS also plays an epidemiological role in the form of bacterial ooze, which is generated when *E. amylovora* builds to such high levels within the parenchyma that it ruptures the plant epidermis and a mixture of bacteria encased in EPS exudes from the injury. Insects such as *Delia platura* feed on ooze and become potential vectors in the process. The goal of this study was to investigate interactions between *D. platura* and *E. amylovora* to better understand how insects facilitate shoot blight. We demonstrate for the first time that *D. platura* can successfully initiate new infections in mechanically damaged apple shoots, and that EPS aids in adherence of *E. amylovora* to the insect surface. We show that flies can carry the bacteria externally for at least five days and that they shed a constant daily rate. We also show that *E. amylovora* strains differing in virulence do not behave differently when associated with *D. platura* and that consumption of *E. amylovora* has no effect on insect survival. Our data demonstrate that flies can be efficient facilitators of new shoot blight infections, but the field conditions that make this type of transmission possible require further investigation. We posit that the relationship between *E. amylovora* and *D. platura* is at least commensal and possibly mutualistic.

Introduction

Fire blight is a bacterial disease of rosaceous plants caused by *Erwinia amylovora* that leads to significant losses in apple and pear production worldwide (Van der Zwet et al. 2012). The bacterium can colonize any natural opening or injury on the plant surface and infects every host tissue type (Norelli et al. 2003), spreading systematically throughout the plant via the
intercellular spaces of the cortical parenchyma (Bogs et al. 1998, Momol et al. 1998, Malnoy et al. 2012, Slack et al. 2017). An important virulence factor required for colonization and systematic movement of \textit{E. amylovora} is exopolysaccharide (EPS) production (Denny 1995, Koczan et al. 2009). Exopolysaccharides are bacterially produced sugars that serve various functions during infection (Nimtz et al. 1996). \textit{E. amylovora} EPS has two known components, amylovoran (Goodman et al. 1974), which makes up the bulk of the EPS, and levan, which is a minor component that enhances virulence (Koczan et al. 2009). Amylovoran is key to \textit{E. amylovora} biofilm formation in apple xylem vessels and mutant \textit{E. amylovora} lacking the genes responsible for amylovoran production do not form biofilms (Koczan et al. 2009). Biofilms form across the xylem lumen and block nutrient movement and water uptake, which leads to wilting symptoms characteristic of fire blight (Sjulin and Beer 1978, Bogs et al. 1998, Koczan et al. 2009). Levan also plays a role in biofilm formation and is hypothesized to aid in bacterial localization to host tissues (Geier and Geider 1993, Koczan et al. 2009). Additionally, \textit{E. amylovora} EPS has been hypothesized to protect the bacterium from recognition by host plant defenses (Geier and Geider 1993, Denny 1995, Geider 2004). Multiple mechanisms have been proposed to explain this recognition phenomenon, including sequestration of toxic plant compounds and inhibition of contact between hypersensitive response-inducing harpins on the \textit{E. amylovora} surface and plant cells (Denny 1995).

In addition to their role in virulence, EPSs produced by \textit{E. amylovora} serve an important epidemiological function (Slack et al. 2017). The clogging of xylem vessels via bacterial and EPS aggregation leads to the rupture of xylem walls, allowing bacteria to colonize the parenchyma (Bogs et al. 1998). Continued \textit{E. amylovora} growth and EPS production in the parenchyma leads to the buildup of a bacterial mass encased in EPS that creates pressure on the
surrounding plant tissue (Slack et al. 2017). This pressure buildup ultimately causes a rupture of the plant epidermis through which bacteria encased in the EPS matrix exude (Slack et al. 2017). Epidemiologically, this ooze serves as primary and secondary inoculum from the early spring through mid-summer, at which time new shoots set terminal buds and green tissue lignifies (Slack et al. 2017). Ooze enhances the long term survival of bacteria encased in it because the bacteria are protected from environmental risks such as desiccation and UV radiation (Hildebrand 1939). High populations of *E. amylovora* in ooze provide ample opportunity for bacteria to be dispersed and allow for significant cell death without compromising the viability of the inoculum source (Slack et al. 2017).

Insects, especially various species of dipterans, have been observed walking and feeding on the sugary ooze, and the interaction between ooze and flies has been identified as a pathway for disease transmission (Stewart and Leonard 1915, 1916, Parker 1936, Slack et al. 2017, Boucher et al. 2019). Despite early skepticism over the materiality of this pathway (Stewart and Leonard 1916), recent research indicates that it may be an important driver of *E. amylovora* transmission (Ordax et al. 2015, Slack et al. 2017, Boucher et al. 2019). Transmission is hypothesized to occur when flies acquire the bacteria while feeding on bacterial ooze and mechanically transmit it to plant surfaces (Stewart and Leonard 1915, Slack et al. 2017, Boucher et al. 2019). The mechanical nature of this mechanism was the primary source of skepticism over its relevance because the types of flies observed feeding on ooze do not generally cause damage to the plants (Stewart and Leonard 1916). This led researchers to believe that *E. amylovora* cells had no viable infection court to colonize when transmitted via this pathway, resulting in a dead end (Stewart and Leonard 1916). However, Slack et al (2017) proposed that the high bacterial titers in ooze allowed insects to acquire enough *E. amylovora* to transmit bacteria for an
extended time before transmitting to a plant with conditions conducive to successful infection. Boucher et al (2019) confirmed that flies can acquire high titers of bacteria but did not evaluate how long insects could retain the bacteria or if the insects could initiate a successful infection in plants that had available infection courts.

While the EPS that constitutes ooze and its contribution to virulence is well characterized (Koczan et al. 2009), relatively little is known regarding the parameters that dictate the epidemiological success of the EPS-insect transmission pathway. The primary objective of this study was to evaluate diptera-\textit{E. amylovora} dynamics that contribute to successful shoot blight infections mediated by \textit{Delia platura}, a frequent insect visitor to bacterial ooze. We sought to define parameters under which flies act as effective disseminators of \textit{E. amylovora} by 1) Quantifying \textit{D. platura} transmission of \textit{E. amylovora} to mechanically damaged apple seedlings as influenced by source of bacteria; 2) identifying differences in \textit{D. platura} survival, bacterial load, and shedding rates by \textit{E. amylovora} strain; 3) describing the population dynamics of \textit{E. amylovora} on the internal and external surfaces of \textit{D. platura} over time based on source of \textit{E. amylovora} acquisition; and 4) evaluating whether acquisition and retention of \textit{E. amylovora} affects survival of \textit{D. platura} relative to unexposed flies.

\textit{Methods}\n\textit{Delia platura colony maintenance}. The \textit{Delia platura} colony was started in the summer of 2018 with wild flies captured in strawberry low tunnel plantings at Cornell AgriTech in Geneva, NY. Flies were captured daily when wild populations were abundant and kept in 24 x 24 x 24” cubic BugDorms (catalog no. 1452, BioQuip Products, Inc., Rancho Dominguez, CA) in a walk-in environmental growth chamber with a 16:8 D:N cycle at 24°C and 50% RH. Adult flies were maintained as described previously (Webb and Eckenrode 1978). Briefly, a 125 mL Erlenmeyer
flask was filled with water, sealed with parafilm, and then threaded with dental wick to provide a water source. A dry diet consisting of 10 parts skim milk powder, 10 parts table sugar, 1 part Brewer’s yeast, and 1 part soy peptone was provided in an open petri dish. An extra petri dish filled with Brewer’s yeast was provided for additional protein (Webb and Eckenrode 1978). Diet and water sources were replaced weekly.

An oviposition bin was created out of two rectangular, 1.9L plastic containers. The lower container was filled about 2.5cm high with water, and the upper container was punctured in the center and threaded with dental wick. The upper chamber was nested in the lower chamber, so the dental wick was in constant contact with the water and filled with about 1.2L of greenhouse sand. Fifty lima beans were pushed about an inch deep into the sand, and 4mL of meat and bone meal fertilizer (Keystone Mills, Romulus, NY) was layered on the surface. The water in the lower chamber kept sand moist, allowing beans to germinate, which stimulated *D. platura* oviposition. Moisture also prevents larval desiccation during development (Webb and Eckenrode 1978). Oviposition bins were transferred twice weekly to a development cage, where 5-10 additional beans and an additional layer of meat and bone meal was added. Larvae in oviposition bins were allowed to develop for two weeks, by which time most had pupated.

Pupae were initially separated from sand and plant debris by slowly adding warm water to the oviposition bin, and then pouring the contents of the bin through a two tiered soil sieve, with a 12.5mm mesh sieve placed above a 0.250mm, 60-mesh sieve to catch pupae. Pupae were then floated in a bin of warm water and skimmed from the water surface (or removed from the bottom of the bin with butterfly forceps if they sunk) to separate pupae from smaller plant debris to maintain cleanliness. Pupae were dried on a paper towel, weighed, and layered onto the surface of an emergence bin. Emergence bins consisted of a 47mL deli cup, punctured at the
center of the base and threaded with dental wick and filled with about 30mL of sand. This deli
cup was nested in a 95mL deli cup filled 2.5cm high with water so the dental wick was in
constant contact with the water. The nested cups kept sand moist and prevented the pupae from
desiccation. Emergence bins were kept in an emergence cage with water and dry diet as
described above. Newly emerged flies were counted and transferred from the emergence cage to
the main colony cages daily and would begin mating and laying eggs in oviposition bins at
around 10 days post emergence. Dead flies were removed from main colony cages daily and
cages were replaced monthly to ensure cleanliness.

**Erwinia amylovora strains.** Two strains were used in this study. Strain Ea273 (ATCC 49946) is
a well described strain isolated from apples in New York (*Malus domestica* cv Rhode Island
Greening) (Sebaihia et al. 2010). This strain was used in every experiment. Strain Ea266 is a
highly virulent strain relative to Ea273 and was isolated from apple (*Malus domestica* cv Rhode
Island Greening) in Ontario, Canada (Norelli et al. 1984). This strain was used in shedding,
survival, and internal load assays. Presumptive positive samples in all experiments were
validated by growing select colonies in LB broth overnight and quick pathogenicity assays
conducted by clipping the center of the youngest leaf on an apple sapling shoot with scissors
dipped in the culture and observing until symptoms emerged for a maximum of 10 days.

**Apple sapling maintenance.** Quarter inch diameter, Brookfield Gala scions (*Malus domestica*)
bench grafted onto EMLA 26 rootstock (Schlabach Nursery, Medina, NY) were potted into six
inch square pots with a 3:1 mixture of LM-3 All Purpose Mix (Lambert, Quebec, Canada) and
greenhouse sand. Plants were transferred to a greenhouse with 14:10 D:N cycle, with 22.22°C
daytime temperature and 18.33°C nighttime temperature. Plants were watered as needed, and any rootstock growth and bloom was removed by hand to promote shoot growth in the scion. We allowed three shoots to grow out of the scion, any additional shoots were removed by hand. Hypoaspis mites (Biobest Group, Westerlo, Belgium) were added to plants weekly to control for thrips. Plants were maintained in the greenhouse for three weeks before use in bioassays.

**Acquisition vial preparation.** Prior to use in experiments, flies were exposed to *E. amylovora* overnight (roughly 18 hours) in acquisition vials. Acquisition vials were made in two different ways as described below, one with *E. amylovora* available from bacterial ooze and the other with pure *E. amylovora* available from a filter disk.

Ooze vials were used to simulate fly feeding on bacterial ooze and were made by pipetting 6mL of Crosse-Goodman (CG) media into individual, sterile plastic vials roughly 23mm in diameter (Crosse and Goodman 1973). CG media is selective for *E. amylovora* and colonies present a distinct cratering morphology (Crosse and Goodman 1973). *Erwinia amylovora* strain Ea273 was grown in LB broth for 8 hours (approximating 1x10⁸ CFU/mL) at 28°C in a shake incubator and 50mL of the resulting mixture was pipetted onto the surface of the CG media in the plastic vials. Bacteria were spread on the media surface by gently rotating the vial in a circle and vials were covered with a paper towel and incubated in an environmental chamber (model I-30BL; Percival Scientific, Inc., Perry, IA) at 28°C for 48 hours. After 48 hours a film forms across the surface of the media, which is the same consistency and chemical make up as bacterial ooze observed in the field (Bellemann et al. 1994, Nimtz et al. 1996). 1-7 day old flies were starved for 5 hours and then 10-15 flies were added per vial. Vials were plugged with cotton and laid on their side in an environmental walk-in chamber with conditions matching
those for colony maintenance. After overnight exposure, all flies were released into a single BugDorm cage used for rearing described above to randomize the flies used in experiments. Filter disk vials were used to simulate epiphytic populations of *E. amylovora* that a fly may encounter (Miller 1972, McManus and Jones 1994), or as a standard testing method in survival assays (Chakrabarti et al. 2012). These vials were prepared by pipetting 6mL 5% (w/v) sugar agar into individual, sterile plastic vials roughly 23mm in diameter. Sugar agar was made in batches, boiling 1000mL autoclaved dH₂O before thoroughly mixing in 9g agar and 50g sugar. The mixture was simmered for 15 minutes and allowed to cool in a beaker before being distributed to vials (Siva-Jothy et al. 2018). Bacteria were grown for 8 hours in LB broth at 28°C in a shake incubator and 1mL bacteria were aliquoted into 1.5mL centrifuge tubes and spun in a microcentrifuge at 4°C for 15 minutes at 2,500G to pellet the bacteria. The supernatant was removed, and the pellet was resuspended in 1mL 5% sucrose solution. 100µl of bacteria suspended in sucrose was pipetted onto 23mm filter disks and allowed to dry. Filter disks were placed on the surface of the solidified sugar agar using sterile forceps and 1-7 day old flies were prepared as described above.

**Delia platura** mediated transmission of *E. amylovora* to apple saplings with and without ooze. Three-week-old Gala saplings were removed from the greenhouse, and two of the three shoots were removed from each sapling by hand. A sterilized, 5mm hole punch was used to make four wounds in each of the four youngest leaves of each sapling to simulate tissue damage and create wounds necessary for *E. amylovora* infection. Thirty-five inoculated flies were removed from the cage and released into a 2L paint strainer (W. W. Grainger, Inc., Lake Forest, IL), containing a moist piece of dental wick as a water source for the flies. The strainer was then
bagged onto the sapling shoot so it covered only the damaged leaves and sealed at the open end with a twist tie. The strainer bag was balanced so that the water wick was at the base to ensure that the weight of the bag did not bend and snap the young shoot. Controls were treated as described above, but instead of being bagged with flies, the youngest leaf of each control was clipped at its center with scissors dipped in an 8-hour *E. amylovora* culture for positive controls and sterile dH₂O for negative controls.

Saplings were then transferred to a mist chamber, arranged about 1 meter apart from each other to avoid cross-contamination, and held at 24°C with flies bagged to the plants for a 48-hour transmission period. During the transmission period, mist was turned on for 2 hours each day to maintain a relative humidity of roughly 80%. After the transmission period, strainer bags were removed from the plants and flies were discarded. Plants were incubated in the mist chamber for an additional 8 days, with the mist on for 3-4 hours per day to maintain 85-100% relative humidity. After 10 days, plants were removed from the chamber for disease assessments. Two measurements were taken; 1) disease incidence, a binomial measure of whether symptoms were present; and 2) severity, in which the length of the fire blight lesion was measured in proportion to the total length of the shoot.

We tested 50 total trees for both acquisition treatments (30 for *E. amylovora* ooze and 20 for *E. amylovora* culture on a filter disk), setting up a maximum of 25 trees per week to avoid overcrowding the mist chamber and to prevent cross contamination. For the ooze treatment, 10 replicates were tested with 5 positive controls and 5 negative controls, which was repeated for a second round after the disease assessment for the first round was completed. The same blocking pattern was replicated for the filter disk treatment, but we added 5 ooze replicates as an additional positive control in each round. Due to this blocking pattern, the ooze treatment is the
only treatment with replicates in all blocks. A generalized linear model with binomial
distribution analysis of disease incidence in ooze replicates by block indicated no blocking
effects for this treatment, so all ooze replicates were used in the final analysis. If any negative
controls exhibited positive symptoms in a given round, all replicates in that round were
composted and restarted. However, only one negative control tested positive in all experiments,
and this was likely due to transportation of negative control plants on the same tray as positive
control plants. All positive controls tested positive and were used to compare severity across
treatments. Separately, 30 flies from the ooze acquisition method and 20 flies from the filter disk
acquisition method were analyzed for the initial *E. amylovora* dose as described below. All flies
for initial dose of the ooze method were collected from the BugDorm used to set up the first
block of the ooze method transmission experiment. Flies for initial dose of the filter disk method
were collected in two groups, one from each BugDorm used to set up the two blocks of the filter
disk method transmission experiment. A Wilcoxon rank-sum test showed no differences between
the two blocks of filter disk flies, so data points were aggregated for comparison to ooze
acquisition method flies.

Differences in incidence between treatments were analyzed in R using the lme4 package
with a generalized linear mixed-effects model and a binomial distribution (Bates et al. 2015, R
Core Team 2019). Treatment (ooze or no ooze) was the only fixed effect and block was the only
random effect. The model was fit by maximum likelihood and direct comparisons between
treatments were made using the estimated marginal means with the emmeans package in R
(Lenth 2020). Differences in severity between treatments (including control) were analyzed
using a linear mixed-effects model with treatment as a fixed effect and block as a random effect.
The model was fit by restricted maximum likelihood, and pairwise comparisons within treatment
were made using the Tukey HSD method in the emmeans package (Lenth 2020). Differences in initial dose between acquisition methods (filter disk and ooze) were analyzed using a Wilcoxon rank-sum test for the \( \log_{10}(x + 1) \) transformed CFU values in R (R Core Team 2019). Data were visualized using the ggplot2 package in R (Wickham 2016).

**Shedding of *E. amylovora* over time by *D. platura***. One to 7-day old flies were exposed to either *E. amylovora* strain Ea273, Ea266, or a 5% Sucrose control using the filter disk acquisition vial method described above. All exposed flies were released into a single cage for each treatment, and ten flies for each treatment were captured in individual shedding vials. Shedding vials were made by pipetting 125 µl of sugar agar into the cap of a sterile 1.5mL microcentrifuge tube to provide a food source for the flies. Flies were kept in shedding vials for 24 hours, after which they were transferred to a new, sterile shedding vial every 24 hours for a total of 4 days. Flies were sexed after the 4-day testing period was completed. On the day the fly was transferred, 1mL of 1xPBS was added to the vial and mixed using a vortex for 20 seconds. Samples were serially diluted and 20 µl of the three most dilute for each sample were drop plated onto CG media plates (Crosse and Goodman 1973). Plates were sealed with Parafilm® and incubated upside down at 28°C for 48 hours. CFU/mL were then calculated by counting the dilution that yielded roughly 30 to 300 colonies for each sample (Siva-Jothy et al. 2018). The minimum detection threshold for this assay was roughly 500 CFU/mL, possible counts below this level were not detectable and considered as zeroes.

We tested 30 flies for each strain plus the sucrose control for a total of 90 flies. This experiment was conducted across 3 weeks, with 10 flies for each of the three treatments tested per week. Data were analyzed in R using a repeated measures hurdle model to account for zero
inflation using the glmmTMB package (Brooks et al. 2017). The count portion of the model was fit with a truncated negative binomial distribution and the zero inflated portion of the model was fit as a logistic regression. The model investigated the effects of strain, day, and sex on 1) the presence or absence of bacteria shed by flies (zero inflated portion) and 2) the total CFU/mL of bacteria shed by flies (count portion). Replicate nested within block was included as a random effect in all model iterations for both portions of the model. Model portions were initially constructed with all factors and interactions and simplified in a nested fashion. Nested models were compared using the Akaike Information Criterion (AIC) via the bbmle package (Bolker and R Development Core Team 2020), and the most parsimonious model (lowest AIC score) was chosen for final analysis (Grab et al. 2018). The count portion of the model ultimately included the fixed effects of all three factors, and the zero inflated portion of the model ultimately included only the day post acquisition as a fixed effect. Post hoc pairwise comparisons using the Tukey HSD method within and among treatments were made for both portions of the model using the estimated marginal means in the emmeans package (Lenth 2020). Data were visualized using the ggplot2 and emmeans packages in R (Wickham 2016, Lenth 2020).

**Internal and external bacterial load on/in *D. platura* over time.** One to 7-day old flies were subject to three *E. amylovora* treatments: 1) Ea273 via the filter disk method; 2) Ea273 via the ooze method; and 3) Ea266 via the filter disk method. A negative control where flies were exposed to 5% sucrose via the filter disk method was also included. 150 flies were exposed to each treatment as described above and released as groups into cages in an environmental chamber with conditions matching those used for colony maintenance. Cages contained dry diet and a water source as described above and flies were never exposed to *E. amylovora* after the
initial acquisition phase. Immediately upon release, 10 flies from each cage were captured in individual, sterile 1.5mL microcentrifuge tubes to determine the initial dose of bacteria acquired by the flies. For both Ea273 treatments, flies were subjected to external wash by adding 1mL 1xPBS to the tube and mixing the fly for 30 seconds using a vortex (Thomson et al. 2017). The fly was removed from the wash with sterile forceps and transferred to a sterile 1.5mL microcentrifuge tube containing 100µl 70% ethanol for 1 minute for surface sterilization. The external wash was diluted, plated, and counted as described above.

After insects were sterilized, the ethanol was removed from the tube with a pipette, and the sample was rinsed in 100µl 1xPBS for 1 minute. The PBS rinse was then removed with a pipette and spot checked to ensure proper surface sterilization by plating methods described above. Flies were homogenized in 200µl 1xPBS with autoclaved plastic pestles and samples were serially diluted, plated, and counted as described above. Ea266 samples were not evaluated for external load and were processed as described above starting with surface sterilization. Sucrose samples were immediately homogenized in 200µl of 1xPBS and treated as described as a negative control. All sucrose replicates tested negative.

The above procedure was repeated daily to measure depreciation of bacterial load over time. External load measurements for both Ea273 treatments were terminated after 5 days, and internal load measurements for Ea273 ooze treatments were also terminated after 5 days. Internal load measurements for Ea273 filter disk, Ea266 filter disk, and sucrose were terminated after 7 days. This procedure was repeated two additional times, resulting in 30 fly replicates per day post acquisition per treatment.

The effect of *E. amylovora* location (internal or external), acquisition method (ooze or filter disk acquisition), and day post acquisition (1-5) on CFU acquired by *D. platura* was
analyzed using a hurdle model to account for zero inflation in the glmmTMB package in R (Brooks et al. 2017). The count portion of the model was fit using a truncated negative binomial and the zero inflated portion was fit as a logistic regression. Model simplification and selection was conducted as described above and all iterations of both model portions included replicate nested within block (when the experiment was run) as a random effect. Both model portions included fixed effects of all three factors and two-way interaction terms for treatment by location, treatment by day, and location by day. Initial dose (counts for 0 DPA) were not included in this analysis because all samples were positive for flies subject to the ooze acquisition method, which led to a lack of variation necessary to compare probability of infection at 0 DPA between acquisition method treatments. Likewise, three-way interactions were excluded from the model selection process because all flies subject to the ooze acquisition method at 1 and 3 DPA were positive for *E. amylovora*, again leading to a lack of necessary variation to include the term. Post hoc pairwise comparisons were made for both portions of the model using the Tukey HSD method in the emmeans package (Lenth 2020).

The effect of *E. amylovora* strain (ea273 or ea266) and day post acquisition (0-7) on the internal load of *E. amylovora* in *D. platura* was analyzed using a hurdle model to account for zero inflation in the glmmTMB package in R (Brooks et al. 2017). The count portion of the model was fit using a truncated negative binomial and the zero inflated portion was fit as a logistic regression. Model simplification and selection was conducted as described above and block was included as a random effect in all model iterations. The final model included DPA as the only fixed effect in the zero inflated portion and the count portion included strain, DPA, and a strain by DPA interaction as fixed effects. Post hoc pairwise comparisons were made using the Tukey HSD method as described above.
Survival of *D. platura* after infection with *E. amylovora*. One to 7-day old flies were subject to three infection treatments via the filter disk method: 1) Ea273, 2) Ea266, and 3) Sucrose control. 120-130 flies were inoculated for each treatment as described above and were then released into individual cages according to treatment containing dry diet and a water source (Webb and Eckenrode 1978). Flies were released as groups into cages in an environmental chamber with conditions matching those used for colony maintenance. 10 flies were immediately removed from the cage to determine initial dose as described above and to ensure no contamination in the sucrose control. Each day for 29 days, cages were checked for dead flies, which were removed, counted, and discarded. Diet and water were replaced every 3 days to ensure flies did no reacquire bacteria. Cages were rotated to a new spot in the environmental chamber every day to control for possible location effects. After 29 days, the remaining live flies were counted, and the proportion of flies alive on each day was determined using the sum of the flies remaining at the end of the test period and the total number of dead flies over 29 days. This experiment was replicated 2 additional times and data were pooled and analyzed with the survival and survminer packages in R using a Cox proportional hazards model that included the effects of treatment and replicate on fly survival (Kassambara et al. 2019, Therneau 2020).

**Results**

**Transmission incidence and severity.** *Delia platura* that acquired *E. amylovora* via ooze successfully initiated infections in 95% of cases, while *D. platura* that acquired *E. amylovora* via filter disks successfully initiated infections in 30% of cases (Figure 3.1). Based on differences in estimated marginal means, flies that acquired *E. amylovora* via ooze were significantly more likely to successfully initiate an infection relative to flies that acquired *E. amylovora* via filter.
disks (Z-ratio = -3.448, \( P < 0.001 \)).

Mean \textit{E. amylovora} severity in apple saplings exposed to flies that acquired \textit{E. amylovora} from ooze was 0.77/1.00, compared to 0.25/1.00 in apple saplings exposed to flies that acquired \textit{E. amylovora} from filter disks (Figure 3.2). Control plants had a mean disease severity of 0.97/1.00. Fire blight severity was higher in controls relative to both the ooze treatment (t-ratio = 2.569, \( P = 0.033 \)) and the filter disk treatment (t-ratio = 6.977, \( P < 0.0001 \)) and disease severity was statistically higher in the ooze treatment relative to severity when bacteria were acquired via filter disks (t-ratio = -4.180, \( P < 0.0001 \)).

The initial dose of \textit{E. amylovora} acquired by flies from ooze ranged from 7.3-8.6 Log\textsubscript{10} CFU/mL with a median value of 8.2 Log\textsubscript{10} CFU/mL, while the initial dose of \textit{E. amylovora} acquired by flies from filter disks ranged 5.7-7.3 Log\textsubscript{10} CFU/mL with a median value of 6.7 Log\textsubscript{10} CFU/mL (\( W = 600, P < 0.0001 \), Figure 3.3).

**Shedding of \textit{E. amylovora} by \textit{D. platura}**. There was a main effect of day on the predicted probability that a fly will shed \textit{E. amylovora}, which declined gradually over time. Flies were equally likely to shed bacteria 1 and 2 days post acquisition (DPA) (t-ratio = 1.754, \( P = 0.299 \)), with a significant drop in predicted probability between 2 and 3 DPA (t-ratio = 2.723, \( P = 0.035 \)), and a non-significant drop in predicted probability between 3 and 4 DPA (t-ratio = 1.871, \( P = 0.244 \)) (Figure 3.4). Flies had a significantly lower probability of shedding bacteria 3 and 4 DPA compared to 1 DPA (t-ratio = 3.637, \( P = 0.002 \) and t-ratio = 4.294, \( P = 0.0002 \), respectively), and significantly lower probability of shedding 4 DPA compared to 2 DPA (t-ratio = 3.727, \( P = 0.001 \)).

Numerically, there was a slight decline in CFU counts of positive flies over time, but the
difference in CFU between 1 DPA and 4 DPA (the first and last days of testing) was not significant (t-ratio = 2.081, \( P = 0.164 \), Figure 3.5). There was no effect of *E. amylovora* strain on counts of bacteria shed by *D. platura* (t-ratio = 0.260, \( P = 0.795 \), Figure 3.5). There was no effect of sex on shed bacteria count (t-ratio = -1.566, \( P = 0.119 \)).

*E. amylovora* load dynamics on/in *D. platura*. There was a treatment by location interaction on the predicted probability of *E. amylovora* presence such that flies subject to the ooze acquisition method were more likely to be externally contaminated relative to flies subject to the filter disk acquisition method (t-ratio = -5.205, \( P < 0.0001 \)), but flies subject to either treatment were equally likely to be internally contaminated (t-ratio = 0.494, \( P = 0.961 \), Figure 3.6). Flies subject to the filter disk treatment were more likely to be contaminated internally relative to externally (t-ratio = -3.749, \( P = 0.001 \)), but flies subject to the ooze treatment were more likely to be contaminated externally relative to internally (t-ratio = 4.196, \( P < 0.001 \), Figure 3.7).

Flies had a higher probability of external contamination relative to internal contamination at 1, 2, and 3 DPA, but a higher probability of internal contamination relative to external contamination at 4 and 5 DPA. None of the differences between internal and external contamination probability on a given day were significant (for pairwise comparisons across all days: t-ratio = -2.050 – 2.737, \( P > 0.05 \)). There was a decline in probability of external contamination over time as evidenced by significant differences between the first day of testing and the 4th (t-ratio = 3.493, \( P = 0.018 \)) and 5th (t-ratio = 4.400, \( P < 0.001 \)) day of testing (Figure 3.8). Flies were equally likely to be externally contaminated from 1 to 3 DPA (for pairwise comparisons of 1-3 DPA: t-ratio = 0.565 – 2.149, \( P > 0.05 \)), from 2 to 4 DPA (for pairwise comparisons of 2-4 DPA: t-ratio = 0.565 – 2.287, \( P > 0.05 \)), and from 4 to 5 DPA (t-ratio =
Flies were equally likely to be internally contaminated across time as evidenced by no statistical differences between any day (for pairwise comparisons across all days: t-ratio = -0.477 – 1.912, P > 0.05).

When location of bacteria was controlled, flies had a higher probability of *E. amylovora* contamination when acquiring bacteria from ooze relative to filter disks at 1 DPA (t-ratio = -3.234, P = 0.042), but were equally likely to be contaminated regardless of acquisition method on all other days (for pairwise comparisons at 2-5 DPA: t-ratio = -2.908 – 0.289, P > 0.05). Flies were equally likely to contain *E. amylovora* across all days when acquiring bacteria from filter disks (for pairwise comparisons across all days: t-ratio = -0.678 – 2.026, P > 0.05) indicating a steady presence over time from 2-5 DPA when acquiring bacteria from ooze (for pairwise comparisons at 2-5 DPA: t-ratio = 0.531 – 2.724, P > 0.05). When acquiring bacteria from ooze, flies were more likely to carry bacteria at 1 DPA than at 4 DPA (t-ratio = 3.445, P = 0.022) and 5 DPA (t-ratio = 3.681, P = 0.01), but were equally likely to carry bacteria from 1-3 DPA (for pairwise comparisons from 1-3 DPA: t-ratio = 0.647 – 1.977, P > 0.05) indicating a decline in the presence of bacteria over time.

Within acquisition method treatment, internal bacterial counts were significantly higher than external bacterial counts in flies that acquired *E. amylovora* from filter disks (t-ratio = -18.880, P < 0.0001) and from ooze (t-ratio = -17.543, P < 0.0001, Figure 3.9). This pattern was consistent on all days (for pairwise comparisons across all days: t-ratio = -12.684 – -9.367, P < 0.0001). Internal *E. amylovora* counts between the two acquisition method treatments were significantly different such that flies that acquired bacteria from ooze had higher overall counts relative to flies that acquired *E. amylovora* from filter disks (t-ratio = -3.448, P = 0.003). External counts followed the same pattern (t-ratio = -5.320, P < 0.0001).
External bacterial loads declined over time with significant differences at 1 and 3 DPA, 1 and 4 DPA, 1 and 5 DPA, 2 and 4 DPA, 2 and 5 DPA, 3 and 4 DPA, and 3 and 5 DPA (for pairwise comparisons of noted days: t-ratio = 3.412 – 7.317, \( P < 0.05 \), Figure 3.10). There were no significant differences at 1 and 2 DPA, 2 and 3 DPA, and 4 and 5 DPA (for pairwise comparisons of noted days: t- ratio = 0.475 – 2.437, \( P > 0.05 \)). Internal bacterial loads declined over time such that 1-2 DPA were significantly different from 3-5 DPA (t-ratio = 4.127 – 7.028, \( P < 0.05 \)), where the days within each group do not differ (t-ratio = -0.280 – 1.589, \( P > 0.05 \), Figure 3.10).

Total bacterial loads varied between acquisition method treatments at 4 DPA (t-ratio = -4.470, \( P < 0.001 \)) and 5 DPA (t-ratio = -3.344, \( P < 0.05 \)), with flies that acquired \( E. \ amylovora \) from ooze carrying higher loads than flies that acquired bacteria from filter disks. Numerically, total loads in flies that acquired bacteria from ooze were higher than filter disk flies at 1-3 DPA, but the differences were not significant (t-ratio = -2.180 - -0.977, \( P > 0.05 \)). Total loads in flies that acquired \( E. \ amylovora \) from filter disks did not vary on consecutive days (t-ratio = 0.484 – 3.006, \( P > 0.05 \)), but there was a significant difference between total load on the first and last day of testing (t-ratio = 6.833, \( P < 0.0001 \)), indicating a decline in total load over time. The same pattern held for flies that acquired bacteria from ooze (for differences on consecutive days: t-ratio = -0.284 – 2.586, \( P > 0.05 \); for differences between first and last day of testing: t-ratio = 5.869, \( P < 0.0001 \)).

There was no effect of \( E. \ amylovora \) strain on the internal load of flies that acquired bacteria from filter disks within day (for comparisons between strains within day: t-ratio = -2.816 – 1.648, \( P > 0.05 \), Figure 3.11). Strain Ea273 loads were stable over three distinct periods such that 0-2 DPA were not different from each other, 3-5 DPA were not different from each other,
and 5-7 DPA were not different from each other (Figure 3.12). See Figure 3.12 for statistical differences within strain. Strain Ea266 internal loads were stable from 0-2 DPA before dropping at 3 DPA (Figure 3.12). The mean internal load at 3 DPA was significantly different from mean internal load at 1 DPA, but not significantly different from any other day. There was a numerical increase in internal load between 3 and 4 DPA, which was not significant, and internal load at 4 DPA was significantly different from 6 DPA but no other. At 5 DPA, internal load was significantly different from 0-2 DPA, but not statistically different from all other days. At 6 DPA, internal load was significantly different from 0-2 DPA and 4 DPA, but not significantly different from all other days. At 7 DPA, internal load was significantly different from 0-2 DPA, but not different from 3-6 DPA.

**Survival of *D. platura* after infection with *E. amylovora*.** Although flies fed either strain Ea266 or strain Ea273 tended to die at slightly higher rates than flies fed sucrose, this was not statistically significant (Ea266: $z = 0.975, P = 0.330$; Ea273: $z = 1.260, P = 0.208$, Figure 3.13). There was a significant difference between replicates such that survivability in block two was significantly higher than the reference block (block one) ($z = -12.437, P < 0.0001$). Survivability between the reference block and block three did not differ ($z = -1.758, P = 0.079$).

**Discussion**

The goal of this study was to detail basic parameters that drive the success of *Erwinia amylovora* transmission by *Delia platura*. To our knowledge, we are the first to establish that *D. platura* can transmit *E. amylovora* to mechanically damaged shoots and we support the theory that dipterans that are not considered pests of apple can cause losses in an orchard when the proper environmental circumstances align for fire blight outbreaks (Emmett and Baker 1971,
Ordax et al. 2015, Slack et al. 2017). We underscore the importance of *E. amylovora* EPS in this transmission mechanism by showing that flies that acquire the bacteria from non-ooze sources are less likely to successfully initiate infection in saplings. Ooze aids in adherence of high levels of *E. amylovora* to the external surface of the fly and provides a likely food source that leads to the establishment of *E. amylovora* on and inside *D. platura*. Flies can eliminate the acquired *E. amylovora* load, demonstrated by a lower probability of infection over time and gradually decreasing bacterial loads over time. However, we demonstrate that *D. platura* that have yet to clear their bacterial load shed at a relatively constant mean daily load for at least four days following acquisition, and based on these results we suggest that flies can initiate new infections for at least that long after exposure. Additionally, we show that internal bacterial loads are larger and more persistent than external bacterial loads over time. There was no effect of *E. amylovora* strain on bacterial shedding and load dynamics in flies, or on insect survival in flies fed different strains of *E. amylovora*. Flies fed *E. amylovora* did not differ in likelihood of survival relative to flies fed a sucrose control. Overall, our study demonstrates that the insect-microbe dynamics at play between *D. platura* and *E. amylovora* can make *D. platura* effective facilitators of new infections. Further, we suggest that the interaction between *E. amylovora* and *D. platura* is at least commensal and possibly mutualistic under certain environmental conditions, wherein the bacteria benefits by transmission to new hosts and the fly benefits by using bacterial ooze as a food source.

This study adds to the growing body of research that underscores the role of flies in insect mediated transmission of *E. amylovora* (Ordax et al. 2015, Slack et al. 2017, Boucher et al. 2019). While flies have been implicated in the disease cycle since the early phases of *E. amylovora* research, their role was discounted in favor of other groups of insects such as
pollinating bees or hemipterans such as *Lygus lineolaris* or *Empoasca fabae* (Stewart and Leonard 1916, Parker 1936). We show that a fly species with worldwide distribution, *D. platura* (Higley and Pedigo 1984), can initiate new infections and that these flies do not need to generate their own entry courts to do so because they can disseminate the bacteria into wounds already present on the plant. Under field conditions, wounds generally come in the form of hail or storm damage (van der Zwet et al. 2012), but they can also be caused by herbivorous insects (Gutbrodt et al. 2012). Future research should evaluate the relative risk of different damage types on fly mediated transmission, as different damage types can initiate different plant defense responses (Smith et al. 2009, Gutbrodt et al. 2012), which could affect the likelihood of *E. amylovora* establishment.

We conducted this study using a high density of flies to account for the mechanical nature of the proposed transmission mechanism. Interactions between flies and damaged plant tissue are relatively random in this scenario (Stewart and Leonard 1916, Slack et al. 2017), so we sought to increase the likelihood of a random encounter between an infected fly and an entry court by using high densities of flies. Moreover, *D. platura* exhibit frequent and widespread swarming behavior, so high densities of this species at one location is not unlikely (Miller and McClanahan 1960). Additional research should consider the effect of fly density on the likelihood of *E. amylovora* infection because *D. platura* produces 3-5 generations per growing season, leading to regular fluctuations in population density (Higley and Pedigo 1984).

In our study, filter disk acquisition was used as a method to simulate dispersed or epiphytic populations of *E. amylovora* that *D. platura* may encounter on an apple sapling or shoot. Rainstorms can dissolve ooze, dispersing the bacteria to surrounding leaves and shoots where *E. amylovora* can survive for variable periods of time depending on environmental
conditions (Miller 1972, McManus and Jones 1994). *Delia platura* adults feed on moisture droplets available to them on plant surfaces as well as flower nectar and aphid honeydew (Miller and McClanahan 1960). In our research, we have readily observed *D. platura* walking along newly diseased succulent apple shoot growth, probing the surface, and pausing to feed even when *E. amylovora* ooze is not visibly present. These observations led us to believe that it was possible for flies to acquire *E. amylovora* from non-ooze sources in the field. The disease severity of *E. amylovora* infections initiated by *D. platura* that acquired bacteria from filter disks was significantly lower than those initiated by flies that acquired bacteria from ooze. This result was likely driven by a lower overall incidence of infection initiated by flies fed *E. amylovora* via filter disks, as samples that did not develop symptoms naturally had a severity of zero. The few positive samples in the filter disk trial did result in high severity, but the sample size of positive saplings was not large enough to adequately compare to the ooze trial.

The lower incidence of infection initiated by flies fed via filter disks could have resulted from higher overall doses acquired from ooze relative to filter disks. Both internal and external bacterial loads in flies fed on ooze were higher across time, indicating that the trend observed in initial dose was consistent across time. It is possible that the relative inefficiency of the fly mediated transmission mechanism necessitates flies acquire loads surpassing a CFU threshold that acquisition from filter disks or epiphytes do not readily meet. While it takes relatively few *E. amylovora* cells to initiate an infection under controlled conditions (Crosse 1972), flies would need to shed bacteria at temporally and spatially appropriate moments to successfully initiate an infection. Thus, higher initial loads could increase the likelihood of shedding bacteria at the right time, though this is not always the case (Pace et al. 2017).

Another possible mechanism driving higher disease incidence could be that flies fed on
ooze were more likely to be externally contaminated and had higher external loads than flies fed on filter disks. In this scenario, bacteria adhered to the surface of the fly would be deposited onto the plant surface during grooming (Zhukovskaya et al. 2013), or be transferred at the point of contact from the insect surface during movement (Miller 1972, Rossmann et al. 2018), whereas internal bacteria would have to be defecated or regurgitated with ample time available to do so (Pava-Ripoll et al. 2012). This agrees with suggestions made in previous studies that *E. amylovora* on the insect surface is the key source of new infections generated by insects (Parker 1936, Miller 1972, Slack et al. 2017). While we cannot definitively state that externally located bacteria drive new infections based on our data, we suggest that this source is the most readily available and contributes to the greatest likelihood of successful transmission in damaged apple saplings in conjunction with high bacterial loads afforded to flies by ooze.

We show that the likelihood of external contamination decreases by roughly 20-60% over the five-day testing window and based on these data we suggest that flies are beginning to clear their external loads within the given time frame. In contrast, the probability that a fly will be infected internally did not change over the testing window. Internal bacterial loads depreciated over time, so it is likely that these loads were high enough that it took the average *D. platura* longer than five days to begin to clear. Taken together, these dynamics suggest that insect mediated transmission of *E. amylovora* will be most successful within five days of acquisition, with the highest likelihood of successful transmission coming within 48 hours of acquisition.

We demonstrate that flies shed a constant mean daily rate of *E. amylovora* despite a decreasing likelihood over time that they would shed live bacteria. The decreasing likelihood of shedding over time indicates that some flies were clearing the infection, resulting in higher zero counts later in the time sequence. A possible limitation of this study leading to the decreasing
likelihood of shedding is that the vials were conducive to growth of other microbes that individual flies could be carrying, which could have out competed \textit{E. amylovora} before the vials were tested and resulted in false negatives. Moreover, this assay was conducted in a confined space of 1.5mL, so while our data depict an estimated daily total CFU that a fly can shed, future research should evaluate the relative amount of \textit{E. amylovora} transferred from internal and external sources during defined behavioral periods. This type of study would better approximate how much bacteria \textit{D. platura} can shed at one moment, providing a more defined idea of how individual and groups of flies contribute to infection success.

We found no effect of \textit{E. amylovora} strain on various insect-microbe dynamics. Strains Ea266 and Ea273 had similar mean daily shedding rates and similar internal loads over time. Internal loads of both strains depreciated over time at similar rates. There was no effect of strain on survival, as flies fed either strain did not differ significantly from flies fed a sucrose control. Related bacteria in the \textit{Enterobacteriaceae} family occasionally exhibit differences in insect-microbe dynamics between strains (Pava-Ripoll et al. 2012), but this was not the case for the dynamics we studied. Our goal in comparing multiple strains was to gage the effect of strain virulence on \textit{D. platura-\textit{E. amylovora}} interactions. Strain Ea266 is highly virulent relative to Ea273 (Norelli et al. 1984), and since virulence is correlated with higher EPS production (Ayers et al. 1979), we hypothesized that virulent strains may have higher shedding rates and internal loads over time and lower survivability than less virulent strains. We considered the dynamics we tested as the key ones associated with insect mediated transmission, so based on our data, it is unlikely that virulence would alter the transmission capabilities of insects, though more strains should be studied to confirm the trend we observed.

This study reveals no evidence that \textit{E. amylovora} is pathogenic to \textit{D. platura}. Some plant
pathogens in *Enterobacteriaceae* are known to be pathogenic to alternative insect hosts (Nadarasah and Stavrinides 2011). For example, *Dickeya dadantii* causes soft rot in various cropping systems and resembles *E. amylovora* in that it does not have a specific insect vector but can be transmitted by insects that interact with diseased plants (Grenier et al. 2006). This bacterium exhibits pathogenicity in the pea aphid, *Acyrthosiphon pisum*, but insect pathogenicity is not thought to play a role in epidemiology of *D. dadantii* as it relates to plants (Grenier et al. 2006). Based on our data, we suggest that the strains evaluated in this study are not pathogenic to *D. platura*. However, we cannot rule out active use of *D. platura* by *E. amylovora* as an alternative host due to the presence of non-phytopathogenic type III secretion systems in the *E. amylovora* genome (Zhao et al. 2009). Type III secretion systems (T3SS) generally deliver effector proteins to host cells that cause pathogenicity (Preston 2007), but can also mediate interactions between insects and bacterial endosymbionts (Dale et al. 2001). *E. amylovora* contains three known T3SS, only one of which is required for pathogenicity in plants (Zhao et al. 2009). The remaining T3SS are hypothesized to be involved in interactions between *E. amylovora* and insect hosts (Zhao et al. 2009, Boucher et al. 2019). We suggest here that if the non-phytopathogenic T3SS play a role in *E. amylovora*-*D. platura* interactions, then that role is not pathogenic and likely involves enhancing bacterial survival in the insect gut, as it does in the *Pantoea stewartii*-*Chaetocnema pulicaria* pathosystem in maize (Correa et al. 2012, Boucher et al. 2019).

Overall, this study is a key step in advancing our understanding of the ecology of insect mediated transmission of *E. amylovora* as it pertains to shoot blight. The importance of ooze in secondary spread of the pathogen by insects cannot be understated. Ooze contributes to the establishment of high bacterial loads on *D. platura*, which we believe enhances transmission
efficiency. When ooze is not present in the orchard, it may still be possible for insects to acquire the pathogen, though it is likely that acquisition and transmission efficiency are greatly reduced. We also show that this pathogen can survive for extended periods in *D. platura*, possibly broadening the window during which a fly could initiate infection, though further study is necessary to better understand the transmission dynamics of internalized *E. amylovora* and the molecular interactions at play between *E. amylovora* and *D. platura*. Additional future studies should focus on transmission by *D. platura* under field conditions. Our study serves as a proof of concept that cements the role of orchard dwelling Diptera in the transmission of *E. amylovora*, but environmental conditions will drive the success of insect mediated transmission across temporal and landscape scales.

**Acknowledgements**

We thank Françoise Vermeylen of the Cornell Statistical Consulting Unit for guidance and instruction throughout data analysis. This research was funded by NSF-GRFP grant #DGE-1650441; Northeast SARE Graduate Student Grant #GNE16-115-29994; New York State Department of Agriculture and Markets Apple Research Development Program grant #C200849; Federal Capacity Funds #2016-17-199, the Grace Griswold Endowment, and the Arthur Boller Research Fund.
REFERENCES


Bolker, B., and R Development Core Team. 2020. bbmle: Tools for general maximum likelihood estimation.


Correa, V. R., D. R. Majerczak, E. D. Ammar, M. Merighi, R. C. Pratt, S. A. Hogenhout,


R Core Team. 2019. R: A Language and Environment for Statistical Computing.


Therneau, T. M. 2020. A package for survival analysis in R.


Figure 3.1: Percentage of Gala apple saplings positive for *E. amylovora* when exposed to pathogen by 35 *D. platura* that acquired bacteria through ooze or filter disks (no ooze). The two acquisition methods were meant to simulate possible sources of *E. amylovora* acquisition in the field, with ooze being the most readily available and filter disks simulating epiphytic or dispersed sources of *E. amylovora*. ***, P < 0.0001.**
Figure 3.2: Mean ± 95% CI disease severity in Gala apple saplings. Black points represent individual severity scores for each replicate, and darker shading of those points indicates a greater accumulation of scores at that point. Letters above each factor level indicate significant differences from each other as determined by Tukey HSD adjusted pairwise comparisons.
Figure 3.3: Median + IQR of initial dose in \( \log_{10} \) CFU/mL between flies that acquired *E. amylovora* from ooze and from filter disks. Black points represent the \( \log_{10} \) CFU/mL of tested individuals and darker shading indicates accumulation of individuals at that point. Medians were compared using a Wilcoxon rank sum test, which showed that flies that acquired *E. amylovora* from ooze had significantly higher initial doses than flies that acquired *E. amylovora* from filter disks (\( W = 600, P < 0.0001 \)). ***, \( P < 0.001 \).
Figure 3.4: Predicated probability ± 95% CI that a fly will shed *E. amylovora* on a given day.

Predicted probability gradually decreases over time, suggesting that flies are clearing acquired bacterial loads below detection thresholds as DPA increases. DPA is a strong predictor of bacterial shedding by flies, letters above predicted probability values indicate which differences between probabilities are significant, with the same letter indicating non-significance.
Figure 3.5: Mean ± 95% CI of *E. amylovora* shed by *D. platura* in Log$_{10}$ CFU/mL separated by *E. amylovora* strain. Black points represent individual CFU counts from replicates. Flies that tested negative (resulting in a CFU count of zero) are not shown. There was no effect of strain on count and no effect of day on count as evidenced by an insignificant change in count between 1- and 4-days post acquisition.
Figure 3.6: Predicted probability ± 95% CI that a fly will be contaminated with *E. amylovora* externally or internally based on acquisition method. Data compares the likelihood of a fly being contaminated when acquiring bacteria from filter disks or from ooze within location. ***, $P < 0.0001$. NS, not significant.
Figure 3.7: Predicted probability ± 95% CI that a fly subject to one of two acquisition methods will be contaminated with *E. amylovora* externally or internally. Data compares the likelihood of a fly being contaminated externally or internally within acquisition method. **, $P \leq 0.001$. 
Figure 3.8: Predicted probability ± 95% CI that a fly will be internally or externally contaminated with *E. amylovora* by day post acquisition (DPA). The likelihood of external contamination decreased over time while the likelihood of internal contamination remained constant over time. Letters above each point indicate significant differences within location as determined by Tukey HSD adjusted pairwise comparisons.
Figure 3.9: Mean ± 95% CI of *E. amylovora* load on internal and external surfaces of *D. platura* in Log$_{10}$ CFU/mL separated by acquisition method. Shaded points show Log$_{10}$ CFU/mL counts of individual replicates, colored by location on *D. platura*. Data compares internal and external load within acquisition method. Internal loads were higher than external loads in both acquisition methods, and this pattern was consistent across all days as determined by Tukey HSD adjusted pairwise comparisons.
Figure 3.10: Estimated marginal mean ± 95% CI of total *E. amylovora* colony forming units on external or internal surface of *D. platura* over time. Letters indicated significance in bacterial load by day, depicting a gradual decline in both internal and external load over time. Internal loads were higher than external loads on all days as determined by Tukey HSD adjusted pairwise comparisons.
Figure 3.11: Mean ± 95% CI of internal *E. amylovora* load in *D. platura* in Log$_{10}$ CFU/mL separated by strain. All flies acquired *E. amylovora* through the filter disk method and only internal loads were measured. Differences in internal loads between strains were not significantly different within day. Bacterial loads dropped gradually over time. Shaded points show Log$_{10}$ CFU/mL counts of individual replicates, colored by strain fed to *D. platura*. 
Figure 3.12: Estimated marginal mean ± 95% CI showing change in internal load of *E. amylovora* in *D. platura* over time separated by bacterial strain. Data show pattern change in bacterial load over time within strain. Letters above each time point indicate significant differences between loads from other days within strain as determined by Tukey HSD adjusted pairwise comparisons.
Figure 3.13: Probability of survival over time of *D. platura* fed one of two *E. amylovora* strains or a sucrose control as determined by Cox proportional hazards model. While survival for flies fed *E. amylovora* regardless of strain was slightly lower than flies fed sucrose, the differences between either strain and the control were not significant (Ea266: $z = 0.975$, $P = 0.330$; Ea273: $z = 1.260$, $P = 0.208$).
CHAPTER 4

THE EFFECT OF ERWINIA AMYLOVORA INFECTION IN APPLE SAPLINGS AND FRUIT ON THE BEHAVIOR OF DELIA PLATURA

Matthew Boucher,1* Rowan Collins,1 Stephen Hesler,1 Kerik Cox,2 and Greg Loeb1

1Department of Entomology, Cornell AgriTech, New York State Agricultural Experiment Station, Cornell University, Geneva, New York, 14456 USA

3Section of Plant Pathology & Plant Microbe Biology, School of Integrated Plant Sciences, Cornell AgriTech, New York State Agricultural Experiment Station, Geneva, New York, 14456 USA

*Corresponding author: M. T. Boucher; Email: mtb245@cornell.edu
Department of Entomology, Cornell AgriTech, College of Agriculture and Life Sciences, Cornell University, 15 Castle Creek Drive, Geneva, NY 14456

A version of this manuscript has been submitted to Environmental Entomology
**Abstract**
Phytopathogen mediated behavior change in insect vectors is well documented in systems with high vector specificity, but whether similar behavioral changes occur in systems with many vectors is not well studied. *Erwinia amylovora* bacterium is a pathogen of pome fruit such as apple and pear and produces a bacterial ooze that attracts many insect vectors that mechanically transmit the pathogen after feeding. The goal of this study was to evaluate the effect of *E. amylovora* on the behavior of *Delia platura*, a fly with a worldwide endemic presence known to transmit *E. amylovora*. We show that *D. platura* prefer diseased, oozing fruit to mock inoculated fruit in choice tests, and that preference subsides when bacterial ooze is removed from the infected fruit. When given a choice between a diseased sapling and a healthy sapling at various time points after inoculation, flies did not exhibit a preference. We show that the volatiles of infected fruit are not attractive to *D. platura*, indicating the diseased fruit odor is not responsible for the observed preference for infected fruit. Flies did not differentiate between sapling odors until infected trees had died, at which point they preferred healthy tree odors. Collectively this work shows that *E. amylovora* can have minor effects on the behavior of *D. platura*, but it is not yet understood whether these behavioral changes impact transmission. This study highlights the challenges in understanding plant-insect-microbe interactions in a low vector specificity pathosystem.

**Introduction**
Insect-vectored bacterial plant pathogens exhibit a variety of mechanisms to ensure acquisition and transmission by insects (Nadarasah and Stavrinides 2011, Eigenbrode et al. 2018). These mechanisms are complex, acting directly on the insect and indirectly via the host plant, and in combinations ranging from molecular interactions to behavioral manipulation of the vector (Eigenbrode et al. 2018). The complexity of vector-phytopathogen interactions is
exemplified by the relationship between the causal agent of Stewart’s bacterial wilt in maize, *Pantoea stewartii*, and its flea beetle vector, *Chaetocnema pulicaria* (Melsh) (Correa et al. 2012). The bacterium contains a specialized type III secretion system (T3SS) that enhances the persistence of *P. stewartii* in the gut of the flea beetle (Correa et al. 2012). T3SS are needle-like structures that intercellular pathogens use to translocate pathogenicity causing effector proteins into host cells (Troisfontaines and Cornelis 2005). T3SS are common in Proteobacteria, with seven distinct phylogenetic groups (Troisfontaines and Cornelis 2005). Of these groups, six are known to facilitate infection in animals while the seventh facilitates infection in plants (Troisfontaines and Cornelis 2005). The *P. stewartii* genome codes for two T3SS, one of which is in the plant infection group while the other is in an animal infection group (Correa et al. 2012). When *C. pulicaria* was challenged with *P. stewartii* mutants lacking the animal grouped T3SS, persistence of the bacteria in the gut of the insect was significantly reduced, underscoring the coevolutionary nature of the relationship between the phytopathogen and the vector (Correa et al. 2012).

Additional vector-phytopathogen complexities related to vector behavior are evident in the *Erwinia tracheiphila* pathosystem. *E. tracheiphila* causes bacterial wilt in cucurbit crops and is primarily vectored by the cucumber beetle *Acalymma vittatum* (Luperini) (Yao et al. 1996). The beetles feed on cucurbit leaves and mate in the flowers, vectoring the pathogen by defecating infective frass into flowers while mating (Shapiro et al. 2012). *E. tracheiphila* infection reduced production of volatiles from flowers and enhanced production of volatiles from wilting leaves, and beetles exhibited a preference for wilting leaves and healthy flowers (Shapiro et al. 2012). These bacterially induced changes 1) enhanced the likelihood of a beetle feeding on infected leaves relative to healthy leaves; and 2) reduced the likelihood of beetles mating in
flowers of diseased plants relative to flowers of healthy plants (Shapiro et al. 2012). These
indirect interactions exhibit how plant phenotype manipulation by the pathogen increases the
likelihood of successful transmission to new hosts by the vector (Shapiro et al. 2012).

Studies investigating the relationship between insects and plant pathogens have mainly
been conducted in systems with high vector specificity, meaning that these pathogens only have
one or two known insect vectors (Purcell 1982). However, many phytopathogens exhibit medium
or low vector specificity and further research describing the relationship between vectors and
microbes under these circumstances is required (Eigenbrode et al. 2018, Rossmann et al. 2018,
Sitz et al. 2019). *Erwinia amylovora* is a devastating pathogen of pomaceous crops such as apple
and pear and exhibits low vector specificity (van der Zwet et al. 2012, Rossmann et al. 2018). *E.
amylovora* can infect any tissue in the tree, including blossoms, fruit, and succulent shoots
through natural openings or damage associated with severe storms, management practices, and
insect feeding (Norelli et al. 2003). The bacterium invades the intercellular spaces of the plant
and produces an exopolysaccharide (EPS), which creates a sugary matrix that encases bacteria
aiding in the formation of biofilms and subverting host plant defenses (Koczan et al. 2009).
Eventually, *E. amylovora* builds to high enough populations within plants tissues that the
pressure exerted on the epidermis causes a rupture, and *E. amylovora* encased in EPS ooze out
of the wound (Slack et al. 2017). This bacterial ooze serves as inoculum for new infections (van
der Zwet et al. 2012), and insects, primarily Diptera, have been known to feed on ooze for over
100 years (Stewart and Leonard 1916).

Transmission of *E. amylovora* by flies is considered mechanical, wherein flies feed on
ooze and acquire the bacteria then deliver it to plant surfaces via shedding or defecation, after
which *E. amylovora* can persist until it has the opportunity to invade a wound or natural opening
Various families of Diptera have been studied in relation to the transmission of *E. amylovora* (Stewart and Leonard 1916, Gossard and Walton 1922, Parker 1936, Emmett and Baker 1971, Ordax et al. 2015, Boucher et al. 2019), giving the pathogen a cosmopolitan suite of potential vectors. Despite low vector specificity, previous researchers suggested that the relationship between insects and *E. amylovora* has coevolutionary ties (Koczan et al. 2009, Zhao et al. 2009, Slack et al. 2017). The discovery of two T3SS in the *E. amylovora* genome that are phylogenetically grouped with the T3SS of animal pathogens and not required for pathogenicity in plants led to suggestions that those T3SS mediate interactions between *E. amylovora* and insects (Zhao et al. 2009). The high titers of *E. amylovora* in bacterial ooze were hypothesized to have developed in response to the inefficiency of mechanical transmission by insects during the pre-agriculture evolution of the pathogen (Slack et al. 2017). One study demonstrated that flies strongly preferred oozing bark chips to molasses and hypothesized that the fermenting odors of the oozing cankers were attractive to flies (Thomas and Ark 1934).

The goal of this study was to use adult *Delia platura* (Meigen) to investigate whether *E. amylovora* infection in apple saplings and in apple fruit causes changes in insect behavior that could enhance acquisition and transmission of the bacterium. This anthomyiid is distributed worldwide and the larvae are considered to be a pest of germinating seeds (Miller and McClanahan 1960, Higley and Pedigo 1984). Adult flies feed on water droplets, honeydew, and flower nectar and seek out wooded habitats such as apple orchards when temperatures exceed 29°C (Miller and McClanahan 1960, Higley and Pedigo 1984). Moreover, adult *D. platura* were recently observed feeding on *E. amylovora* ooze (Boucher et al. 2019), and have been shown to transmit the pathogen to healthy apple saplings from this source. Broadly, we sought to
understand whether plant pathogens with low vector specificity facilitate insect-microbe-plant interactions following similar patterns exhibited by plant pathogens with high vector specificity. Specifically, we sought to: 1) evaluate *D. platura* preference for diseased or healthy fruit; 2) determine if bacterial ooze induces preference for diseased fruit; 3) establish whether *D. platura* exhibit preference for diseased apple saplings at various time points post inoculation; and 4) observe whether *D. platura* exhibit preference for the odor produced by diseased fruit and saplings.

**Methods**

*Delia platura* colony maintenance. This colony was started in summer 2018 with wild *D. platura* captured in low tunnel strawberry plantings at a single Cornell AgriTech research farm in Geneva, NY. Flies were collected on days when *D. platura* were likely to be active (between 15.6°C and 29.4°C) between May and September (Miller and McClanahan 1960). Adult flies were confined to 24 x 24 x 24” cubic BugDorms (catalog no. 1452, BioQuip Products, Inc., Rancho Dominguez, CA) in a walk-in environmental growth chamber with a 16:8 D:N cycle at 24°C and 50% RH and maintained on diet as previously described (Webb and Eckenrode 1978). The diet included two separate sources, a petri dish filled with brewer’s yeast powder for protein, and a dry mixture of brewer’s yeast (1 part by weight), soy peptone (1 part by weight), skim milk powder (10 parts by weight), and table sugar (10 parts by weight) (Webb and Eckenrode 1978). A water source was provided by threading a parafilm sealed 125 mL Erlenmeyer flask with dental wick. Water and diet sources were replaced weekly or as needed. Colony cages were switched out once a month and washed thoroughly to avoid disease outbreaks and poor growing conditions. Dead flies were removed from cages daily.

Adults were provided oviposition boxes in a 1.9 L plastic container filled with 1.2 L of greenhouse sand modified from Webb and Eckenrode (1978). A total of 50 lima beans were
pushed into the sand and a 4 mL layer of meat and bone meal fertilizer (Keystone Mills, Romulus, NY) was dispersed across the top of the sand. This container was threaded with dental wick through a hole in the center of its bottom and nested into an identically sized container containing roughly 300 mL tap water to moisten the sand and to encourage germination of the beans, which stimulates oviposition in *D. platura* (Weston and Miller 1989). Oviposition boxes were replaced twice weekly, and old boxes were moved to a development cage, where 5-10 more beans were added along with another layer of meat and bone meal. Most larvae pupated after 14 days (Webb and Eckenrode 1978).

Two weeks after oviposition boxes had been removed from colony cages, pupae were separated from sand and plant debris by running warm water (~49°C) over the surface of the bin and pouring through a two tiered soil sieve, with a 12.5mm mesh sieve to catch plant debris placed above a 0.250mm, 60-mesh sieve to catch pupae. This was done continuously until boxes were empty. Pupae were floated in warm water (~49°C) and skimmed from the surface, and butterfly forceps were used to remove extra debris stuck to the pupae. Some pupae sank to the bottom, and those pupae were collected using butterfly forceps. Pupae were washed an additional time in warm water (~49°C) and set to dry on a paper towel, after which the dry weight was measured for tracking colony production. Pupae were then spread across the surface of moist sand in a 47 mL deli cup to avoid desiccation and placed in a separate 24 x 24 x 24” cubic BugDorm with food and water sources as described above. Newly emerged flies were transitioned into colony cages daily or weekly depending on which age cohorts were being used for experiments.

**Fruit preference.** *Delia platura* fruit preferences were tested in a 2-choice bioassay using an
arena design based on *Drosophila suzukii* fruit preference assays (Cha et al. 2019). The arena was constructed from 2 L cylindrical, lidded plastic buckets roughly 15 cm in diameter with opaque walls (Figure 4.1). Two 3x5 cm rectangular windows were cut into the sides directly across from each other and covered with 1 mm mesh fabric (Outdoor Wilderness Fabrics, Caldwell, ID) to allow for air flow in the bucket. An 8 mm in diameter section was cut out of the center of the lid and the resulting hole was covered with 1 mm mesh fabric. During experiments, a folded half piece of paper towel was placed over the lid to avoid stimulating flies with light or objects outside of the arena. Two 5 mm diameter holes were made using a hole punch about 1.5 cm from the top lip of the bucket. Holes were placed directly across from each other and a 5 mm wooden dowel was secured through the holes, running across the middle of the bucket. This dowel was used to suspend two fruit “swings” that each contained a single fruit for the flies to choose between. Fruit “swings” were constructed by bending two standard size paper clips into an “S” shape, and puncturing them through the lid of a 44 mL plastic shot glass opposite each other so that the bottom loop of the “S” of each paper clip held the lid. Paper clips were secured to the lids with glue, and the swing was hung on the wooden dowel from the top loop of the paper clip. Fruit swings were kept 5 cm apart for all replicates. Fruit were suspended within the arena in near each other because infected and healthy fruit can be found within fruit clusters in orchards. With this design, flies had the opportunity to walk or fly to the fruit after being released into the arena.

Flies were released into arenas through a 5 mm hole drilled into the base of the bucket at its center. The cap of a 15 mL conical tube with a 5 mm hole drilled through its center was glued to the external surface of the bucket so the hole drilled through the middle aligned with the hole at the center of the bucket base. The cap’s threading was facing outward so a 15 mL conical tube
containing flies could be screwed onto the cap. Flies would then crawl or fly out of the conical tube into the arena, ensuring introduction into the arena was not biased towards one side and so flies were not significantly jostled. All experiments were conducted in an environmental chamber with conditions matching those used in colony maintenance.

Immature apples (cv. Gala) used in this assay were prepared as previously described (Boucher et al. 2019). Fruit were collected from fire blight free blocks at a single Cornell AgriTech research farm in Geneva, NY in the spring of 2019 when they reached roughly 2-3 cm in diameter and held in cold storage (4°C) until inoculation with *E. amylovora*. Single colonies of *Erwinia amylovora* strain Ea273 (originally isolated from *Malus × domestica* cv. Rhode Island Greening in New York) maintained on Crosse-Goodman (CG) medium were grown overnight in LB broth (catalog no. 12795-027; Invitrogen) at 28°C in a shake incubator (Crosse and Goodman 1973, Norelli et al. 1984). Fruit were surface sterilized by wiping fruit surface thoroughly with 95% ethanol, and then soaking in a 10% bleach solution for 10 minutes. After soaking, fruit were removed and allowed to air dry under a laminar flow hood. Once dry, fruit were cut in half, and any that appeared to contain rot at the center were discarded. The exposed flesh of the fruit was scored 6 to 8 times using a sterile dental pick, and 100 µl of *E. amylovora* grown in LB broth was pipetted onto the flesh surface. The sterile dental pick was then used to collect *E. amylovora* colonies maintained on CG media, which were rubbed across the fruit flesh surface, dispersing the LB broth in the process. The fruit was allowed to absorb the liquid flesh side up in the laminar flow hood for 5 minutes, before being placed flesh side down on a piece of filter paper that had been soaked in sterile, distilled water in a 180 mL deli cup. The cup was capped with a plastic lid, sealed with parafilm, and incubated at 28°C in a growth chamber with high humidity (>90%) for 7 – 14 days, until ooze droplets were abundant on the skin of the fruit.
Mock inoculations were done according to the same process, but sterile LB broth was used and extra colonies from CG media were not added. For fruit used in ooze removed experiments, ooze was washed from the fruit by rinsing with sterile, distilled water, wiping the surface dry, and then repeating 4 times to ensure all ooze was removed from the surface rather than dispersed across the surface. We could not control the severity of infection on the immature fruit halves, meaning that some infections at 8 days could have reached the desired severity that other fruit would need 10 days to reach. However, all fruit used on a given day were inoculated on the same date (e.g. on a given testing day, all fruit used were 10 days old), and we selected fruit to roughly match in severity, discarding fruit that was over or under our standard. In general, we chose fruit that had roughly 20 – 50 opaque ooze droplets on the skin, with 2 – 3 patches of yellow brown to brown skin. Any fruit that was dark black, shriveling, or growing mold was discarded. Any fruit that did not produce significant amounts of ooze was discarded. Prior to use in preference assays, the flesh side of the fruit was covered with parafilm, as flies in pre-trials that located the flesh of the fruit often roosted permanently on the flesh, which could have confounded the results.

On the morning of each experiment day, 6 (3 males, 3 females), 5-7 day old D. platura per replicate were removed from the colony and transitioned into a cubic BugDorm containing only a water source in the environmental chamber that experiments would be conducted in so flies could acclimate to the chamber for 4 – 6 hours. At 2pm, fruitlet halves were placed on the fruit swings and arenas were sealed. Six flies (3 males, 3 females) were captured into a sterile 15 mL conical tube, and the tube was screwed onto the cap that had been glued to the bottom of the arena and left in place until the end of the experiment. Over the next two hours, every two minutes, the number of flies on each fruit was recorded. The time period and observation intervals were chosen based on previous experiments finding high rates of activity during this
time (Hough-Goldstein and Bassler 1988). At the end of two hours, flies and fruit were
discarded, and all parts of the arena were rinsed with 95% ethanol and washed with warm soapy
water. Total counts of flies on each fruit in the arena were tabulated.

We conducted three independent preference assays with 20 replicates in each assay: 1) mock inoculated fruit vs oozing fruit; 2) mock inoculated fruit vs inoculated fruit with ooze
removed; and 3) oozing fruit vs inoculated fruit with ooze removed. We also conducted controls
for each fruit type (fruit with same conditions on both sides) and another with no fruit to test for
any “side” biases in the arenas. We ran 10 control replicates for each, totaling 40 control
replicates, and there were no side biases in any of the controls. The fixed effect of treatment (the
two fruit types used in each assay) on count of flies landing on each fruit in the arena was
analyzed in R with a generalized linear mixed effects model and a negative binomial distribution.
Replicate nested within block was included as a random effect and the analysis was conducted
using the glmmTMB package (Brooks et al. 2017, R Core Team 2019). Comparisons between
fruit treatments were made using estimated marginal means in the emmeans package (Lenth
2020). Data were visualized in R using the ggplot2 package (Wickham 2016).

**Shoot preference.** *Delia platura* preference for diseased or healthy apple saplings were tested in
groups of 100, 5-7-day old flies in 93 cm x 47.5 cm x 47.5 cm BugDorm cages (catalog no.
4S4590, MegaView Science Co., Ltd., Taiwan) using the 47.5 cm square as the base. The arena
design was based on similar preference bioassays conducted on various Diptera (Hough-
2019). All experiments were conducted in an environmental chamber matching the conditions
used for colony maintenance, and flies were transitioned from the colony chamber to the
experiment chamber in a cage containing diet and a water source 12-15 hours prior to testing so flies could acclimate to the chamber. In preparation for experiments, apple saplings (cv. Brookfield Gala (*Malus domestica*) bench grafted onto EMLA 26 rootstock) 6.35 mm in diameter (Schlabach Nursery, Medina, NY) were potted into 15.24 cm square pots with a 3:1 mixture of LM-3 All Purpose Mix (Lambert, Quebec, Canada) and greenhouse sand and maintained in a greenhouse for 3 weeks with a 14:10 D:N cycle and 22:18°C D:N temperature cycle. Plants were watered every other day or as needed, and all rootstock growth and bloom was removed by hand. Hypoaspis mites (Biobest Group, Westerlo, Belgium) were used as a biocontrol for thrips in the greenhouse. After three weeks of growth, saplings were inoculated with *E. amylovora* or mock treated. *E. amylovora* inoculum was prepared in LB broth as described above. Two of the three shoots growing on the sapling were removed, and the youngest leaf on the remaining shoot was bisected with the scissors what was dipped into the inoculum. Mock inoculated saplings were treated the same way, but sterile LB broth was used for the scissor dip. Saplings were then transferred to a mist chamber and arranged on the floor of the chamber about 1 meter apart to avoid cross contamination. The chamber was set to 24°C, and plants were kept in the chamber for 2, 5, or 10 days depending on the assay being conducted. The mist in the chamber was turned on for 3 – 4 hours per day or as needed to maintain a relative humidity between 85% and 100%.

Two potted apple saplings were placed 13 cm apart, 2 cm from the sides of cage and 5 cm from the back of the cage. Two liter paint strainer bags were used to sheath the pots (W. W. Grainger, Inc., Lake Forest, IL) to keep flies away from the soil such that only ~10 cm of scion and the entire shoot were available to the flies. Two webcams (model no. C615, Logitech, Lausanne, Switzerland) were attached to ring stands with rubber bands so that each camera was
facing a single sapling, and adjusted for height on the ring stand so that the entire shoot was
visible. The front edge of the ring stand base was 10 cm in front of the sapling, such that the back
dge of the ring stand was flush with the front edge of the base of the cage. The webcam cables
were run through the upper arm sleeve and connected to a laptop computer running ImageJ
image processing software (NIH, Rockville, MD) with the Webcam Capture plugin installed.
Using this plugin, webcams were programmed to take a photo every minute for 8 hours from
8:30am – 4:30pm for a total of 480 frames per sapling (960 total frames per rep). Flies were
collected into a single vial and released into the cage by placing the vial at the exact center of the
cage base and removing the cap immediately prior to starting the camera feed. A large piece of
black cloth was placed over the top of the arena such that it hung 30 cm on all sides to avoid
stimulating flies with light and chambers were closed off for the duration of the experiment to
avoid disturbing or interrupting fly behavior. The location of diseased and healthy plants was
switched every other testing day to ensure no side biases were present.

The number of flies on each sapling shoot was counted for every frame captured. Only
flies that were visible were counted, and if we could not tell if a fly was on a shoot or not, it was
not counted. If part of the fly was visible (e.g. if the fly was on the back-facing side of the shoot),
that fly was counted as present on the shoot. We conducted three independent assays to evaluate
if preferences change as disease symptoms become more severe: 2 days post mock inoculation
(DPMI) vs 2 days post inoculation (DPI); 2) 5 DPMI vs 5 DPI; and 3) 10 DPMI vs 10 DPI.
Plants were never reused (e.g. plants used in 2 DPI trials were discarded, not used for 5 DPI
trials) and 10 replicates of each assay were conducted. We spot checked for side biases in the
assay by running 5 control replicates using healthy, 3-week-old saplings and no side biases were
found. The number of flies in each frame for a given sapling was summed and data were
analyzed and visualized as described for fruit preference assays.

**Odor preference.** Individual *D. platura* were tested for preference between diseased and healthy shoots or diseased and healthy fruit using a glass Y-tube olfactometer (Cha et al. 2011, Wong et al. 2018). The olfactometer was 3 cm in diameter, with a 30 cm long base and 5 cm long arms. 3.175 mm inert plastic tubing (W. W. Grainger, Inc., Lake Forest, IL) was secured to an airline tap with a hose clamp and connected to an activated charcoal filter to purify the air. The filter output was connected via tubing to a round bottom flask containing dH2O to humidify the air, which was then passed through a Y shaped splitter to divide the flow in two. Each arm of the splitter was connected via tubing to a flow regulator (catalog no. 97004-640, VWR International, Radnor, PA) and air was pushed into each odor source chamber at 1 L min⁻¹. Odor sources were connected via tubing to specialized glass inserts that slid into the arms of the Y-tube and sealed the flow, so all air was pushed down to the base of the Y (Figure 4.2). This apparatus was set up in an environmental chamber matching the conditions using for colony maintenance. A black canvas was erected around the Y-tube to avoid stimulating flies with human movements.

Two independent assays were conducted to compare fruit odor preferences: 1) mock inoculated fruit vs inoculated fruit; and 2) fresh fruit vs inoculated fruit. Mock and infected fruit were prepared as described above, and fresh fruit was prepared by halving room temperature, immature fruit that had been removed from cold storage on the morning of experimentation. Fruit odor sources were contained in 75 mL glass jars, with a plastic lid fitted with input and output sources. Tubing was run from the flow regulator to the jar, then separately from the jar to the Y-tube. Air was run through the system for at least 1 hour prior to experimentation to ensure no odors associated with handling the fruit were present when flies were tested.
Three independent assays matching those conducted for shoot preference were conducted to evaluate shoot odor preference. Shoots were prepared as described above and contained in large (406 mm x 444 mm oven bags (Reynolds, Inc., Richmond, VA) that had been punctured on both sides with a #5 cork borer (Raguso and Willis 2005). Each hole in the bag was fitted with a plastic fitting so tubing could be attached, with one acting as the input, receiving air after it passed through the flow regulator, and the other acting as the output, pushing air into the Y-tube. Only shoots and a wax covered section of the scion were contained in the bag, which was sealed tightly at its open end with twist ties. Bags were held upright by attaching binder clips to the top of the bag and the running wire that was secured to a wall through the clips, which prevented bending and breaking of shoots that could have led to the emission of damage associated volatiles. Once the bags were in place, the bag inflated naturally and plants were left with air running for 1 hour to flush the chamber of volatiles associated with handling (Cha et al. 2008).

On each testing day, 25, 5-7 day old *D. platura* were isolated at 8am in individual plastic vials that had the bottom replaced with mesh fabric so air could flow out of the Y-tube when vials were attached. Vials were then plugged with cotton and laid on their side in the testing chamber. Replicates were tested from 2 – 4pm to match previous experiments (Hough-Goldstein and Bassler 1988). Flies were sexed, the cotton plug was removed, and the vial was inserted into the base of the Y-tube. Flies were timed for 5 minutes, after which they were removed. Flies that had not made a choice were recorded. Replicates were terminated before 5 minutes if it was determined that the fly had made a choice. We counted choices when flies exhibited settling behavior. In this assay, that behavior was defined as having entered an arm and remained in that arm for greater than 30 seconds. If a fly remained for 30 seconds, it generally stopped walking and roosted for extended time. The leads pushing air into the Y-tube were switched to the
opposite arm after every replicate was tested to avoid side biases, and all glassware that a fly could contact were cleaned after every 5th replicate. Glassware was cleaned by first soaking for 10 minutes in warm water (49°C) mixed with powdered enzymatic detergent (catalog no. 04-358-23, Fisher Scientific, Waltham, MA), then rinsed with 95% EtOH using a squirt bottle and rinsed again with acetone before being set to dry completely. The same odor sources were used for every replicate tested on a given day and then discarded.

We conducted 50 replicates for each of the five assays, with a replicate defined as a fly that successfully made a choice. This usually required running 60-70 total flies to reach 50 successes. We also conducted a 50-replicate control in which only humidified air was pushed through the apparatus to demonstrate that flies would be active in the given experimental conditions. Overall preferences for odor sources tested were analyzed in R using a chi-square goodness of fit test with the null hypothesis that flies would choose each treatment in a 1:1 ratio (R Core Team 2019). Sex based preferences for odor sources were analyzed the same way. Data were visualized in R as described above.

Results

Fruit preference. D. platura exhibited a preference for diseased fruit when given a choice between infected fruit and mock inoculated, healthy fruit (Figure 4.3A: t-ratio = 2.077, P = 0.045). Flies given a choice between infected fruit and infected fruit with no ooze exhibited no preference (Figure 4.3B: t-ratio = 0.875, P = 0.388). No preference between infected fruit with ooze removed and mock inoculated fruit was found (Figure 4.3C: t-ratio = 0.069, P = 0.946).

Shoot preference. D. platura did not exhibit preference between infected and mock infected saplings that had been allowed 2 days to develop symptoms (Figure 4.4A: t-ratio = -0.032, P = 0.975), or after 5 days of symptom development (Figure 4.4B: t-ratio = -0.129, P = 0.899), or at
Odor preference. 75% of *D. platura* responded to odor stimuli when presented with a choice between an infected sapling and a mock infected sapling after 2 days of symptom development (DPI). There was no difference between choice of sapling (Figure 4.5A: $\chi^2 = 1.28, P = 0.337$) and no effect of sex on sapling choice ($\chi^2 = 0.335, P = 0.774$). 71% of *D. platura* responded to odor stimuli when presented with a choice between an infected sapling and a mock infected sapling after 5 days of symptom development. There was no difference between choice of sapling (Figure 4.5B: $\chi^2 = 0.08, P = 0.883$) and no effect of sex on sapling choice ($\chi^2 = 2.826, P = 0.156$). 86% of *D. platura* responded to odor stimuli when presented with a choice between an infected sapling and a mock infected sapling after 10 days of symptom development (DPI). Significantly more flies chose the mock inoculated sapling than the infected one (Figure 4.5C: $\chi^2 = 9.680, P = 0.003$) and there was no effect of sex on sapling choice ($\chi^2 = 0.397, P = 0.764$).

When presented with a choice between an infected immature apple fruit and a mock inoculated immature apple fruit, 83% of *D. platura* responded to odor stimuli. Significantly more flies chose the mock inoculated fruit (Figure 4.6A: $\chi^2 = 13.52, P = 0.002$) and there was no effect of sex on fruitlet choice ($\chi^2 = 0.967, P = 0.500$). When presented with a choice between an infected immature apple fruit and a fresh immature apple fruit, 85% of *D. platura* responded to odor stimuli. There was no difference between fruit choice (Figure 4.6B: $\chi^2 = 0, P = 1$) and there was no effect of sex on fruit choice ($\chi^2 = 0.739, P = 0.551$).

**Discussion**

We show that *D. platura* prefer immature apple fruit infected with *E. amylovora* when given a choice between an infected fruit and a mock infected fruit and that this preference subsides when ooze droplets are removed from the fruit surface. When given a choice between
infected immature fruit and infected immature fruit with ooze droplets removed, *D. platura* again exhibit no preference for either choice. When given a choice between infected fruit odor and mock infected fruit odor, *D. platura* strongly preferred odors of mock inoculated fruit. However, this preference subsided when flies were given a choice between infected fruit odor and fresh fruit odor. *D. platura* exhibited no preference between infected and mock infected apple saplings at 2-, 5-, and 10- days post inoculation with *E. amylovora*, and no preference between infected and mock infected sapling odors at 2- and 5-days post inoculation with *E. amylovora*. Flies did exhibit a preference for odor of mock infected apple saplings at 10 DPI. Based on these results, we suggest that infection with *E. amylovora* has the capacity to alter insect behavior and that the mechanism of that behavior change is not likely to be odor driven.

We did not expect to find that *D. platura* preferred the odor of mock inoculated fruit over the odor of infected fruit, especially given previous research that suggested that fermenting odor of *E. amylovora* infected tissue is attractive to flies (Thomas and Ark 1934). These data could indicate that the odor produced by *E. amylovora* infected fruit is deterrent, or that the odor produced by mock inoculated fruit is attractive. Given that 1) the preference for non-infected fruit odor subsided when flies were given a choice between fresh fruit odor and *E. amylovora* infected fruit odor, and that 2) flies preferred diseased fruit when given access to the fruit, we do not believe that infected fruit odors are deterrent to *D. platura*. One possible explanation for the observed preference for mock inoculated fruit volatiles could be production of attractive volatiles by the microbial community present on the fruit (Farré-Armengol et al. 2016). Microbes and their by-products are capable of producing volatile blends that elicit behavioral responses from insects (Becher et al. 2012), and *D. platura* are responsive to microbe associated cues (Hough-Goldstein and Bassler 1988, Weston and Miller 1989). In our study, mock treated fruit were
maintained in warm, humid conditions matching those of infected fruit for the same amount of
time, providing ample opportunity for colonization of the fruit by airborne microbes (Teixidó et
al. 1999). In contrast, fresh fruit were kept in cold storage until the day of testing, which slows
microbial proliferation on the fruit surface (Shen et al. 2018). Although cold storage does not
eliminate microbial growth, it could reduce the strength of the volatile signal emitted by
maintaining lower microbial populations on the fruit (Mercier and Wilson 1994).

* D. platura* prefer diseased, oozing fruit over mock inoculated fruit, which indicates that
* E. amylovora* can cause behavioral changes in some vectors that could enhance acquisition. We
hypothesized that the accumulation of flies on diseased fruit relative to mock inoculated fruit
occurred because of bacterial ooze, which is the primary medium through which insects interact
with and acquire *E. amylovora* in the field (van der Zwet et al. 2012). Flies feed on ooze for
extended lengths of time and likely acts as a type of arrestant, keeping flies on the fruit if they
discover an ooze droplet. To demonstrate the arresting qualities of ooze, we compared *D. platura*
preference for infected fruit with ooze removed relative to mock inoculated fruit and diseased,
oozing fruit. We predicted that: 1) flies would prefer oozing, infected fruit over infected fruit
without ooze; and that 2) there would be no difference in preference when given a choice
between infected fruit with ooze removed and mock infected fruit. We found no preference in
both assays, potentially confounding our understanding of the role of ooze in *D. platura* choice.
However, we cannot rule out that ooze and the sugars that constitute it were completely removed
from the fruit despite our best efforts to do so. The remnants of bacterial ooze on the fruit could
have diluted possible preference for an oozing fruit relative to a diseased fruit with ooze
removed, as flies could have spent time feeding on the remnants. Biologically, this scenario may
approximate what occurs in the field after a rainstorm and suggests that the overall behavioral
effect of *E. amylovora* ooze on *D. platura* is relatively weak and is easily interrupted.

Saplings infected with *E. amylovora* appear to have no behavioral effect on *D. platura* under the conditions we tested. Infected shoots on saplings turn brownish black over time and wilt, developing a characteristic shepherd’s crook appearance (Koczan et al. 2009). Other *Delia* spp. use plant shape and color to identify suitable hosts, but *D. platura* is hypothesized to rely more strongly on odor cues than vision and is able to identify a wide range of potential hosts (Gouinguené and Städler 2006). Our data align with this theory of *D. platura* host finding, as the changes in sapling shoot appearance caused by *E. amylovora* infection did not alter the behavior of *D. platura*. Importantly, apple saplings are not considered ovipositional hosts for *D. platura* adults, serving only as a potential refuge from high temperatures and as a food source location (Miller and McClanahan 1960, Higley and Pedigo 1984). Thus, the cues generally employed by *D. platura* during host finding, and more broadly those cues that are taken advantage of in pathosystems with higher vector specificity may not apply to a lower vector specificity system such as this one (Mann et al. 2012, Shapiro et al. 2012, Eigenbrode et al. 2018).

Infection with *E. amylovora* has been shown to alter the volatiles released by apple saplings (Cellini et al. 2018), and our data suggest that these volatile changes do not have a behavioral effect on *D. platura*. We believe that *D. platura* preference for mock infected plant odors after 10 days of symptom development is primarily due to the state of the diseased plant. At this stage, the diseased plant has no green tissue and the shoot is dry, shriveled, and blackened. We predict here that those plants are no longer producing sufficient volatiles for the insect to register in comparison to a healthy plant. While the exhibited preference may be a by-product of plant death, the fact that the fly prefers mock infected saplings benefits *E. amylovora*, as the fly is more likely to interact with healthy shoots and to transmit the bacteria to a new host.
if the fly is infectious. Overall, the odor of diseased saplings does not play a role in location of
diseased tissue in this specific interaction, but we cannot rule out the use of these odors by other
potential vectors. Future research should focus on this aspect of insect mediated transmission of
*E. amylovora*.

This study underscores challenges associated with understanding interactions between
insects and a phytopathogen with low vector specificity (Rossmann et al. 2018). The phenotypic
changes caused by *E. amylovora* may be used differently by the various insects that can transmit
the pathogen, as the degree of behavior change exhibited by the insect could relate to the life
history of the insect in question (Mauck et al. 2012, Biere and Bennett 2013, Biere and Tack
2013). Moreover, low vector specificity could itself be a mechanism ensuring acquisition and
transmission by insects. In this scenario, it may be more beneficial for the pathogen to rely on the
plant-insect-microbe interactions of the broader community to bring insects into contact with the
pathogen rather than actively recruiting insects to diseased locations (Sitz et al. 2019). For
example, *D. platura* are known to forage on hemipteran honeydew (Miller and McClanahan
1960), and could do so on a shoot that was simultaneously infested with *E. amylovora* ooze and
hemipterans (Stewart and Leonard 1916). The indirect interaction between *D. platura* and
another insect in the community could thus bring the fly into close contact with *E. amylovora*.
Furthermore, *E. amylovora* is readily dispersed by wind and rain (Thomson 1986), and the
presence of additional transmission mechanisms may reduce selection for traits that promote
insect behavior changes in high vector specificity systems.

Collectively, this study demonstrates that changes in plants associated with *E. amylovora*
infection have minor behavioral effects on a mechanical vector but does not show whether these
behavioral effects enhance transmission or how the broader ecological community affects
transmission. Community level effects have been hypothesized in related bacterial pathosystems (Sitz et al. 2019), and behavioral manipulation is an important component of many high specificity systems but may be muted or nonexistent in low specificity systems (Mauck et al. 2012). Understanding of the factors and mechanisms underlying transmission for low vector specificity systems are understudied relative to high-vector specificity systems even though they represent economically devastating crop diseases. Further research is necessary to better understand the extent to which complex behavioral and community interactions contribute to the success of low vector specificity pathogens.

Acknowledgements

We would like to acknowledge the efforts of Françoise Vermeylen at the Cornell Statistical Consulting Unit for helpful comments and guidance regarding statistics. This research was funded by NSF-GRFP #DGE-1650441; Northeast SARE Graduate Student Grant #GNE16-115-29994; New York State Department of Agriculture and Markets Apple Research Development Program #C200849; Federal Capacity Funds #2016-17-199, Grace Griswold Endowment, Arthur Boller Research Fund
REFERENCES

Becher, P. G., G. Flick, E. Rozpedowska, A. Schmidt, A. Hagman, S. Lebreton, M. C.

Larsson, B. S. Hansson, J. Piškur, P. Witzgall, and M. Bengtsson. 2012. Yeast, not fruit
volatiles mediate *Drosophila melanogaster* attraction, oviposition and development. Funct.

Biere, A., and A. E. Bennett. 2013. Three-way interactions between plants, microbes and

plants, microbes and arthropods. Funct. Ecol. 27: 646–660.

state on acquisition and accumulation of *Erwinia amylovora* by *Drosophila melanogaster*.

Brooks, M. E., K. Kristensen, K. J. van Benthem, A. Magnusson, C. W. Berg, A. Nielsen, H.
J. Skaug, M. Maechler, and B. M. Bolker. 2017. glmmTMB balances speed and
flexibility among packages for zero-inflated generalized linear mixed modeling. R J. 9:
378–400.

Cellini, A., G. Buriani, L. Rocchi, E. Rondelli, S. Savioli, M. T. Rodriguez Estrada, S. M.
compounds emitted during the pathogenic interactions between apple plants and *Erwinia

evidence for contextual olfactory-mediated avoidance of the ubiquitous phytopathogen


Gouinguené, S. P., and E. Städler. 2006. Oviposition in *Delia platura* (Diptera,


Nadarasah, G., and J. Stavrinides. 2011. Insects as alternative hosts for phytopathogenic
bacteria. FEMS Microbiol. Rev. 35: 555-575


R Core Team. 2019. R: A Language and Environment for Statistical Computing.


Shapiro, L., C. M. De Moraes, A. G. Stephenson, and M. C. Mescher. 2012. Pathogen effects on vegetative and floral odours mediate vector attraction and host exposure in a complex


Figure 4.1: Diagram of fruit preference arena is not to scale. Infected and mock infected immature fruit were suspended from the top of the arena using fruit “swings” and flies were introduced into the arena through a hole in the center of the arena base. The goal of this design was to mimic the spatial patterns a fly was likely to encounter under field conditions.
Figure 4.2: Diagram of Y-tube apparatus is not to scale. Air (top left) is passed through a charcoal filter to purify the air, then humidified and split to two flow regulators that push air at equal rates across both odor choices, which are subsequently pushed into the short arms of the Y. The odors then push to the base of the Y and the fly walks upwind and chooses an arm.
Figure 4.3: Mean ± 95% CI of total *D. platura* counts on apple fruit when given a choice between A) a fruit infected with *E. amylovora* and a mock inoculated fruit; B) a fruit infected with *E. amylovora* with ooze removed and a mock inoculated fruit; and C) a fruit infected with *E. amylovora* and a fruit infected with *E. amylovora* with ooze removed. Black points show total counts of flies on fruit for individual replicates. There were significantly more flies counted on infected fruit when given a choice between infected fruit and mock inoculated fruit (A: $P = 0.045$), but not when given a choice between infected fruit with ooze removed and mock inoculated fruit (B: $P = 0.388$), or when given a choice between infected fruit and infected fruit with ooze removed (C: $P = 0.946$). *, $P < 0.05$. 
Figure 4.4: Mean ± 95% CI of total *D. platura* counts on apple saplings when given a choice between a sapling infected with *E. amylovora* and a sapling that was mock inoculated at three time points post inoculation (DPI). Black points show total count of flies on saplings for individual replicates. There was no statistical difference in fly choice between infected and mock infected saplings after 2 days of symptom development (A: \( P = 0.975 \)). There was no statistical difference between infected and mock infected saplings after 5 days of symptom development (B: \( P = 0.899 \)). There was no statistical difference between infected and mock infected saplings after 10 days of symptom development (C: \( P = 0.410 \)).
Figure 4.5: Frequency of *D. platura* that chose each odor source when presented with a sapling infected with *E. amylovora* and a mock inoculated sapling of the same day post inoculation (DPI). There was no preference for either sapling at 2 DPI (A: *P* = 0.337) and 5 DPI (B: *P* = 0.883), but *D. platura* preferred mock inoculated plants at 10 DPI (C: *P* = 0.003). **, *P* < 0.01.
Figure 4.6: Frequency of *D. platura* that chose each odor source when presented with an immature fruit infected with *E. amylovora* and A) a mock inoculated immature fruit of the same day post inoculation, or B) a fresh immature fruit. *D. platura* preferred mock inoculated fruit over infected fruit (A: \( P = 0.002 \)) but exhibited no preference when offered a fresh fruit and an infected fruit (B: \( P = 1.000 \)). **, \( P < 0.01 \).