# CHEMICALLY-MEDIATED HOST PLANT LOCATION BY THE GRAPE BERRY MOTH, *PARALOBESIA VITEANA*

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Michael Scott Wolfin

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# CHEMICALLY MEDIATED HOST PLANT LOCATION BY THE GRAPE BERRY MOTH, *PARALOBESIA VITEANA*

Michael Scott Wolfin, Ph. D.

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This dissertation explores the chemically-mediated mechanisms for host plant discrimination in specialist phytophagous insects using the grape berry moth (GBM; *Paralobesia viteana*)-grape, *Vitis* spp. complex as a model. The GBM–grape complex represents an excellent system to explore the chemically-mediated mechanisms of host plant location because the GBM is an ovipositional specialist, meaning gravid females discriminate between host and non-host plants. Additionally, synthetic blends of host plant volatiles have already been shown to attract GBM females in the flight tunnel (Cha et al. 2008). Therefore the GBM-grape complex was used to test the existing theories regarding host plant location. I used flight tunnel assays to observe GBM responses to host and non-host odor sources (cut shoots, extracts, and synthetic blends), and isolate and identify the volatiles that elicit the observed behavior. All antennally active compounds found in grape shoots were also present in non-host plants. Moths displayed higher levels of upwind flight than expected to non-host sources, suggesting discrimination is not occurring at a distance. I used additional flight tunnel assays, to investigate cues necessary to elicit landing on an odor source (water vapor and visual cues). Individual and paired stimuli did not elicit landing, and landing only occurred when plant volatiles, a visual cue, and water vapor were all present, suggesting the cues have a synergistic effect. Interestingly, moths flew upwind a low percentage of the time in response to water vapor alone suggesting the

moths can use nonspecific cues to locate a host. In a final study, I explored whether microorganisms living on the surface of plant shoots produced the behaviorally active compounds. Volatile collections of surface sterilized plant shoots indicated the surface microorganisms did not significantly contribute to the volatile profile of the grape shoots as all of the peaks in the volatile profile of sanitized shoots were found in the profile of control shoots. In flight tunnel assays, moths responded similarly to sanitized shoots as they did to control shoots suggesting surface microorganisms did not play a significant role in the production of previously identified blend of behaviorally active volatiles.

BIOGRAPHICAL SKETCH

Michael Scott Wolfin was born on July 22nd, 1989 to his two loving parents, Madelyn and Steven Wolfin. Seemingly born to be parents, Maddie and Steve did everything in their power to raise a happy son. Together they sang songs (like namanama ningo), watched Disney movies (like the Jungle Book) and played games (like the great mouse detective). As much fun as he had with his parents, just a few years later, Maddie and Steve did the best thing they could have done for Michael when they gave him a little brother, James Ithan on April 15th, 1993. Michael was very proud and excited to be a big brother, and still remembers the feeling when his kindergarten teacher told him that the day had finally come. Michael and James became best friends from the first second they saw each other, and remain best friends to this day. Maddie and Steve created a strong, loving, tight-knit family that parents can only dream to have. And lucky for all of them, some dreams come true.

That is no surprise however, because of how fantastic Michael's grandparents were. Just as Michael believes his parents were born to be parents, he also believes his grandparents were born to be grandparents. One of his earliest memories is a conversation he had with his father's mother, Grammy Annette, about what he wanted to be when he grew up. Luckily, at a very young age, Michael had already narrowed it down to two careers: He either wanted to be a truck driver or an inventor. At the time, he was leaning toward being a truck driver because truck driver's got paid to see

the world (or so he thought). However, Grammy told him that being an inventor would probably be more fun. She called him "The Thinker", because when she first saw him in the hospital his hand was on his head in a pensive pose, and she was *certain* he was thinking about meeting his grandparents (and who would dare suggest otherwise?). Grammy Annette was very proud of all of her grandchildren, and would routinely tell them that "her buttons were popping [with pride]". Another early memory is when his father's father, Poppy, taught him how to carry important things like glasses. Sadly, Poppy passed away too early.

Michael was also close with his grandparents on his mother's side. It would be incomplete to discuss Grammy Marsha and Poopah individually, as they were inseparable. Together they loved family and bridge. Poopah (sometimes referred to as Mr. Backwards), was the kindest person Michael has ever known. Michael believes Poopah should be everyone's role model, and one day hopes to be half the man Poopah was. Poopah also enjoyed making videos. Poopah's best video is a tribute to Steve, recognizing him for the exemplary father he is to Michael and James. Grammy Marsha, like Grammy Annette, was a great grandmother. She often told the story of when she found out Maddie was pregnant with Michael, she went to the park to play with the children to "practice" playing with the children to be ready for when he arrived. Michael and Grammy Marsha remained close after Poopah's passed away, and throughout his college and graduate school years. She would always ask Michael when he would graduate, and when he would get married. Sadly, Grammy Marsha passed away only 5 short months before Michael defended his dissertation, and became engaged to be married.

Michael had a very happy childhood. He attended P.S. 221 in Queens, NY starting in first grade. Michael met a group of best friends with whom he remains close to this day (Eric, Todd, and Zach). One thing all of his friends shared in

common was that they loved everything sports. They loved playing and watching football and baseball. One of Michael's favorite memories to this day are Sunday afternoons watching the Jets games with his brother, father, Uncle Pickle, and his cousins. Although most of the time the Jets did not contribute the entertainment, laughs, or in general good feelings, the Jets game was a family event that Michael always looked forward to.

Michael's favorite thing to do was go to the 811 schoolyard a few blocks from his house and play in pick-up baseball games. Some of the fondest memories Michael had growing up were spent playing until the sun went down. Jamie Wholler (the all-time career leader in home runs at the Park) was the "big kid" at the time, and made sure everyone was included and had a good time. The best football games were played "behind Brian's back", which was a slender patch of grass in between some apartment buildings. The neighborhood kids played there so often, the maintenance staff planted a tree in the middle of the "field" in an attempt to dissuade them from playing there (it didn't work). Other memories of the Park days include the time Todd ate two slices of pizza, 14 garlic knots, and a 2-liter of coke, the time Eric hit a home run and hit the top of the handball court, and every game he got to play with James. Some say Michael's athleticism peaked in 7$^{th}$ grade. In addition to playing sports, his friends played MLB Showdown, a dice-based baseball card game that they still continue to play. In middle school, Michael won an essay contest detailing the impact sports had on his life. He won a laptop and a trip for his family to attend the Empire State Games. Additionally, Michael also won the Korean Heritage essay contest, despite his complete lack of Korean heritage. Also in middle school, Michael was introduced to two life long friends: Julie and Mark. In 2004, the Wolfin family adopted a cat, Rascal (Pookie). He was a very cute kitten.

In addition to sports, Michael's budding interest in science continued to grow.

He spent countless hours with James digging in the backyard searching for bugs and worms. His favorite class in elementary school was Mrs. Wachs' science class, and his curiosity for science continued to grow in middle school. He developed a love of discovery. He first noticed this about himself following a lesson exploring the effect of simple table salt on the freezing and melting temperatures of water. Michael joined the Science Olympiad in middle school, and won a handful of medals in the astronomy events. At the time he wanted to be an astronomer because of how fascinating outer space is.

Michael attended Cardozo High School, and played on the junior varsity baseball team. He also played football with the Queens Falcons and was introduced to another role model, Coach Tim. Michael discovered his love for flag football in high school. Michael played on highly competitive teams that travelled all over the northeast to compete in tournaments. The DAC Predators (now Marksmen) won tournaments and leagues in Nassau County, Suffolk County, Queens, Booklyn, the Bronx, New Jersey, and Pennsylvania. He really enjoyed organizing neighborhood vs. neighborhood tackle football games. Specifically, every year, Michael organized his annual Turkey Bowl event the Friday after Thanksgiving. His Little Neck team played in their Turkey Bowl game for over a decade.

Michael experienced a bit of a disconnect with science in high school after some bad science teachers in middle school, and a particularly bad experience with a chemistry teacher in high school. He then made the decision chemistry was likely not the science for him. However, he was lucky enough to take an elective class, Sports Medicine, in his junior year and meet Scott (Mr.) Olson. Mr. Olson saw potential in Michael that he didn't know he had, and encouraged him to go into science. Mr. Olson was instrumental in Michael's decision to go to SUNY Cortland. The advice Michael was given still resonates with him to this day. Michael's college choices

were down to two schools: SUNY Cortland and SUNY Stony Brook. Mr. Olson told him that the choice barely mattered, and that he would be successful wherever he went. Mr. Olson told Michael that he was already a big fish in a big pond (referring to Cardozo), and he might benefit from being a big fish in a smaller pond (SUNY Cortland, which oddly enough had a similar number of students to Cardozo). He also said 'Go out there and split some wood or something in Upstate New York… It's a big world out there, go out there and get some new experiences!'. Michael took Mr. Olson's advice and attended SUNY Cortland.

The most important thing to happen in high school was the final addition to their family. Because Pookie turned out to be a mean cat, the family wanted to add a friendlier pet. In September of 2005 the family added Lexi (Pie) to the family, and thus the family was truly complete. She brought happiness to the Wolfin family that didn't know they could be even happier

Looking back, Michael considers his time at SUNY Cortland the best 5 years of his life. Michael entered college as an 'undecided' major, but took some science classes in an attempt to figure out which science would be his major. He declared a biology major his freshman year. However, his love of science and discovery was renewed when he took Dr. Frank Rossi's organic chemistry class. That class opened his eyes to how vast and interesting science can be. He also declared a chemistry major at the end of his sophomore year. He also established a solid core of friends that got through tough times together such as Advanced Labs and Thermodynamics. There were lots of late nights together, and they may have gotten into their share of mischief. Michael was also a founding member of the Rho- Iota Chapter of the Kappa Sigma Fraternity. The friendships he created through that organization will last a lifetime. He will always cherish the road trips to "Nawlins" with that group, and hopes they continue the biennial trips with that group of Hobnobbers. And of course,

Cortaca was always a blast (Woo!).

Most of the time Michael could be found working in the chem club room, hanging out at the Kappa Sigma house, or doing both at his favorite bar, the Study Hall. When he wasn't in the chem club room or in the lab, odds are he was working on something with a whiskey sour (with just a dash of grenadine) at the Study Hall. He made lifelong friends at that bar, and he believes he would not have graduated from SUNY Cortland without the support of his "Study Buddies". RIP Study Hall, you will be missed.

Towards the end of his sophomore year he started working on a research project in Dr. Rossi's lab. Although that project wasn't a good fit, a year later Michael met Dr. Terrence Fitzgerald, an entomologist. Michael immediately started working on a project studying the chemical and behavioral ecology of the cactus moth, *Cactoblastis cactorum*, co-advised by Dr. Rossi. Michael learned valuable skills while working on this project. Michael got his first experience observing behavior, and with bioassay guided fractionation. Additionally, Michael also gained first hand experience using advanced chemical instrumentation such as a high performance liquid chromatograph, a proton NMR, and a gas chromatograph-mass spectrometer (GCMS), and thus his relationship with gas chromatography began. Michael became the acting GCMS technician at SUNY Cortland, becoming the primary contact for all use and maintenance of the instrument. Through this project, Michael realized his true passion for chemical ecology, and decoding the sensory world. His work on this project has led to three publications to date (and possibly more to come). Encouraged by Dr. Rossi, Dr. Fitzgerald, and Dr. Timothy Baroni, Michael made the decision to apply to graduate school to work on a Ph. D.

Michael initially applied to three graduate programs. He wanted to work with Rob Raguso in the Neurobiology and Behavior Department at Cornell University,

Tom Baker in the Entomology Department at Penn State, or Jocelyn Millar at the University of California- Riverside. Because Michael would be a December graduate, he was also looking for employment to remain in Cortland with his friends for the spring semester, and gain additional experience working in his field. He applied for a laboratory technician job in Rob Raguso's lab at Cornell University. This turned out to be one of the best decisions he'd ever made. The interview went well over an hour, and from his perspective, was less of an interview and more of a pleasant conversation discussing science, responsibility, and future goals. Although not initially chosen for the job, Stephanie (the previous technician) switched career paths, and on the same day as his last undergraduate final, Rob offered him the job. Michael graduated SUNY Cortland with bachelors degrees in chemistry and biology, and also received the Top Graduating Senior award in the chemistry department, and the Outstanding Student Research in the Biological Sciences award, the only time both awards have been won by the same student.

Michael received invitations from Penn State and UC-Riverside to attend their interview weekends, but was rejected from the Neurobiology and Behavior Department at Cornell. However, Rob spoke with Dr. Charles Linn, Jr. (Charlie) in the entomology department, and arranged an interview. In the meantime, the entomology department at Cornell also rejected Michael's application. Michael met with Charlie and Dr. Greg Loeb in Geneva to discuss the potential of working with them at the New York State Agricultural Experiment Station (NYSAES). At the conclusion of the day, Charlie gave Michael a copy of the grant proposal for the project he'd be working on. He was instructed to read the grant proposal on the plane back from his interview at UC Riverside. Michael enjoyed his visits to both Riverside and State College, but his decision was made after reading the grant proposal. He knew that this project would be the type of project he wanted to work on throughout

his academic career. He informed Charlie that he wished to pursue graduate school at the Station, and the following August he started graduate school there.

Michael entered graduate school with little concept of what "outreach" was, but was excited to be funded by an Extension/Outreach Assistantship. Michael enrolled in Dr. Linda Rayor's Naturalist Outreach class, and discovered his true passion for sharing his excitement for science with the public. Armed with his trusty sidekick, Rex (a bearded dragon), Michael went to a total 26 different venues, giving 200 talks, reaching over 8,000 members of the community. Michael will continue to engage the community with science outreach for the rest of his academic career.

On February 3rd, 2016, Michael's world changed forever when he attended a Super Bowl party at his old fraternity house in Cortland. He sat next to a beautiful brunette who appeared to be heavily invested in the game. Although Michael tried to talk to her during the game, she remained focused on the Super Bowl. Her attention was also focused on the halftime show, Beyonce, so unfortunately for Michael, it appeared he would need a miracle to talk to her. And then it happened- an improbable and unexplainable event- the Super Bowl experienced a blackout early in the 3rd quarter of the game. This awarded Michael the time he needed to talk to this girl. However, she had no interest in him. Eventually, his friend put her on the spot and directly asked her if she would go on a date with Michael. She almost accidentally agreed, and Michael took her on a date the following week. On the night before his Ph. D. defense (11/19/2017), Michael asked that girl, Mary Elizabeth Simoncelli to marry him, and to his delight, she said 'YES!!!!!!!!!!!'

Michael has loved his time spent working on his Ph. D. at the NYSAES. He has had many wonderful experiences there, and really fell in love with the Finger Lakes Region. He really enjoyed working on his research project exploring the cues associated with host plant location and discrimination, and hopes to continue to research this topic for the rest of his academic career. Additionally, Michael was happiest when he was advising undergraduates in his lab. Michael is very excited for the next step- a post doc in Dr. Tom Baker's lab at Penn State. He eventually hopes to be a professor, and positively impact his student's lives in a similar way he has been impacted by his advisors.


This dissertation is dedicated to family- from those who are no longer with us: Poppy, Poopah, Grammy Annette, and Grammy Marsha; to the most influential people in my life thus far: Mom, Dad, and James; and to the family still to come, Mary, my wonderful fiancé, and the family that awaits us.

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I have been lucky enough to be positively impacted by a great number of diverse people in my life. I would first like to thank Charlie for everything he has done for me over the years. Charlie, thank you for believing in me, supporting me, and for always pushing me to be better than I am. My experiences in graduate school have had their ups and downs, and none of this would be possible without you. Your careful attention to detail has taught me to think critically about everything I do. Thank you for having the confidence that I would work through my mistakes, and supporting me though times of frustration. Your 'glass is *always* half full' mentality has changed how I approach most situations, and I now swear by your mantra of having 'good karma' in everything I do.

I'd like to thank the members of my committee- Rob, Greg, and Bruce for their support as well. Rob, thank you for believing in me, and working as hard as you do for your students. Your support, guidance, and vision have led me to my current path, and I would not be where I am without you. Your comprehensive and ecological perspectives have challenged me to think more broadly about the impacts of my work. Greg, you have truly been a second advisor to me in Geneva. You have challenged the way I think about my research, and expanded my perspective from the bench to the field, and how the work can be applied to solve problems. Rob and Greg, I have always been impressed by, and appreciative of the care and attention you both give to all of your various responsibilities, especially your students. Bruce, your Principles of Neurophysiology class was the best class I have ever taken. I built skills in electrophysiological techniques, and had fun doing so. Thank you for working with me all of these years.

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I'd like to thank the undergraduate students who have helped me out through the years. Thank you Sara, Ronnie, and Yuxi for all of your hard work, and also for the experience of mentoring you. I hope you had as much fun as I did.

Working here at the NYSAES hase given me a better understanding of how research gets done well. I sincerely believe that technicians are the cornerstone of a good research program. From the bottom of my heart, I would like to thank Shinyoung Park, Steve Hesler, and Callie Musto for your support during my dissertation research. This would not be possible without you. I'd also like to thank Wendy Kain for her daily conversations at coffee break. I attribute much of my good karma for the flight tunnel to those coffee breaks. On the same note, I'd like to thank Chris Olmstead for sharing my passion for the Mets, and talking baseball with me to no end. Too bad we didn't get that championship in 2015, but thanks for enjoying the ride with me. Additionally, thanks for being such a good listener when I had problems on my mind. You make the Station a better place everyday. I'll miss you.

I'd also like to thank Holly King for being really, really good at her job, and also for being the cheerful and friendly person that she is. Thank you also for allowing me to "Trick or Treat" in your office year round. Nancy left big shoes to fill, and you have made my graduate experience better, and I think you make the Station a better place.

I'd like to thank my research advisors at SUNY Cortland. Thanks to Dr. Fitzgerald for introducing me to the fascinating world of insect behavior. Your careful

advise of "watch the insect" will stay with me forever. I wouldn't be here without Dr. Rossi. Thank you so much for all of the support you've given me over the last decade. From dropping me from your research program (I deserved it), to agreeing to co-advise me again, to allowing me the freedom to own the GCMS, and always pushing me to be better, I really appreciate everything you have done for me. Your dedication to your students is truly admirable, and I am lucky to have you as an advisor and friend. I hope to one day build relationships with my students the way you routinely do with yours.

I'd like to thank all of my friends for helping to make me who I am as well. From my earliest childhood friends, Todd, Zach and Eric, to Julie and Mark, thanks for putting up with me all of these years. It's been fun, and I couldn't have done any of this without you. Thanks also to my football buddies Pete and Colin, and the 306 Bishop Hall connection- Tyler. Thanks to all of my college buddies: the chem club- Steve, Hooten (Mike), Tyler, and Tyler. A special thanks to Josh for being my support through that rough senior year- you single handedly prevented a major breakdown. I hope we can be friends again someday. Thanks to all of my Fraternity Brothers, and first to the 7 Who Stayed: Hotsauce, Hudson, Jonny Glass, Larry The Chin, Tim (you're not really a Towel in my eyes), and Brad. We've been through a lot together, and you all have a special place in my heart. Thanks to the rest of the Founders and the Nawlins crew for some of the best memories and laughs I'll ever have, Woody, Sticky, Hotsauce, Hudson, Hollywood, Zhuo, LK, and El Tornado. And finally, thanks to Ryan, Jamie, and the rest of Study Hall for their support as well. I wouldn't be the person I am without all of you. I'd also like to thank Matt and Erik for being my 'Barton Buds', I know we'll stay in touch.

I don't think the number of pages exist for me to thank my family. Thank you to my grandparents for being the positive influences and role models you all were, you

will be missed. Mom and Dad, you have been my biggest supporters, cheerleaders, teachers, and role models throughout my life. I couldn't fathom better parents. You've allowed me to grow into the person I am today showing me love and support every step of the way. You taught me to persevere through hardships and never give up. From a young age you always encouraged me to follow my dreams, and this dissertation is a direct result of everything you have done for me. You gave James and I a wonderful childhood, and prepared us to be happy, well-adjusted adults, scientists, friends, boyfriends, husbands, fathers, and in general, members of society. I hope we were as much of a pleasure to raise as it is to be your sons, and I can't thank you enough for everything you've done. Mom and Dad, I hope I can be half the parents you are, and thank you for being such loving parents.

I'd also like to thank my brother James for everything. From 'getting' me, to tolerating me, to supporting me, and when appropriate, either encouraging me or knocking me down a peg. You're my best friend, quarterback, role model, and person I lean on the most. You're the best little brother a big brother could ever hope to have, and I hope I've affected your life as positively as you have affected mine. Uncle Pickle and Dad have been great role models for us, and I hope we'll remain best friends for the rest of our lives.

And finally, I'd like to acknowledge Mary Elizabeth Simoncelli. Mary, you have been a great partner throughout my graduate school experience, and you have supported me through all of it. Thank you for your encouragement and always pushing me to be better than I am. Whether I had a a bad day or a good day you were there for me at the end of the day. You are intelligent, beautiful, and kind to me, and I can't wait to spend the rest of my life as your husband. I love you for the person you are, I love how we've grown together as a couple, and I can't wait for your family to officially be my family. We have a great life waiting for us together Mary Liz. For

everything, from the bottom of my heart, thank you.

Quick Laughs:

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# PREFACE

The sections in this preface describe key topics in the literature central to insect host plant location. The first section justifies the importance of locating an appropriate host plant, and explains the costs associated with oviposition 'mistakes'. The second section outlines the cascade of behaviors involved in host plant location. The third section describes the potential uses of host plants by virgin females, mated females, and males with examples from the literature. The fourth section provides a set of detailed protocols for isolating the chemical compounds that elicit insect behavior. This section highlights four case studies involving different insects, chemical isolation and identification procedures, electrophysiological techniques, and behavioral assays. Additionally, this section covers the technological advances required for the efficient and thorough study of plant volatiles that mediate insect behavior. The fifth section describes four models for host plant location, with examples from the literature, and commentary on the strengths and weaknesses of each model. The sixth section introduces the study system, and justifies its use as a model system to study host plant location and discrimination.

*Challenges for phytophagous larvae, and the importance of minimizing oviposition mistakes*

It is crucial to the reproductive success of a gravid female phytophagous insect to oviposit on an appropriate plant because in many cases the larvae have relatively low levels of motility and relatively

narrow diet breaths (Mattson et al. 1988, Schoonhoven et al. 2005, Berenbaum and Feeny 2008, van Griethuijsen and Trimmer 2014). Feeding specialization is likely mediated by an insect's ability to tolerate or resist plant secondary metabolites (Brues 1920, Ehrlich and Raven 1964, Powell 1980, Mitchell 1981, Ehrlich and Murphy 1988, Jaenike 1990, Kliebenstein 2012, Carmona and Fornoni 2013, Kessler 2015). Specialist phytophagous larvae can adapt to resist or tolerate toxic plant compounds produced by a specific plant (Metcalf 1986, Shonle and Bergelson 2009, Agrawal et al. 2012). For example, milkweed plants, *Aslepias* spp*.,* contain high concentrations of cardiac glycosides that are toxic to many insects (Holzinger and Wink 1996). However, monarch butterfly larvae, *Danus plexippus*, are specialized to feed on milkweed plants, and have developed tolerance to these toxic compounds, allowing them access to an otherwise unavailable food source (Holzinger and Wink 1996, Agrawal and Konno 2009). Additionally, significant evidence exists that nutrient availability also plays a major role in host plant specialization (Waldbuer and Friedman 1991, Bernays et al. 1994, Chambers et al. 1996, Singer and Stireman 2001, Behmer 2009). For example, specialist herbivores can more efficiently convert food to growth, and also maintain higher rates of development compared to generalists (Lee et al. 2006). Such specificity can, however, also negatively affect fitness, as ovipositional "mistakes" can cause larvae to "perish" (Dethier 1959, Ehrlich and Raven 1964, Davis and Cipollini 2014). Because of these costs, the oviposition behavior of gravid females has evolved to, depending on the degree of host specialization, be able to discriminate against non-host plants, and select an appropriate host (Nylin

and Janz 1996, Janz and Nylin 1997, Berenbaum and Feeny 2008, Reisenman et al. 2009, Bruce and Pickett 2011, Riffell 2012).

*A cascade of behaviors involved in olfactory-mediated host plant location*

Although it has been suggested that the cascade of behaviors involved in host plant selection is initiated while the insect is already flying (Finch and Collier 2000), for the purposes of this preface, the cascade begins with a quiescent insect, and appetitive searching behavior begins the cascade (Ramaswamy 1988, Visser 1988, Schoonhoven et al. 2005, Cardé 2016). These behaviors are shown in Figure i. Unlike later behaviors in the cascade, the 'take flight' response is not necessarily chemically mediated (Edwards 1962, Sanders and Lucuik 1975, Dreisig 1980). Appetitive flight is part of the insect's natural activity, and can be non-directional or have a directional component that could be related to wind flow, allowing the insect to, for example, fly in a cross wind pattern to optimize contact with airborne plumes (Schoonhoven et al. 2005). The take flight response involved in sex pheromone mediated mate location in male moths, for example, is stimulated by favorable environmental conditions, such as a photoperiod regulated decrease in light intensity (Linn et al. 1992, 1994, 1996, Gadenne et al. 2016). Male cabbage looper moths, *Trichoplusia ni*, took flight 2-3 hours after the beginning of scotophase, and remained in flight for the duration of scotophase. The decrease in light intensity caused a decrease in octopamine levels, which correlated with changes in locomotor activity and sensitivity to pheromone (Linn et al. 1992, 1994, 1996). Male and female silver Y moths, *Autographa gamma*, have a similar take flight response to the

changing photoperiod (Dreisig 1980). Female Saddle-backed looper moths, *Ectropis crepuscularina*, Western tent caterpillar moths, *Malacosoma pluviale*, silver spotted tiger moths, *Halisodota argentata*, and spruce budworm moths, *Choristoneura anagasta*, all took flight in response to changing levels of light corresponding with a natural photoperiod (Edwards 1962). Interestingly, different species took flight at different light intensities (Edwards 1962, Dreisig 1980). Mated spruce budworm females showed peak flight periods 2-3 days post eclosion, and three hours before both sunset and sunrise (Sanders and Lucuik 1975).

Once flying, male and female moths are more likely to detect a favorable odor plume and initiate the next behavior in the cascade (Phelan et al. 1991, Murlis et al. 1992, Finch and Collier 2000, Sasso et al. 2009, Faucher et al. 2013, Cardé 2016), the initiation of oriented flight along the odor plume in the upwind direction (Figure i-2 purple; Schoonhoven et al. 2005, Cardé 2016). The upwind flight response has been extensively studied in male moths responding to sex pheromone (Vickers and Baker 1994, Cardé and Willis 2008, Cardé 2016), and it is likely that females use a similar mechanism to locate their host plant (Mechaber et al. 2002, Riffell et al. 2009a). The detection of a favorable odor plume stimulates the moth to fly upwind mediated by visual cues (Kennedy 1940, 1983, Kennedy and Marsh 1974, Murlis et al. 1992, Cardé and Willis 2008, Baker and Hansson 2016), also known as optomotor anemotaxis. Moths use the image flow of their surroundings to determine upwind direction, proceeding toward the odor source through a succession of left and right turns, or counterturns, within an odor plume (Kennedy 1983), resulting in net movement directly upwind. If the insect loses contact with the odor

plume, it gradually lengthens the time between counterturns (casting), while making minimal progress upwind (Baker and Haynes 1987). Once the insect has remade contact with the odor plume, it continues its surge upwind. When the flying insect is close to the odor source (1-20 cm), the insect displays a different set of close-range behaviors to prepare for landing, and eventually land on or near the odor source (Figure i-3; Cardé 2016).

Studies on male moth response to sex pheromones have shown that, whereas there are cases of complex chemical interactions involved in close range landing and courtship, the presence of the female produced pheromone is necessary for the courtship displays to occur. However, it is unclear whether the landing response in all cases is chemically mediated either by the physical plume structure, or by the presence of chemical landing cues (Bradshaw et al. 1983, Linn et al. 1987, Cardé 2016). There are examples of low volatility compounds synergizing with sex pheromone to increase male response (Xiao and Honda 2010, Xiao et al. 2012, Yan et al. 2014). Hydrocarbons synergize with sex pheromone to arrest yellow peach moth males, *Conogethes* punctiferalis, close to the odor source (Xiao and Honda 2010, Xiao et al. 2012).

Figure 1. Summary of theories describing insect host location. Brown insects/boxes indicate all theories describe this process (Fraenkel 1959, Finch and Collier 2000, Bruce et al. 2005). Green boxes/ insects are supported by the appropriate/inappropriate landings theory (Finch and Collier 2000), blue boxes/insects are supported by the 'specific blends of ubiquitous compounds theory' (Bruce et al. 2005), and red boxes/ insects are supported by the 'token stimulus theory' (Fraenkel 1959). Intermittent dotted lines indicate an odor plume, and colored dotted lines indicate landing behavior supported by the corresponding theory. In all cases, the insect takes flight in response to abiotic conditions. (1). Oriented upwind flight (2) and landing (3) are elicited by either specific blends of volatile compounds (blue), or species-specific compounds, or no oriented upwind flight occurs (3, green). Nonspecific plant volatiles stimulate the insect to land on the plant (3, green), and initiate post-landing assessments of the plant. Spiral flights (4, green) are performed to determine whether the plant is a host or a non-host. Asterisks indicate the point at which discrimination occurs. Habitat cues may enhance each set of behaviors.

In addition to olfactory cues, visual cues are also important in the landing response (Sparks and Cheatham 1970, Rojas and Wyatt 1999, Tasin et al. 2006, Cha et al. 2008b, Späthe et al. 2013, Luo and Honda 2015). Insects reduce their flight speed as they approach the source and utilize features in their visual field to select a place to land (Cardé 2016). In laboratory bioassays, female moths readily land on a diverse array of artificial substrates (Sparks and Cheatham 1970, Rojas and Wyatt 1999,

Tasin et al. 2006, Späthe et al. 2013, Luo and Honda 2015). Female European grapevine moths, *Lobesia botrana*, landed on a vibrating capillary tube releasing volatiles a statistically similar percentage of time as they landed on control green grape clusters. Female yellow peach moths, *C. punctiferalis*, land on a metal mesh ball wrapped in medical gauze in the presence of host odors (Luo and Honda 2015).

Upon landing, female insects may use additional chemical cues to further assess the plant (Figure i-4) before ultimately accepting (feeding, laying an egg, releasing pheromone) or rejecting it (directed movement away from the plant; Ramaswamy 1988, Kostal and Finch 1994, Finch and Collier 2000). For example, female cabbage root flies, *Delia radicum*, make a series of 'spiral flights', which consist of the insect landing on a host plant leaf, using tarsal chemoreceptors to assess the plant, and repeating the process 4-5 times until ultimately accepting or rejecting the plant (Kostal and Finch 1994, Finch and Collier 2000). Both mated and unmated female tobacco budworm moths, *Heliothis virescens*, bend their abdomen and/or drag their ovipositor on the substrate of the plant before host plant acceptance (oviposition or calling) (Ramaswamy 1988). Additionally, mated female European corn borer moths, *Ostrinia nubilalis*, use tarsal receptors to assess the host plant before laying an egg (Schurr and Holdaway 1970, Derridj et al. 1986).

*A diversity of chemically-mediated host plant interactions*

Phytophagous insects can use host plants to serve a number of different functions such as food sources and mating and oviposition sites. Host plants serve as food sources for both sexes of insects and males and

females can use volatiles as long distance attractants (Bruce and Cork 2001, Cunningham 2004, Raguso 2008a, Alarcón et al. 2010). Depending on the sex and physiological state of an insect, the same set of host plant cues may stimulate or enhance different behaviors. Unmated cotton leafworm females, *Spodoptera littoralis*, displayed significantly higher levels of upwind flight in response to lilac flowers compared to mated females (Saveer et al. 2012). Mating status had no effect on male cotton leafworm moths (Kromann et al. 2014). Therefore, for the remainder of this section, mated females, unmated females, and males are considered separately.

As introduced above, it is crucial that mated female moths oviposit on an appropriate host plant (Dethier 1959, Berenbaum and Feeny 2008, Davis and Cipollini 2014). Mated females can use host plant volatiles to locate an appropriate host (Phelan et al. 1991, Honda 1995, Hern and Dorn 1999, 2002, Yan et al. 1999, Bruce et al. 2005, Piñero and Dorn 2007, Piñero et al. 2008, Bruce and Pickett 2011). However, there is much debate as to the proximate mechanisms for olfactory-mediated host location (Fraenkel 1959, Finch and Collier 2000, Bruce et al. 2005). Mated oriental fruit moth females, *Grapholita molesta*, are attracted to green leaf volatiles produced by peach plants (Piñero and Dorn 2007, Piñero et al. 2008). Mated female codling moths, *Cydia pomonella*, are attracted to synthetic blends of host plant volatiles (Hern and Dorn 2004). Mated grapevine moth females, *Lobesia* botrana, are attracted to synthetic blends of host plant volatiles, and use the volatile blends to assess host plant quality (Tasin et al. 2006, 2007, 2009, 2011, 2012). After landing on the host plant, mated females continue to assess the plant as a potential

host through contact chemoreception as described above before either rejecting a host plant, or laying an egg (Derridj et al. 1986, Ramaswamy 1988, Marion-Poll et al. 1992, Finch and Collier 2000).

In addition to food sources and oviposition sites, host plants can also be locations for courtship and mating (McNeil and Delisle 1989, Landolt and Phillips 1997, Dekker and Barrozo 2016). Pheromone production in corn earworm females, *Helicoverpa zea*, is stimulated by host plant volatiles (Raina et al. 1992), and unmated American sunflower moth females, *Homoesoma electellum,* are stimulated to release pheromone by pollen volatiles (McNeil and Delisle 1989). Ermine moths, *Yponomeutidae spp.*, prefer to call on their host compared to non-host plants, and host plant volatiles increased calling rates (Hendrikse and Vos-Bünnemeyer 1987).

Unmated female cabbage looper and tobacco budworm moths are attracted to host plant volatiles in a wind tunnel (Ramaswamy 1988, Landolt 1989). After landing on the plant, unmated female tobacco budworms use contact chemoreception to further assess a plant (described above) before releasing pheromone (Ramaswamy 1988). Host plant volatiles stimulate female cabbage loopers to release pheromone, and also play a role in the courtship behavior of both sexes (Landolt 1989, Landolt et al. 1994, Landolt and Phillips 1997). Cabbage looper males and unmated females displayed similar upwind flight responses to host plant volatiles (Landolt 1989). The addition of host plant volatiles to cabbage looper female-produced sex pheromone increased the male upwind flight response (Landolt et al. 1994), and also the amount of sex pheromone released by males (Landolt and Heath 1990).

Although males are capable of following a pheromone plume for dozens of meters in the field, females often mate within minutes of releasing pheromone (Witzgall et al. 1999), suggesting males are already near the calling females at the time of pheromone release. Male moths likely use host plant volatiles as habitat odors to increase the likelihood of encountering a sex pheromone plume, and decreasing the flight distance to the female (Webster and Cardé 2016). Male codling moths and virgin cotton leafworm moths, *S. littoralis*, displayed upwind flight to host plant volatiles (Coracini and Bengtsson 2004, Kromann et al. 2014, Light et al. 2014). However, the cotton leafworm upwind flight response was lost post mating, indicating it could be associated with the physiological state of the insect. Interestingly, mating status had no effect on upwind flight toward lilac flowers (a food source), suggesting the male upwind flight response to host plants is also context-dependent.

*Protocols for studying chemically mediated behaviors*

As chemical ecologists, we use an array of instruments and behavioral assays to understand the significance of chemical compounds. This section outlines general protocols for isolating and identifying behaviorally active chemical compounds (Figure ii) highlighting four examples from the literature (Table i). The procedures used to identify the chemical trail pheromones in cactus moth caterpillars, *Cactoblastis cactorum*, (Fitzgerald et al. 2014, 2015), the volatiles that honeybees, *Apis mellifera*, use to discriminate potential hosts (Wright et al. 2002, 2005), the female produced sex pheromone in the cabbage looper (Ignoffo et al. 1963, Berger 1966, Bjostad et al. 1980), and an attractive blend of host

plant volatiles (snowberry, *Symphoricarpos albus*) in the snowberry fly, *Rhagoletes zephyria*, are discussed to compare and contrast the different methods used in each study.

The first step is to identify a specific behavior or behaviors to explore (Figure ii, A). In the first study, cactus moth caterpillars were observed to move *en mass* across cactus pads in a single file line (Zimmerman et al. 2004), and Fitzgerald et al. (2014, 2015, 2016) investigated whether a chemical trail pheromone was involved. In the second study, honey bees were known to use floral odors to discriminate between flowers while foraging (Free 1963, Le Métayer et al. 1997, Chittka and Raine 2006), and Wright et al. (2002, 2005) explored the specific volatile compounds used to discriminate flowers. In the third study, male cabbage looper moths were observed to be attracted to female moths from a distance (Ignoffo et al. 1963, Shorey 1964), and multiple groups explored the use of a female produced long distance volatile attractant (Berger 1966, Bjostad et al. 1980, 1984). In a final example, *Rhagoletis* flies infesting snowberry fruit were shown to be attracted to volatile blends isolated from host fruit (Cha et al. 2017).

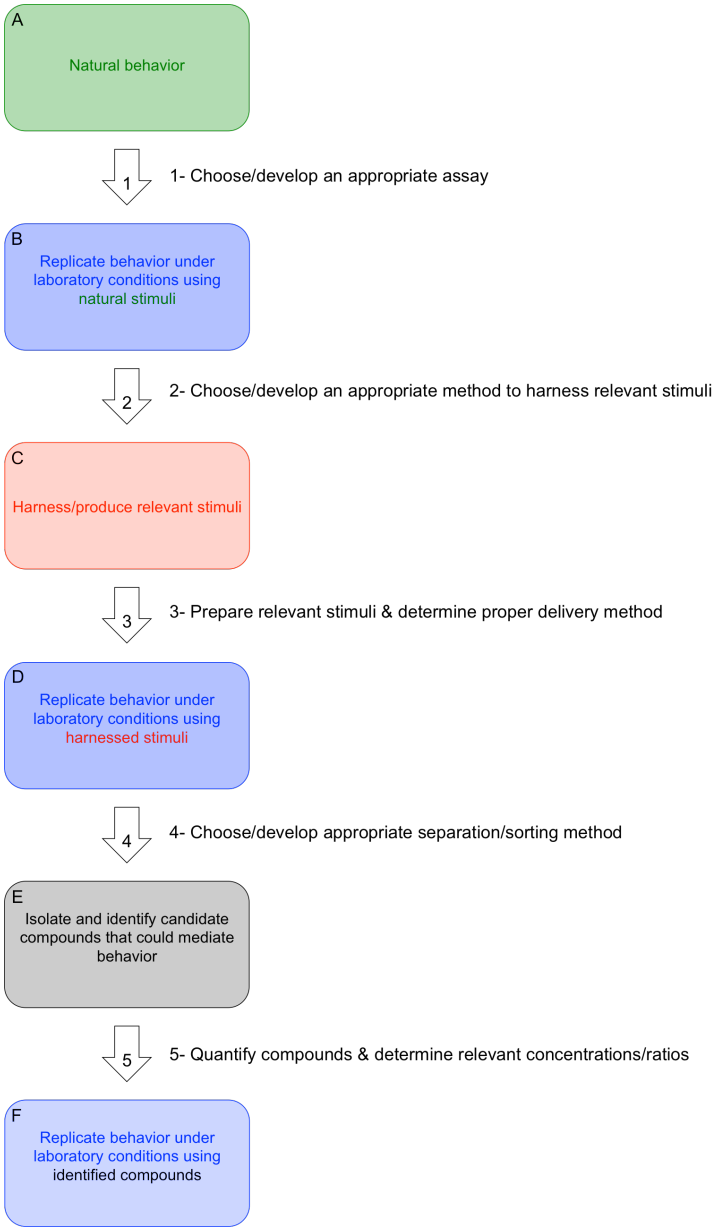


Figure ii. General protocols for isolating and identifying chemicals that elicit behavior. The process begins by identifying behavior(s) to study (A). An appropriate assay is selected to reproduce the behavior(s) (1). Once the behaviors have been replicated under laboratory conditions using natural stimuli (B), appropriate collection methods should be selected to harness and the potentially behaviorally active stimuli, and present it to the insect (2, C, 3). If the harnessed stimuli elicit the focal behaviors (D), separation/sorting methods are necessary (4) to isolate and identify candidate compounds (E). Once the relevant concentrations and ratios have been determined, bioassays should be repeated using synthetic blends of the identified compounds.

Once a key behavior or behaviors have been identified, an appropriate assay must be selected/developed (Figure ii, 1). In an appropriate assay, the insect readily exhibits the focal behavior(s) while allowing the desired variables to be easily manipulated and replicated. In the first study, Fitzgerald et al. (2016) constructed experimental arenas consisting of sections of cactus pads in a 1 oz. cup to observe the potential trail following behavior under laboratory conditions. In the second case, Wright et al. (2002, 2005) used gas chromatography coupled proboscis extension reflex (PER) assays to determine the behaviorally active volatiles used in host plant discrimination. In the third case, a combination of flight cage tests and field observations to record the behavioral responses of male cabbage looper moths to calling females (Berger 1966, Bjostad et al. 1980). In the last case, Cha et al. (2017) used flight tunnel assays to observe the responses of snowberry flies to adsorbent extract collections from snowberry fruit.

Table i. Summary of procedures used to identify behaviorally active chemicals in each case study. References: 1=Zimmerman et al. 2004; 2=Fitzgerald et al. 2016; 3=Free 1963; 4=Ignoffo et al. 1963; 5=Shorey 1964; 6=Cha et al. 2017; 7=Fitzgerald et al. 2014; 8=Wright et al. 2005; 9=Berger 1966; 10=Bjostad et al. 1980; 11=Fitzgerald et al. 2015.

| # | System | Natural Behavior | Behavioral Assay | Relevant Stimuli | Collection Method | Sorting Mechanism |
|---|---|---|---|---|---|---|
| 1 | *C. cactorum* trail pheromones | Trail following[1,2] | Slit trail assays[7] | Non-volatile compounds | Full body extract; gland extract[7] | Bioassay guided fractionation[7,11] |
| 2 | *A. meliffera* flower discrimination cues | Host choice[3] | GC-PER[8] | Volatile compounds | Dynamic headspace collection[8] | GC-PER[8] |
| 3 | *T. ni* sex pheromone | Oriented upwind flight; courtship behavior[4,5] | Flight tunnel assays[9,10] | Volatile compounds | Dynamic headspace collection; gland extract[9,10] | Bioassay guided fractionation[9,10] |
| 4 | *R. zephyria* host plant cues | Oriented upwind flight[6] | Flight tunnel assays[6] | Volatile compounds | Dynamic headspace collection[6] | GC-EAD[6] |

After the behavior has been replicated using natural stimuli (Figure ii, B), it becomes necessary to harness/produce the relevant stimuli for use as a chemical source in the bioassays (Figure ii, C). In the first case, Fitzgerald et al. (2014) reproduced the natural stimuli using multiple techniques. Caterpillars were dragged along pieces of filter paper, and the response to the residue was recorded. Because trail following was observed to the cuticular residue, whole body extracts were prepared by grinding 10 caterpillars in solvent, and gland extracts were prepared by vortexing 15 pairs of mandibular glands in solvent. The extracts were applied to a substrate (index card), and the behavioral response was recorded. In the second case, Wright et al. (2005) used a dynamic headspace procedure to prepare volatile extracts from 195 flowers (Raguso and Pichersky 1995). Volatile compounds were trapped on activated charcoal, eluted with solvent, and then used in coupled gas chromatography-proboscis extension reflex assays (Le Métayer et al. 1997). In the third case, extracts were prepared by soaking at least 2000 excised female pheromone glands in solvent overnight. These extracts were concentrated, loaded on to filter paper and used as an odor source in behavioral wing-fanning assays or field tests (Berger 1966, Bjostad et al. 1980). In the fourth case, as in the second, Cha et al. (2017) used a dynamic headspace procedure to prepare volatile extracts from whole snowberry fruit. Volatile compounds were trapped on activated charcoal and eluted with solvent. These extracts were concentrated, loaded on to rubber septa, and used as an odor source in flight tunnel assays.

If the chemicals presented in the assays elicit the key behaviors (Figure ii, D), the next step is to isolate and identify candidate compounds that could mediate the observed behaviors (Figure ii, E). In many cases, the chemical extracts are complex mixtures of compounds, and thus techniques are necessary to sort and/or separate the compounds to determine their activity (Figure ii, 4). When few compounds are present in the extract, bioassay guided fractionation can be used to separate and identify active candidate compounds. In the case of the cactus moth caterpillars, the mandibular gland extract contained few compounds, and was fractionated by column chromatography by volume. Each fraction was assayed for activity using the same assays used to determine the activity of the extracts. Behaviorally active fractions were fractionated again by time (every 1.5 minutes) using high performance liquid chromatography (Fitzgerald et al. 2014), and bioassayed until it was determined (using NMR and GCMS) that a complex mixture of 2-acyl-1,3-cyclohexane diones elicited the trail following response (Fitzgerald et al. 2014, 2015). In the second case, Wright et al. (2005) used coupled gas chromatography-proboscis extension reflex assays. In these assays, the extract was separated via gas chromatography (GC), and the separated compounds were blown over the honey bee antenna, where the proboscis extension reflex was observed. A compound was considered behaviorally active if it stimulated the insect to extended its proboscis. Eight behaviorally active compounds were identified via GCMS (Wright et al. 2005). Gas chromatography coupled behavioral assays are only appropriate when a single compound elicits a behavioral response.

However, in the majority of cases, blends of compounds are necessary to elicit a complete behavioral response. For example, in the third case, cabbage looper pheromone gland extracts were separated by column chromatography by volume. Each fraction was assayed for activity using the same assays used to determine the activity of the extracts. Behaviorally active fractions were fractionated using gas chromatography, and each fraction contained a single peak (Berger 1966). Each fraction was assayed, and it was determined using gas chromatography (GC) and synthetic techniques that (*Z*)-7-dodecenyl acetate was a long distance sex pheromone. However, it was later determined that this pheromone was incomplete, and the chemical analysis was limited by the technology available at the time (*see below*; Bjostad et al. 1980, 1984). Bjostad et al. (1984) found that a 6-component blend elicited maximal levels of oriented upwind flight, as well as the entire cascade of courtship behaviors a male exhibits to a calling female. In the fourth case, an electrophysiological screening technique was necessary to eliminate compounds from consideration (*explained below*; Figure ii 4). Cha et al. (2017) used gas chromatography coupled with electroantennographic detection (GC-EAD) to identify antenally active compounds. Using this technique, the snowberry fruit volatile extract was separated via gas chromatography, with the separated compounds then blown individually over the fly antenna. An electrical potential was generated (and recorded) if the compound could be detected. Nine antenally active compounds were identified via GCMS, and a blend was created and used as a source in flight tunnel assays (Cha et al. 2017). It is important to note that using GC-EAD, the behavioral significance of the

antenally active compounds is not known, only that the insect is capable of detecting the compounds.

Once the active compounds have been identified, the next step is to replicate the key behavior(s) using synthetic compounds. (Figure ii F). During this stage of bioassays, the optimal concentrations of the active compounds can be determined. In the first case, caterpillars were assayed to synthetic compound at four different concentrations, and maximum responses were observed at 1 mg/mL (Fitzgerald et al. 2015). In the second case, honey bee PER was observed to different flowers that produce different ratios of the active compounds (Wright et al. 2002). In the third case, male moths were assayed to synthetic blends mixed in three different ratios, and maximum responses were observed at 90:10 of (*Z*)-7-dodecenyl acetate to dodecyl acetate (Bjostad et al. 1980). In the fourth case, synthetic blends of snowberry volatiles were prepared using the ratios and concentrations determined via GCMS. Subtraction tests were performed to determine the behavioral significance of selected compounds (Cha et al. 2017). The only synthetic blends that elicited similar upwind flight responses as the extract were the complete blend, and the complete blend without dimethyl trisulfide. Similar behavioral responses to the natural stimulus, the extract of the natural stimulus, and a synthetic blend of active compounds provided evidence that the identified compounds elicit the observed behavior.

<u>Now is the right time to study chemically mediated behavior</u>

As mentioned above, several studies were required to determine the complete cabbage looper sex pheromone (Ignoffo et al. 1963, Berger

1966, Bjostad et al. 1980, 1984, Linn and Gaston 1981). Initially, Berger (1966) identified a single compound from the gland extract. However, as chemical instrumentation improved, Bjostad et al. (1980) identified an additional compound in gland extracts, and Linn and Gaston (1981) determined the optimal concentrations and ratios of the two components for behavioral activity in a flight tunnel assay. However, these early studies were limited by the sensitivity of chemical instrumentation available at the time (packed column gas chromatography; Bjostad 1989). Commercially available capillary GC columns allowed for more accurate resolution of chemically similar compounds. In addition, Bjostad et al. (1984) predicted an optimized 6-component blend based on the biosynthetic pathway of (*Z*)-7-doecenyl acetate. The additional compounds were present in trace amounts and were below the level of detection of the gas chromatographs in previous studies. However, these compounds were detected using a gas chromatograph outfitted with a capillary column.

The use of physiological techniques such as GC-EAD is another major technological advance that has allowed for rapid identification of potential behaviorally active compounds (Olsson and Hansson 2013). Gas chromatography coupled with electroantennographic detection provides a screening mechanism to identify the volatiles that can be detected by an antenna (or antennal pair), and is especially useful to analyze complex plant odor profiles (Raguso 2008b). Without electrophysiological screening, the analysis of volatile compounds for behavioral activity would require hundreds of GCMS analyses, observation hours, and bioassays (Raguso 2008b). Thus, the ability to

eliminate dozens of potential candidate compounds and focusing on only those compounds that the insect can detect using a single technique is a major technological advance. A limitation of this technique, however, is that an electrical response to a compound provides no behavioral information. An EAD active compound may be an attractant, repellant, antagonist, or have no behavioral effect, and thus further behavioral assays are necessary to determine the behavioral significance of a candidate compound.

In addition to GC-EAD, the use of gas chromatography coupled with mass spectrometry (GCMS) is a third technological advance that increases the rapid identification of behaviorally active compounds. The number of necessary chemical analyses is significantly decreased due to the relative ease with which GCMS identifies and quantifies chemical compounds. Before GCMS, unknown chemical compounds were identified using a combination of infrared spectrometry, gas chromatography, and synthetic techniques to analyze derivatives (Berger 1966, Bjostad 1989). However, GCMS provides a single technique to identify a compound. Retention time matches and Kovats Index matches using multiple capillary columns, and mass spectral matches to library searches provide tentative identifications of unknown compounds that can be used to identify the compound (Adams 2007). Identifications have a high degree of certainty when the properties of the candidate compounds match the properties of synthetic standards. Relatively accurate quantification is achieved through the use of internal and external standards (Adams 2007).

A Final Thought on Observing Insect Behavior

I'd also like to point out the attention to detail required for observing insect behavior. Observing natural insect behavior in a laboratory setting requires careful attention to a number of conditions and details. For example, photoperiod, temperature, and relative humidity are all conditions that affect insect behavior. Insects have diverse life histories, and the conditions under which they are assayed need to be representative of natural conditions in order to observe authentic insect behavior. Additionally, special attention needs to be paid to the care and handling of the insects prior to behavioral assays. Insects require an acclimation period to adjust to the bioassay conditions. Finally, and most importantly a routine is necessary for studying insect behavior. Keeping a strict setup schedule can help minimize day to day handling variability and ensure insects of similar quality.

*Theories for host plant selection*

This section outlines the current theories for host plant selection (Figure i; Table ii), highlighting four examples from the literature and commentary on the strengths and weaknesses of each. The four theories described in this section are the 'Token Stimulus' theory (Fraenkel 1959), the 'Ratio-specific blends of volatile compounds' theory (Bruce et al. 2005), the 'Appropriate/inappropriate landings' theory (Finch and Collier 2000), and the 'Habitat odors' theory (Webster and Cardé 2016).

Table ii. Summary of theories for host plant location. 1=Fraenkel 1959; 2=Bruce et al. 2005; 3=Bruce and Pickett 2011; 4=Finch and Collier 2000; 5=Webster and Cardé 2016

| | Theory | | | |
|---|---|---|---|---|
| | Token Stimulus[1] | Ratio-Specific Blends[2,3] | Appropriate/ Inappropriate Landings[4] | Habitat Odor[5] |
| Flight stimulated by: | Appropriate environmental/physiological conditions | | | |
| Oriented upwind flight stimulated by: | detection of plant-specific volatiles | detection of plant-specific ratios of ubiquitous volatiles | No oriented upwind flight | nonspecific habitat cues |
| Landing stimulated by: | detection of plant-specific volatiles | detection of plant-specific ratios of ubiquitous volatiles | detection of nonspecific plant odors | host-specific cues |
| Discrimination occurs through: | detection of plant-specific volatiles | detection of plant-specific ratios of ubiquitous volatiles | multiple consecutive landings on host plant | Not addressed |
| Point of discrimination: | In- flight | In- flight | Post-landing | Close range |

## Token Stimulus

In the landmark paper, 'The Raison d'Etre Substances of Secondary Plant Substances' (1959), Gottfried Fraenkel explained that the role of plant secondary metabolites first evolved as defensive compounds to protect the plant (Dethier 1954, Lipke and Fraenkel 1956, Kennedy 1958, 1965), and that these 'odd compounds' can also be used by insects to discriminate between host and non-host plants. Fraenkel (1959) suggested "the food specificity of insects is based solely on the presence or absence of these odd compounds in plants, which serve as repellents to insects (and other animals) in general and as attractants to those few which feed on each plant species." According to this theory, a flying insect would detect a unique, species-specific volatile compound, identify the source as a host plant, and initiate oriented upwind flight towards the odor source (Figure i). It is important to note, that according to Fraenkel

(1959), the same compounds may be used by insects as repellants to identify a plant as a non-host plant. Therefore, the point of host plant discrimination occurs upon the detection of the species-specific volatile compound.

There are examples of insects using taxonomically specific compounds to locate a host (*see Table 1 in* Bruce et al. 2005). Insects across three orders (Coleoptera, Diptera, Lepidoptera) that feed on brassicaceous plants, *Brassicaceae spp.*, can use isothiocyanates to locate their host (Nottingham et al. 1991, van Loon et al. 1992, Blight et al. 1995, Baoyu et al. 2001). For example, the species-specific volatile compound allyl isothiocyanate alone elicited upwind flight in 95% of the male and female diamondback moths flown in a flight tunnel, *Plutella xylostella*, (Baoyu et al. 2001). Additionally, cabbage seed weevils, *Ceutorhynchus assimilis*, were attracted to synthetic blends of EAD active volatiles found in oilseed rape leaves (Evans and Allen-Williams 1998). The level of attraction was equivalent to the negative control when the isothiocyanate compounds were removed from the blend using subtraction flight tunnel assays, although the authors did not report the response to only isothiocyanate compounds in the flight tunnel. In an olfactometer, Bartlet et al. (1993) showed cabbage seed weevils did not display a preference for isothiocyanate compounds compared to complete extracts (singly or as a blend). Field tests with cabbage seed weevils (Smart and Blight 1997) showed slightly higher trap catches in traps baited with isothiocyanate compounds compared to those baited with other volatiles (except phenylacetonitrile).

There are limited examples of the 'token stimulus' theory outside

of *Brassicaceae*. In field tests, three species of *Arctiid* moths were attracted to traps baited singly with (S)-(+)-hydroxydanaidal and (R)-(-)-hydroxydanaidal, which are species-specific compounds found in asters, *Asteraceae*, (Krasnoff and Dussourd 1989). Additionally, compounds specific to *Allium spp.* stimulated oviposition in the onion fly (*Delia antiqua*) in cage assays (Matsumoto and Thorsteinson 1968). These compounds also attracted the flies in field tests (Vernon et al. 1978, Judd and Borden 1990). Finally, there are studies suggesting that blends of species-specific compounds, along with common green leaf volatiles, may play a role in host plant location. For example, maximal carrot fly (*Psila rosae*) field trap catches were observed to a binomial mixture of *trans*-Asarone (a species-specific volatile common to *Apiaceae spp.*) and (*E*)-2-Hexenal (a common green leaf volatile) (Guerin et al. 1983). A shortcoming of this theory, however, is that the examples are limited, especially compared to the number of studies that support the 'ratio-specific' odor recognition theory (Bruce et al. 2005, Bruce and Pickett 2011).

Ratio specific blends of common compounds

A more recent theory suggests that insects use ratio-specific blends of ubiquitous plant volatiles to locate the host plant (Bruce et al. 2005, Bruce and Pickett 2011). Bruce et al. (2005) explains that host plant recognition occurs through the detection of common plant volatiles in species-specific ratios. Common plant volatiles are defined as certain fatty acid derivatives, phenyl propanoids, and terpeniods (Bruce et al. 2005, *see Table 2*). Bruce and Pickett (2011) expanded on this theory to

explain that these compounds act synergistically, meaning the activity of a blend of compounds exceeds that of the summed activity of individual components (Bruce and Pickett 2011). Additionally, because a blend is comprised of multiple components, an insect may also recognize a subset of the components of a blend as a host blend. This behavioral plasticity is important because the plant volatile profile is dynamic and can change in response to different stresses (Kessler 2015). Furthermore, ratios of blend components could indicate plant quality (Tasin et al. 2011, Späthe et al. 2013). Discrimination could occur through the detection of either a) incomplete blends, b) blends in incorrect ratios, or c) non-host volatiles (that are also ubiquitous). According to this theory, a flying insect would detect a species-specific blend of common volatile compounds, identify the source as a host plant, and initiate oriented upwind flight towards the odor source (Figure i). Conversely, if a non-host blend is detected using either a, b, or c (above), oriented upwind flight is not initiated. Therefore, the point of host plant discrimination occurs upon the detection of a correct blend or incorrect blend.

Although a plant volatile profile can consist of over 100 compounds (Visser 1986), there are many electrophysiological studies showing that insects detect a small subset of ubiquitous plant volatiles (*Reviewed in* Bruce et al. 2005 *see Table 2*, Pickett et al. 2012 *see Table 1*). Electrophysiology data has been summarized for 27 insects across 5 orders to 21 common plant volatiles (Bruce et al. 2005). Out of the 322 electrophysiological recordings (245 combinations were not performed), 97% of the tested compounds could be detected by the selected insects. This large data set indicates that diverse clades of insects can detect the

same plant compounds, which supports evidence that the olfactory receptors are highly conserved across the class *Insecta* (Bruce et al. 2005). The optimal blends/ratios of these compounds in specific blends for insect species likely differ. More importantly, electrophysiological assays are used to screen candidate compounds (*see above*), and do not imply a behavioral function. Behavioral assays are necessary to determine the behavioral significance of these antenally active compounds.

In addition to electrophysiological studies, there are many behavioral studies that support the use of ubiquitous volatiles to locate a host (Bruce et al. 2005, Bruce and Pickett 2011). For example, 9 volatiles were identified from sacred datura, *Datura wrightii*, to be antenally active to tobacco hornworm moths, *Manduca sexta*, (Riffell et al. 2009). A synthetic blend of these 9 compounds elicited upwind flight and feeding responses similar to the plant extract in flight tunnel assays. Additionally, subtraction assays were performed, and 3 separate subsets of the complete active blend were identified to elicit similar levels of behavior, suggesting these volatiles could be used in host plant location (Riffell et al. 2009b). However, 5 additional subsets of the complete volatile blend elicited lower levels of upwind flight and feeding, suggesting the presence of 'essential' volatile compounds (or combinations of volatile compounds) to indicate an acceptable host.

A second example of insect host location using specific blends of host volatiles is the *R. pomonella*-host plant complex (Zhang et al. 1999, Nojima et al. 2003a, 2003b, Linn et al. 2003, 2012, Cha et al. 2011c, 2011b, 2012, Powell et al. 2012). The apple maggot fly has three host races that display a preference for their natal host, which they locate using

different blends of ubiquitous volatile compounds (Zhang et al. 1999, Nojima et al. 2003b, 2003a, Linn et al. 2003). This discrimination is facilitated through the detection of antagonistic compounds found in non-host blends (Linn et al. 2003). Interestingly, the addition of 3-methylbutan-1-ol (an essential component of the hawthorn blend) to the otherwise attractive apple blend significantly reduced upwind flight of flies to the apple blend. Similarly, the addition of butyl hexanoate in high concentrations (as found in the apple blend) to the otherwise attractive hawthorn blend significantly reduced upwind flight of flies to the hawthorn blend. The fact that butyl hexanoate was present in the attractive hawthorn blend, but antagonized oriented flight in the hawthorn host race when the concentration was increased to that of the apple blend suggests insects can use ratio-specific blends to discriminate between potential hosts.

A weakness, however, of the 'ratio specific blends' theory is that many of the studies presented lack complete behavioral studies to synthetic blends of antenally active compounds (Bruce et al. 2005; *see Table 2*). Only 3 of the 20 studies contained behavioral assays to synthetic blends (Blight et al. 1997, Baoyu et al. 2001, Zhao and Kang 2002). Six studies that lacked behavioral assays were followed up by additional studies with such assays (Dickens 1986, Evans and Allen-Williams 1998, Honda et al. 1998, Casaña-Giner et al. 2001, Bruce and Cork 2001, Gregg et al. 2010). Dickens (1986), however, only assayed a single antenally active component (not a blend). The remaining studies supported the 'ratio specific blends' theory with electrophysiological recordings coupled with behavioral assays to either plant material and/or

chemical extracts of plant material. As explained previously, however, the behavioral significance of the antenally active volatiles remain unknown. In spite of this potential weakness studies continue to be published with behavioral assays to synthetic blends of EAD active compounds, which offer more widespread support for this theory (Alagarmalai et al. 2009, Sasso et al. 2009, Williams et al. 2010, Riolo et al. 2012, Cha et al. 2012a, 2017, Zhang et al. 2014, Samantaray 2016).

A theory of appropriate/inappropriate landings

Finch and Collier (2000) proposed a theory that focused on host plant acceptance rather than on the oriented upwind flight of an insect to a particular plant. A major difference between the appropriate/inappropriate landings theory and the two previous theories is the role of host plant volatiles. In the previous theories, host plant volatiles (either unique compounds or specific blends of ubiquitous compounds) initiate oriented upwind flight towards a the odor source (Fraenkel 1959, Bruce et al. 2005). However, in the appropriate/inappropriate landings theory (Finch and Collier 2000), nonspecific plant volatiles (not necessarily from a host plant) stimulate a flying insect to land indiscriminately on a green surface/object (Figure i, green). Upon landing, the insect makes a series of 'spiral flights', or multiple post-landing assessments using gustatory receptors of the plants to discriminate between host (appropriate landings) and non-host (inappropriate landings) plants (Kostal and Finch 1994). Before accepting a host plant, an insect must make a series of consecutive appropriate landings, as an inappropriate landing indicates the presence of a non-host

plant (Finch and Collier 2000). Therefore, the point of host plant discrimination occurs post landing, and is reinforced with each subsequent appropriate landing.

There are limited examples supporting the appropriate/inappropriate landings theory. The best known theory is the cabbage root fly, *Delia radicum, (*Kostal and Finch 1994, Finch and Kienegger 1997). Kostal and Finch (1994) observed the landing and post-landing behavior of gravid cabbage root flies in response to host plants grown in different backgrounds. These backgrounds consisted of either bare soil, clover, grass, peas, green artificial plants, brown artificial plants, green paper, and brown paper. More than 4 times the females landed on the plants in the bare soil backgrounds than on the plants surrounded by plants. Five times the number of flies landed on the bare soil than on the grass when paired with a host plant, and twice as many females landed on the green paper than the brown paper when paired with a host plant. Additionally, the post-landing behavior was observed to plants grown in soil and plants grown in grass. Plants grown in soil averaged 109 landings per 15 minutes (soil averaged 11 landings per 15 minutes), and plants grown in grass averaged 57 landings per 15 minutes (grass averaged 166 landings per 15 minutes). These assays suggest gravid cabbage root flies use visual cues to land on a green objects/backgrounds. Oviposition studies revealed that gravid cabbage root flies lay more eggs on host plants surrounded by soil than host plants surrounded by artificial plants or paper (regardless of color), and the flies made an average of 4 spiral flights before laying an egg. A follow up study explored the oviposition behavior of 8 different insects across three orders on host

plants in either bare soil or living/dead clover plants (Finch and Kienegger 1997). Undersowing with clover plants reduced oviposition from 39-100%, which the authors suggest is due to the increased number of inappropriate landings.

A strength of the cabbage root fly study is that it was developed through the use of thorough behavioral assays and observations. A potential weakness is that few other examples exist in the literature supporting this study, as the behavioral assays are not as widespread (compared to flight tunnel assays used in the previous theories). Another weakness is that no assays were performed using extracts, synthetic odor sources, or odorless sources, and therefore the definitive role of volatiles remains unknown.

Habitat cues

In a recent paper, Webster and Cardé (2016) suggest that insects use nonspecific habitat cues to identify a favorable habitat. Once in the favorable habitat, insects can then use more species-specific cues to locate a host (Webster and Cardé 2016). Habitat cues differ from host cues in the fact that they are generally not species-specific, are released in large quantities, can be detected at long distances, and are associated with host-specific cues (Webster and Cardé 2016). Webster and Cardé (2016) suggest that habitat cues elicit general upwind movement, localized searching behavior, and enhanced response to host odor cues. Insects can use these habitat cues to increase the probability of detecting specific host cues.

Examples of habitat cues can include $CO_2$, differences in relative

humidity, and green leaf volatiles. For example, blood-feeding insects use $CO_2$ as a long-range orientation cue (Benton and Lee 1965, Fallis and Raybould 1975, Voskamp et al. 1999, Pinto et al. 2001, Barrozo and Lazzari 2006, Lacey et al. 2014). Foraging phytophagous insects may also use $CO_2$ to discriminate between nectar sources. Thom et al. (2004) demonstrated that tobacco hornworm moths preferred artificial flowers with elevated $CO_2$ levels compared to those with ambient levels of $CO_2$. Further studies are necessary to explore the role of $CO_2$ as a long-distance attractant in phytophagous insects.

Humidity may also play a role in insect habitat location. The role of humidity has been explored in tobacco hornworm moths (*Manduca sexta*) in a foraging context (Wolfin et al. in prep). Von arx et al. (2012) showed that during the first 30 minutes of anthesis, the floral headspace of newly opened *Oenothera cespitosa* flowers produced local humidity levels ~4% higher than ambient conditions, and moths approached flowers with the elevated humidity over those with ambient humidity levels. The increased number of flower visits indicates the moths can detect small differences in relative humidity, and these differences can affect moth in-flight behavior.

Ubiquitous, nonspecific green leaf volatiles may also play a role in insect habitat location. Tobacco budworm moths, *Heliothis virescens,* displayed increased attraction to, and laid more eggs on tobacco plants supplemented with synthetic Germacrene-D (a common green leaf volatile) compared to control tobacco plants that do not produce Germacrene-D (Mozuraitis et al. 2002). An insect might use any or all of these cues to locate a favorable habitat, and then to search for and select a

specific host plant. Additional studies are necessary to fully understand the role of nonspecific plant cues as a long distance olfactory attractant.

Summary of theories

The four theories for host plant location are summarized in Table ii. Some aspects of these models are similar. For example, the token stimulus and ratio-specific blends theories involve the same cascade of behaviors (Figure i). In both models, the insect takes flight due to favorable environmental conditions, and initiate oriented upwind flight upon detecting a favorable odor plume. The insect follows the plume, and lands on (or near) the odor source. However, the major difference between the two models is the widespread abundance of the volatiles that elicit the behaviors. In the token stimulus model, unique compounds drive the oriented upwind flight, whereas in the ratio-specific blends model, ubiquitous compounds drive the oriented upwind flight.

Some aspects of these theories are not mutually exclusive. For example, both the ratio-specific blends theory and the habitat odors theory suggest ubiquitous volatiles initiate oriented upwind flight towards the odor source. The difference between these two theories is the point at which discrimination occurs. In the ratio-specific blends theory, the insect recognizes the host plant upon the detection of the favorable blend, whereas in the host odors theory, the insect discriminates using close range cues. Additionally, both the appropriate/inappropriate landings theory and the habitat odor theory suggest plant volatiles can be nonspecific cues, and plant discrimination occurs at close range. The major difference between these two models is the role of the nonspecific

plant volatiles. In the appropriate/inappropriate landings theory, the nonspecific plant volatiles initiate indiscriminate landing on a green object/surface, whereas in the habitat odor theory the nonspecific volatiles initiate oriented upwind flight to a general location where a host plant may be located.

A Final Thought on Insect Host Location

A common theme to all theories is the separation of 'long range' and 'close range' cues. However, there is very little discussion of the distances (and scales) associated with 'long range' and 'close range' cues. In fact, I would argue that it is not suitable to associate these different cues (and their associated behaviors) with finite distances because of the diverse life histories of insects. For example, host plant location can occur within a flower patch where an insect can travel centimeters to choose a host (von Arx et al. 2012), to the landscape scale, where an insect can travel kilometers to locate a favorable habitat (Janzen 1984, Powell and Brown 1990). Therefore, rather than define 'close range' and 'long range' by the distance travelled, I propose that this distinction be made by the behaviors exhibited. Regardless of distance travelled, if an insect displays oriented upwind flight to locate a host, then the scale should be considered 'long range orientation'. Similarly, if an insect does not display oriented upwind flight, then the scale is considered to be 'close range'. For example, the token stimulus theory, ratio-specific blends theory, and habitat odor theory all involve oriented upwind flight toward the odor source, and would then be considered 'long range'. Conversely, the appropriate/inappropriate landings theory simply involves

non-directional flight until volatiles stimulate landing behavior, which would all therefore be considered ‘close range’ behaviors.

*Grape berry moth (Paralobesia viteana)-grape plant (Vitis spp.) complex as a model to study host plant location*

The grape berry moth (GBM; *Paralobesia viteana*) is a tortricid moth native to the eastern United States (Taschenberg and Carde 1974), and is an important pest of cultivated grape, *Vitis spp.,* (Williamson and Johnson 2005). The GBM is an ovipositional specialist, laying its eggs almost exclusively on grape clusters and leaves (Clark and Dennehy 1988). The animal ranges from 1-2 generations in southern Ontario to 3-4 in Arkansas (Williamson and Johnson 2005), and has three generations in the Finger Lakes Region of New York State, U. S. (Hoffman et al. 1992). Although male flight activity is currently monitored by pheromone traps (Taschenberg and Carde 1974), these traps are poor predictors of female flight activity (Weigle et al. 1998). Cha et al. (2008a) showed that GBM females displayed oriented flight toward host plant material in a flight tunnel. A blend of eleven behaviorally active compounds were identified to attract GBM females in the flight tunnel, and two different 7-component blends were found to elicit equivalent behavior under the same conditions (Cha et al. 2008b).

The GBM–grape plant complex represents an excellent system to explore the proximate olfactory mechanisms of host plant location: First, the GBM is a specialist, which means gravid females must discriminate between host and non-host plants to ensure the survival of their larvae

(Davis and Cipollini 2014). Second, synthetic blends of host plant volatiles have already been shown to attract GBM females in the flight tunnel (Cha et al. 2008b). These blends are comprised of ubiquitous plant volatiles, and appear to be ratio-specific, as changing ratios diminish flight tunnel responses (Cha et al. 2008b, 2011a). In the following chapters the GBM system is used to test the 'ratio-specific blends' theory. In Chapter 1, I used flight tunnel assays to observe GBM responses to non-host plants, and isolate and identify the volatiles that elicit the observed behavior. The results of this study, rather than supporting the 'ratio-specific blends' theory indicate that for this specialist insect that the habitat odor hypothesis might be more applicable. In Chapter 2, I identified the stimuli required to elicit the GBM to land, and also explore the potential role of water vapor in the host-location process. The results of this study indicate that GBM females require visual cues and water vapor to land on an odor source, and that individual or paired stimuli did not elicit equivalent landing responses to the responses to host plant. Additionally, water vapor elicited low levels of upwind flight, and that water vapor paired with synthetic blends also elicited a low level of landing. In Chapter 3, I explored whether microorganisms living on plant tissue contribute to the production of the behaviorally active compounds. The results of this study indicate the plant tissue rather than microorganisms produce the behaviorally active volatiles. The results of these studies indicate that host plant discrimination may not occur at a distance, and that an insect can use additional sensory inputs as it approaches the plant to continue its cascade of behaviors and eventually accept a potential host.

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# CHAPTER 1

# PROXIMATE MECHANISMS OF OLFACTORY-MEDIATED HOST PLANT LOCATION BY A SPECIALIST PHYTOPHAGOUS INSECT, THE GRAPE BERRY MOTH, *PARALOBESIA VITEANA*


***Abstract***

There are contrasting theories regarding the olfactory-mediated mechanisms of host plant location. Here we use the grape berry moth (GBM; *Paralobesia viteana*)-grape plant complex as a model for studying the proximate mechanisms of long distance olfactory-mediated host plant selection by a specialist phytophagous insect. We used flight tunnel assays to observe GBM female in-flight responses to host (grape, *Vitis* riparia) and non-host (apple, *Malus domestica*; and gray dogwood, *Cornus racimosa*,) odor sources (plants, extracts, and synthetic blends). Gas chromatography coupled with electroantennographic detection (GC-EAD) and GC-MS were used to identify the antenally active volatile compounds. All antenally active compounds found in grape shoots were also present in dogwood and apple shoots. Female GBM displayed higher levels of upwind flight to non-host shoots than expected, and flew upwind to host and non-host extracts and synthetic blends at similar levels, suggesting discrimination is not occurring at a distance. However, moths did not land on rubber septum sources releasing the extracts and synthetic blends while landing on host and non-host plant shoots, suggesting not all landing cues were present in the volatile blends. Additionally, mated and unmated moths displayed similar levels of upwind flight responses to all odor sources, suggesting further that plant volatiles are not functioning

only as ovipositional cues. The results of this study, contrary to our original hypothesis for a specialist phytophagous insect, support the conclusion that these insects are using volatile blends to locate a favorable habitat rather than a specific host plant, and that discrimination is occurring within the habitat, or even post-landing.

## *Introduction*

An organism's survival depends on the location of patchily distributed resources. The distribution of plants, for example, is often mediated by patchy resources such as sunlight or soil nutrient availability (Galiano 1985, Cole and Weltzin 2005), as well as competitive interactions between plants. Plants themselves are important resources to herbivores and pollinators, and can be difficult to locate when they are patchily distributed (Miller and Strickler 1984). Phytophagous insects, in particular can use their host plants as food, courtship/mating locations, and oviposition sites (Dethier 1941, Thorsteinson 1953, 1960, Schoonhoven 1968, Van Der Pers et al. 1980, Landolt and Phillips 1997, Schoonhoven et al. 2005, Tilmon 2008), and there is evidence that plant volatiles can play a critical role in the location of a host plant (Fraenkel 1959, Finch and Collier 2000, Bruce et al. 2005, Bruce and Pickett 2011). However, the precise role of these volatiles (and the behaviors they elicit) in the host location process remains poorly understood, and there is much debate regarding the mechanisms of olfactory-mediated host plant location (Fraenkel 1959, Finch and Collier 2000, Bruce et al. 2005).

Four principle theories describing host plant location are illustrated in Figure 1.1. The first, commonly referred to as the 'token stimulus theory', Fraenkel (Fraenkel 1959) suggested that host plant choice is based solely on the presence of 'odd compounds' specific to a particular taxon of plants (Figure 1.1, red, purple). The best example to support this theory is the aphid/mustard plant-*Brassica* spp., complex. Aphids that specialize on mustard plants locate their host using isothiocyanate

compounds (Pickett 1992, Webster et al. 2008, Döring 2014), which are almost exclusively found in *Brassica* spp. (Ahuja et al. 2009). According to this theory, species-specific compounds stimulate an insect to fly upwind and land on the odor source (Figure 1.1, red, purple).

Although there are other examples of 'token stimulus' compounds (Fraenkel 1959), in a second theory Bruce et al. (Bruce et al. 2005) suggested that in the majority of cases, phytophagous insects use mixtures of compounds commonly found in the environment to locate their host plant (Figure 1.1 blue, purple). Electrophysiological studies from insects across five orders have demonstrated the use of ubiquitous plant volatiles for host plant location (*see Table 2 in* (Bruce et al. 2005)). According to this theory, specific ratios of volatile compounds stimulate an insect to fly upwind and land on the odor source (Figure 1.1, blue, purple). Furthermore, if the behaviorally active compounds are common plant volatiles, then an insect would use specific blends of these compounds for host plant discrimination, and volatile compounds unique to non-host plants could have an antagonistic effect.

There are documented cases of insects using antagonist compounds to discriminate between volatile mixtures produced by closely related species or host races. The first involves sex pheromones, where, for example, male moths of the tobacco budworm, *Heliothis virescens*, discriminate between a sex pheromone blend of conspecific females and females of the closely related species *Heliothis subflexa* through the detection of (*Z*)-11-hexadecenyl acetate (Z11-16:OAc). This compound is not a component of the *H. virescens* sex pheromone blend, but is

produced by *H. subflexa* females (Teal et al. 1986, Heath et al. 1991). *Heliothis virescens* males are sensitive to this compound (Vickers and Christensen 2003), and the presence of Z11-16:OAc in an otherwise attractive sex pheromone blend arrests the male upwind flight response (Vickers and Baker 1997).

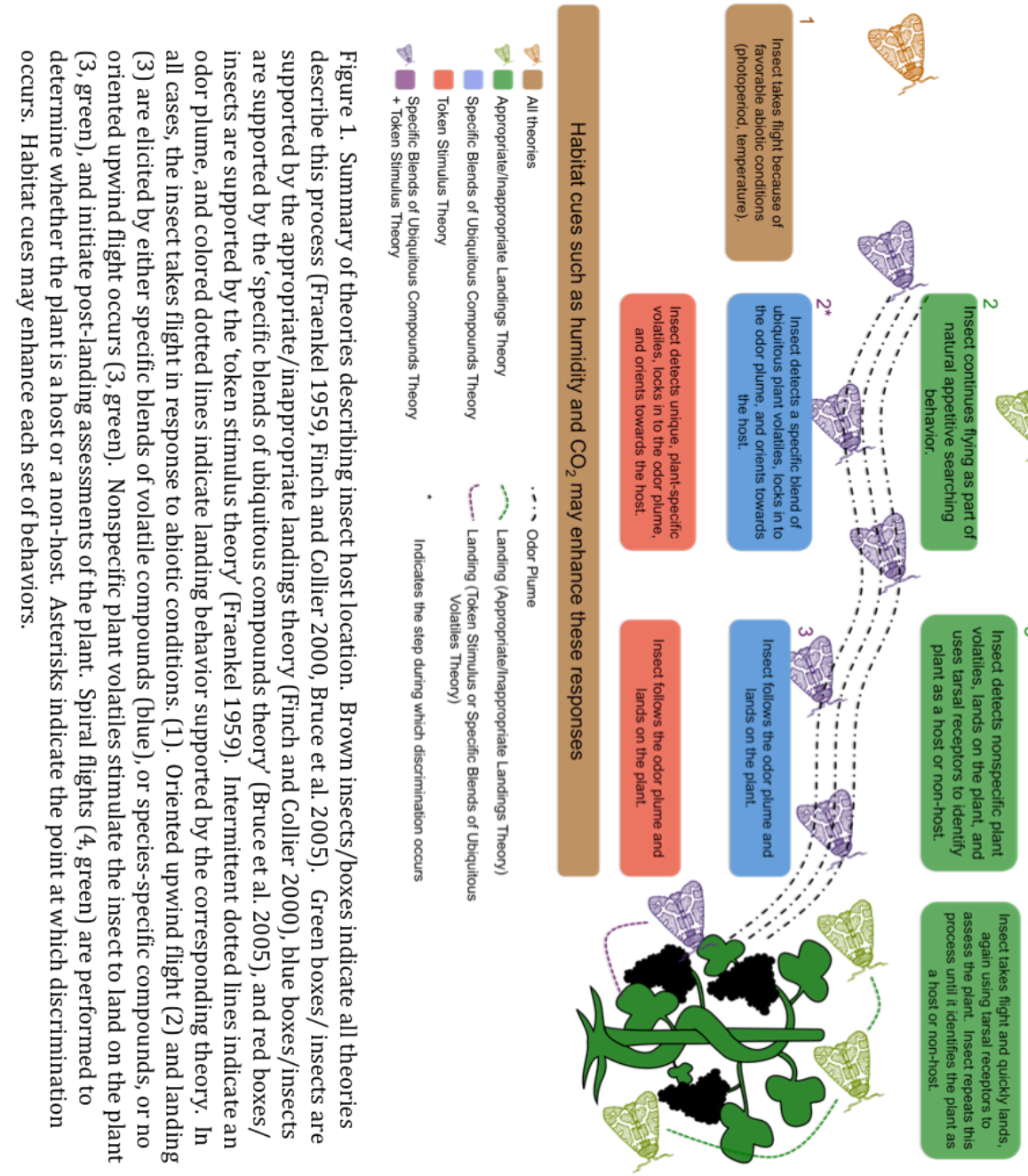



Figure 1. Summary of theories describing insect host location. Brown insects/boxes indicate all theories describe this process (Fraenkel 1959, Finch and Collier 2000, Bruce et al. 2005). Green boxes/ insects are supported by the appropriate/inappropriate landings theory (Finch and Collier 2000), blue boxes/insects are supported by the 'specific blends of ubiquitous compounds theory' (Bruce et al. 2005), and red boxes/ insects are supported by the 'token stimulus theory' (Fraenkel 1959). Intermittent dotted lines indicate an odor plume, and colored dotted lines indicate landing behavior supported by the corresponding theory. In all cases, the insect takes flight in response to abiotic conditions. (1). Oriented upwind flight (2) and landing (3) are elicited by either specific blends of volatile compounds (blue), or species-specific compounds, or no oriented upwind flight occurs (3, green). Nonspecific plant volatiles stimulate the insect to land on the plant (3, green), and initiate post-landing assessments of the plant. Spiral flights (4, green) are performed to determine whether the plant is a host or a non-host. Asterisks indicate the point at which discrimination occurs. Habitat cues may enhance each set of behaviors.

A similar strategy is used in different host races of apple maggot flies, *Rhagoletis pomonella*, to discriminate between host and non-host fruit where mating and oviposition occur. Flies specializing on apple, *Malus domestica*, and hawthorn, *Crataegus mollis*, were flown to host and non-host synthetic blends in the flight tunnel. Flies displayed maximal levels of upwind oriented flight to their host blends compared to non-host blends (Linn et al. 2005). This blend discrimination is facilitated through the detection of compounds found in non-host blends (Linn et al. 2003). For example, the addition of 3-methylbutan-1-ol (an essential component of the hawthorn blend) to the otherwise attractive apple blend significantly reduced upwind flight of flies to the apple blend. Similarly, the addition of butyl hexanoate in high concentrations (as found in the apple blend) to the otherwise attractive hawthorn blend significantly reduced upwind flight of flies to the hawthorn blend. However, these studies also showed that a low (10-30%), proportion of flies in each host race population tested exhibited what was termed 'broad response', flying upwind to their host blend as well as the non-host blend. (Linn et al. 2005). Broad responders could have significant evolutionary importance as a source of genetic variation that could allow for sympatric speciation through shifts to new host plants (Linn et al. 2005, Powell et al. 2012, Clifford and Riffell 2013).

Finch and Collier (2000) proposed third theory that focused on host plant acceptance rather than on the oriented upwind flight of an insect to a particular plant. A major difference between the appropriate/inappropriate landings theory and the two previous theories is the role of host plant volatiles. In the previous theories, host plant

volatiles (either unique compounds or specific blends of ubiquitous compounds) initiate oriented upwind flight towards the odor source (Fraenkel 1959, Bruce et al. 2005). However, in the appropriate/inappropriate landings theory (Finch and Collier 2000) nonspecific plant volatiles (not necessarily from a host plant) stimulate a flying insect to land indiscriminately on a green surface/object (Figure 1.1, green). Upon landing, the insect makes a series of 'spiral flights', or multiple post-landing assessments using gustatory receptors of the plants to discriminate between host (appropriate landings) and non-host (inappropriate landings) plants (Kostal and Finch 1994). Before accepting a host plant, an insect must make a series of consecutive appropriate landings, as an inappropriate landing indicates the presence of a non-host plant (Finch and Collier 2000). Therefore, the point of host plant discrimination occurs post landing, and is reinforced with each subsequent appropriate landing.

In a fourth theory, habitat location is proposed to be an important first step in the host selection process (Webster and Cardé 2016). Because specific host plant(s) may be difficult to locate in a habitat where many plant species exist, an insect might first search for a favorable habitat that is associated with the host plant to increase the probability of finding a host (Figure 1.1, brown; Bell 1990, Meiners 2015, Webster and Cardé 2016). Insects also may use nonspecific habitat cues such as $CO_2$ (Faucher et al. 2013) or differences in relative humidity (Janzen 1987) to aid in the location of a favorable habitat. Green leaf volatiles (GLVs) may also be important habitat cues. Tobacco budworm moths (*H.*

*virescens*) displayed increased attraction to, and laid more eggs on, tobacco plants supplemented with synthetic Germacrene-D (a common green leaf volatile) compared to control tobacco plants that do not produce Germacrene-D (Mozuraitis et al. 2002). An insect might use any or all of these cues to locate a favorable habitat, and then to search for and select a specific host plant.

The grape berry moth, *Paralobesia viteana*, is a tortricid moth native to the eastern United States (Taschenberg and Carde 1974), and is an important pest of cultivated grape (Williamson and Johnson 2005). Moths emerge in natural habitats about one week before they emerge in vineyards, and vineyards adjacent to wooded areas sustain higher levels of damage caused by larvae (Botero-Garcés et al. 2003). Furthermore, moths are found in highest abundances in wooded areas and at the vineyard edge early in the season, and move inward later in the season, suggesting the moths shift from wild to cultivated hosts as the season progresses. The GBM is an ovipositional specialist, laying its eggs almost exclusively on grape clusters and leaves (Clark and Dennehy 1988). It ranges from 1-2 generations in southern Ontario, Canada to 3-4 in Arkansas (Williamson and Johnson 2005). The GBM has three generations in the Finger Lakes Region of New York State, U. S. (Hoffman et al. 1992). Although male flight activity can be monitored with traps baited with synthetic sex pheromone (Taschenberg and Carde 1974), these traps are poor predictors of female flight activity (Weigle et al. 1998). Cha et al. (2008a) showed that GBM females displayed oriented flight toward host plant material in a flight tunnel. A blend of eleven behaviorally active compounds was identified that elicited upwind

oriented flight by GBM females in the flight tunnel, and two different 7-component blends were found to elicit equivalent levels of behavior under the same conditions (*see Table 1 in* (Cha et al. 2008b)). The identified compounds are all common plant volatiles, which supports the 'specific blends of common volatiles' theory for host location (Bruce et al. 2005).

Because of its specialist host status and response to blends of ubiquitous volatiles, the grape berry moth-grape plant complex represents an excellent system to test host plant discrimination theories, especially that proposed by Bruce et al (Bruce et al. 2005) involving ubiquitous blends and antagonist compounds.

The goal of this study was to determine whether GBM females can discriminate host from non-host plants, and whether the discrimination involves detection of non-host antagonist compounds that arrest long distance upwind flight. Apple, *Malus domesticus*, and gray dogwood, *Cornus racimosa*, were chosen as non-host plants because of their overlapping range and phenology with grape (Childers 1961, Hokanson et al. 2001, This et al. 2004, Hadziabdic et al. 2010). We used flight tunnel assays to record the insect's behavioral response to host and non-host plants. We collected volatiles from each plant, and used flight tunnel assays, GC-EAD and GCMS in an iterative process to create a behaviorally active volatile blend for each plant. Contrary to our predictions for this specialist insect we did not find evidence supporting long distance discrimination or antagonism, but rather that females responded equally well to the three plant species. We discuss the results in the context of the other theories discussed above for host plant location in

phytophagous insects.

## *Methods*

### *Insects*

Grape berry moths were reared in cages placed in walk-in environmental chambers at 26°C and 60% RH under a 16:8 L:D photoperiod. Adults were allowed to oviposit on seedless grapes, *Vitis vinifera*, red flame variety. First and second instar larvae were transferred to a diet cup (30 mL, WinCup Inc.) and reared on semi-synthetic diet (Nagarkatti et al. 2000) that consisted of grapes, pinto beans, and commercially available tobacco hornworm diet (Bio-Serve). For behavioral assays, unmated female moths were taken from cohorts set up by placing 10-15 female pupae (near eclosion) in a Plexiglass mating cage (30 cm H ×30 cm W ×30 cm D) and provided with a 50% honey and water solution. Twenty male moth pupae were added to additional mating cages loaded with 10-15 female pupae to assay mated females. For all flight tunnel assays reported below both unmated and mated females were tested to each treatment.

### *Plants*

*Vitis riparia,* a native host species of the GBM in northeastern USA, was used as the host plant for these experiments. Jonagold apple trees, *M. domestica*, and Gray Dogwood, *C. racimosa*, were used as non-host plants for these experiments. All plants were maintained in a greenhouse as in Cha et al. (Cha et al. 2008a) with temperatures

maintained between 21-26 ˚C. Supplemental light was provided to extend the day length to 16 h.

*Adsorbent Sampling*

We used a push–pull collection system to collect headspace volatiles of live grape, apple, and gray dogwood plants. The system was a custom-made, bell-shaped glass chamber (18 cm ID, 10 L) with two air-in adapters (7 mm ID) on the top and four air-out adapters (7mmID) equally distributed at the bottom wall of the chamber. The glass chamber was placed on two pieces of Pyrex glass with a hole (2 cm) in the middle so that the vegetative portion of the plant could be sampled to accommodate a whole, live potted plant. After a plant was set up in the chamber, the chamber was flushed with filtered air (5 L/min) for 24 hours to replace air inside the chamber with filtered air and to stabilize volatile emission from the plant, because we noticed that handling of the plant during set up temporarily induced release of green leaf volatiles. During the collection, flow meters were used to ensure that more filtered air was pushed into the chamber than pulled out through the charcoal filters so as to eliminate possible contamination from outside air. Filtered clean air was pushed into the chamber at 5.0 L/min and volatiles from the headspace of grape shoots were drawn by a vacuum pump onto four activated charcoal filters at 1.2 L/min/filter (ORBO32-small, Supelco Inc., Bellefonte, PA, USA). Adsorbent samplings were made over 4 days in the greenhouse (18:6 L:D). The chamber was washed with acetone, and new ORBO filters were used for a new plant. The volatiles were eluted with 300 µL hexane every 24 h and then combined. The combined extract was concentrated to 1 mL

under a gentle stream of nitrogen gas and kept in a freezer (−20°C) and subjected to GC-EAD and gas chromatography–mass spectrometry (GC-MS) analyses, and flight tunnel bioassays.

*Coupled GC-EAD Analysis*

A Hewlett-Packard 5890 Series II gas chromatograph, equipped with either a DB-1 capillary column (30 m×0.25 mm ID, 0.25 μm film thickness; J&W Scientific), a DB-5 capillary column (30 m×0.25 mm ID, 0.25 μm film thickness; J&W Scientific), or a DB-Wax capillary column (30 m×0.25 mm ID, 0.25 μm film thickness; J&W Scientific) was used for GC-EAD analyses. The oven temperature was programmed from 40°C for 5 min then increased by 15°C min−1 to 250°C. Injector and detector temperatures were set at 280°C and 270°C, respectively. Splitless injection was used with nitrogen as the carrier gas at a flow of 2 mL/min. The column effluent was split in a ratio of 1:1 in the oven to the flame ionization detector and to the heated (270°C) EAD port.

A whole head was removed from a 3-day-old virgin female GBM and mounted on a saline-filled micropipette in an acrylic holder as described in (Nojima et al. 2003, Cha et al. 2008b). Both antennae were positioned in the other saline-filled micropipette. We used an Ephrussi–Beadle insect Ringer as saline (Ephrussi and Beadle 1936). The tips of both antennae were dipped in saline containing surfactant (0.02% Triton X-100) for easy manipulation. The antennal holder was placed inside a humidified cooling condenser maintained at 10°C. A minimum of five different antennal pairs were used to analyze volatiles from plant shoot

extracts. Synthetic blends were prepared according to the ratios in Table 1.2, and diluted with dichloromethane to a concentration of 0.1 mg/mL.

*Chemical analysis*

Chemical extracts were analyzed using an Agilent 5890 gas chromatograph coupled with a 5973n mass selective detector running in EI mode at 70 eV. The GC was equipped with a DB-1 non-polar column (30 m×0.25 mm ID, 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA), or a DB-Wax polar column (30 m×0.25 mm ID, 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA). Helium was used as the carrier gas at a constant flow of 1.0 mL/min. The oven temperature was programmed to hold at 40°C for 5 min then increase by 15°C/min until the oven reached 250°C, and hold at that temperature for 5 min. Volatile compounds were tentatively identified by mass spectral matches to library spectra and confirmed by retention time and mass spectral matches to available authentic standards.

*Chemicals*

(*Z*)-3-hexen-1-yl acetate, ethyl hexanoate, nonanal, racemic linalool, methyl salicylate, decanal, β-caryophyllene, α-farnesene, and were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA), Alfa Aesar (Ward Hill, MA, USA), Fluka (Buchs, Switzerland) or TCI America (Portland, OR, USA). All, except α-farnesene (a mixture of isomers) were greater than 97% purity. The 4,8-dimethyl-1,3(*E*),7-nonatriene was provided by the Chong lab (University of Waterloo, Ontario, CA). Germacrene-D was isolated from golden rod (*Solidago*) as 91%

germacrene-D and 9% β-caryophyllene (by USDA Chemistry Research Unit, Gainesville, FL, USA).

*Flight Tunnel*

The flight tunnel was 2 m in length by 0.6 m in width and 0.6 m in height, with a fan installed at the upwind end to create a steady airflow into the tunnel and an exhaust hood at the downwind end to evacuate odor from the flight tunnel (Cha et al. 2008a, 2008b). Wind speed was set at 0.25 m/s at the wire stand where the moths were introduced into the wind tunnel. A pattern made of dark green paper circles (10 cm diameter) was randomly presented both on a white background glass floor and on the glass ceiling below the light source. During the experiments, the average temperature in the flight tunnel was 23.8 ± 0.07 °C, and the relative humidity ranged from 22% to 71%. Female moths were placed in the flight tunnel room 1 h prior to scotophase. Light intensity was reduced to 25 lx 30 min before dark, and remained at this intensity for behavioral assays. Behavioral assays began 15 minutes prior to scotophase.

The odor source was placed 30 cm from the upwind end of the tunnel. Four- to five-day old females were used in all flight tunnel assays. All insects were discarded after being assayed. Female moths were placed in the flight tunnel individually in a metal screen release cage on a wire stand 1.5 m downwind of the source, and their behavior was observed for 5 min. We recorded whether the insect flew out of the release cage, flew upwind (more than 10 cm of oriented flight towards the source), and landed on the source. Fisher's exact test ($P < 0.05$) was used to compare

the percent response of the GBM females to the different odor sources. A G-test ($P < 0.05$) of independence was used to compare each odor source to the grape shoots and the expected response to a non-host plant.

*Treatments*

The behavioral response of individual moths to control (no odor source; not shown, n = 36) and plant odor sources (summarized in Table 1.1) was observed in the flight tunnel. Freshly cut plant shoots and rubber septa (Thomas Scientific, Swedesboro, NJ, USA) loaded with either synthetic blends or adsorbent extracts were used as odor sources. Shoots were cut 15 cm in length and immediately placed in a water pick as described in previous studies (Cha et al. 2008ab), and were discarded after one flight session. Responses of GBM females to grape shoots were used as positive controls in flight tunnel assays, and the responses of mated and unmated females were observed. Expected response values for a non-host plant was based on responses of female apple maggot (*R. pomonella*) flies to non-host odor sources (Nojima et al. 2003, Linn et al. 2003, Powell et al. 2012). For the current experiments we selected an expected value of 10% response to non-host plants. Septa were loaded with 300 µL of extract or blend and were placed in a fume hood for 1 h to evaporate off the solvent. Septa were stored in a freezer (-20 ˚C) between tests. GC-EAD active blends for each plant were prepared in ratios that corresponded to the ratios of compounds found in the corresponding extract.

Table 1.1 Summary of flight tunnel treatments and number of insects tested.

| Treatments (number of flights) | Grape | Dogwood | Apple |
|---|---|---|---|
| Shoot | 296 | 98 | 148 |
| Extract | 147 | 63 | 109 |
| Blend | 31 | 87 | 99 |

## *Results*

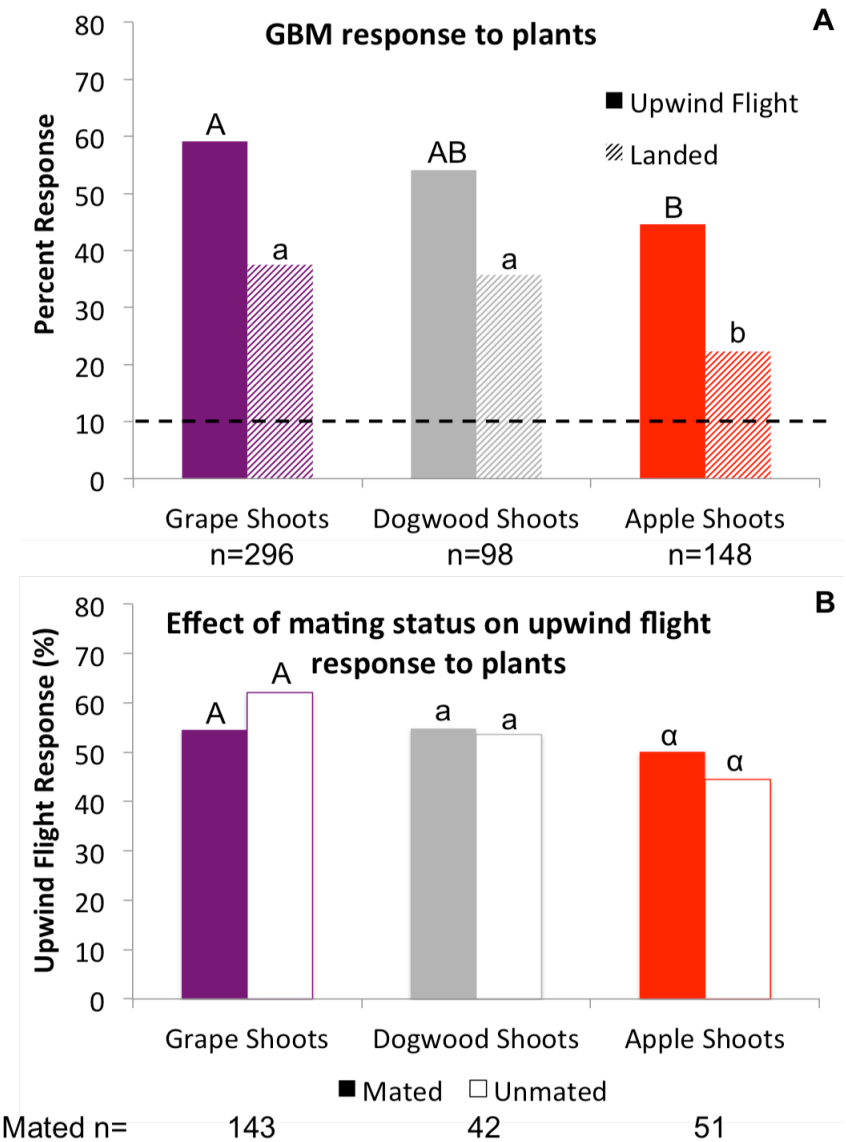
*GBM response to plants*

Grape berry moth females (mated and unmated combined) flew upwind 59.1%, and landed 37.5%, of the time to grape shoots (Figure 1.2A; n = 296). The moths behaved similarly to the dogwood shoots (n = 98), flying upwind 54.1% and landing 35.7% of the time (Fisher's exact test, $P = 0.35$ (Fisher's exact test $P = 0.81$). Female GBM flew upwind (44.6%) and landed (22.3%) in response to apple shoots, a significantly lower percentage of the time (n = 148) than grape (Fisher's exact test, upwind flight $P = 0.005$; landing $P = 0.01$). Females flew upwind similarly to dogwood shoots compared to apple (Fisher's exact test $P = 0.12$), but landed a significantly higher percentage of the time on the dogwood shoots compared with the apple (Fisher's exact test $P = 0.02$). Moths did not fly upwind when no odor source was present (n = 36).

Female GBM flew upwind to and landed on all non-host plants a significantly higher percent of the time than expected, using our 10% threshold for expected broad response individuals (G-test, Dogwood: upwind flight $P < 0.001$, landing $P = 0.002$; Apple: upwind flight $P <$

0.001, landing $P = 0.004$).

When mated and unmated moths are considered separately the results show both groups flew upwind a similar percentage of the time to grape shoots (Figure 1.2B; mated n = 143, 54.6%; unmated n = 153, 61.1%; Fisher's exact test $P = 0.29$), dogwood shoots (mated n = 42, 54.8%; unmated n = 56, 53.6%; Fisher's exact test $P = 0.52$), and apple shoots (n = 51, 50.1%; unmated n = 82, 44.5%; Fisher's exact test $P =$




0.37).

Figure 1.2. GBM response to plants. Flight tunnel response (%) of GBM females to host and non-host plant shoots (Panel A). Different letters (capital for upwind flight response, lower case for landing response) indicate significant differences ($P < 0.05$; Fisher's exact test). The non-host shoots elicited a higher percentage of upwind flight and landing than we expected indicated by dotted line ($P < 0.05$; G-test). Upwind flight of mated and unmated females to plant shoots (Panel B). The same letter (capital for grape shoots, lower case for dogwood shoots, Greek for apple shoots) indicates similar response differences ($P < 0.05$; Fisher's exact test).

*GBM response to extracts*

Grape berry moth females (Figure 1.3A; mated and unmated combined) flew upwind 45.6% of the time in response to the grape extract (n = 147), which was similar to the upwind flight to the dogwood (n = 63, 50.8%, Fisher's exact test $P = 0.55$), and the apple extracts (n = 109, 45.0%, Fisher's exact test $P = 1$). The upwind flight response to the dogwood extract was not significantly different from the response to the apple (Fisher's exact test, $P = 0.53$). All upwind flight responses were significantly higher than the expected value of 10% for broad response individuals (G-test $P < 0.001$).

Female GBM moths did not land in response to the grape or apple extracts, and landed on the dogwood extract only 1.6% of the time.

When considered separately, mated and unmated moths flew upwind a similar percentage of the time to the grape (Figure 1.3B; mated n = 66, 40.9%; unmated n = 81, 49.4%; Fisher's exact test $P = 0.32$), the dogwood (mated n = 19, 47.4%; unmated n = 44, 52.3%; Fisher's exact test $P = 0.79$), and the apple extracts (n = 51, 51.0%; unmated n = 58, 39.7%; Fisher's exact test $P = 0.25$).

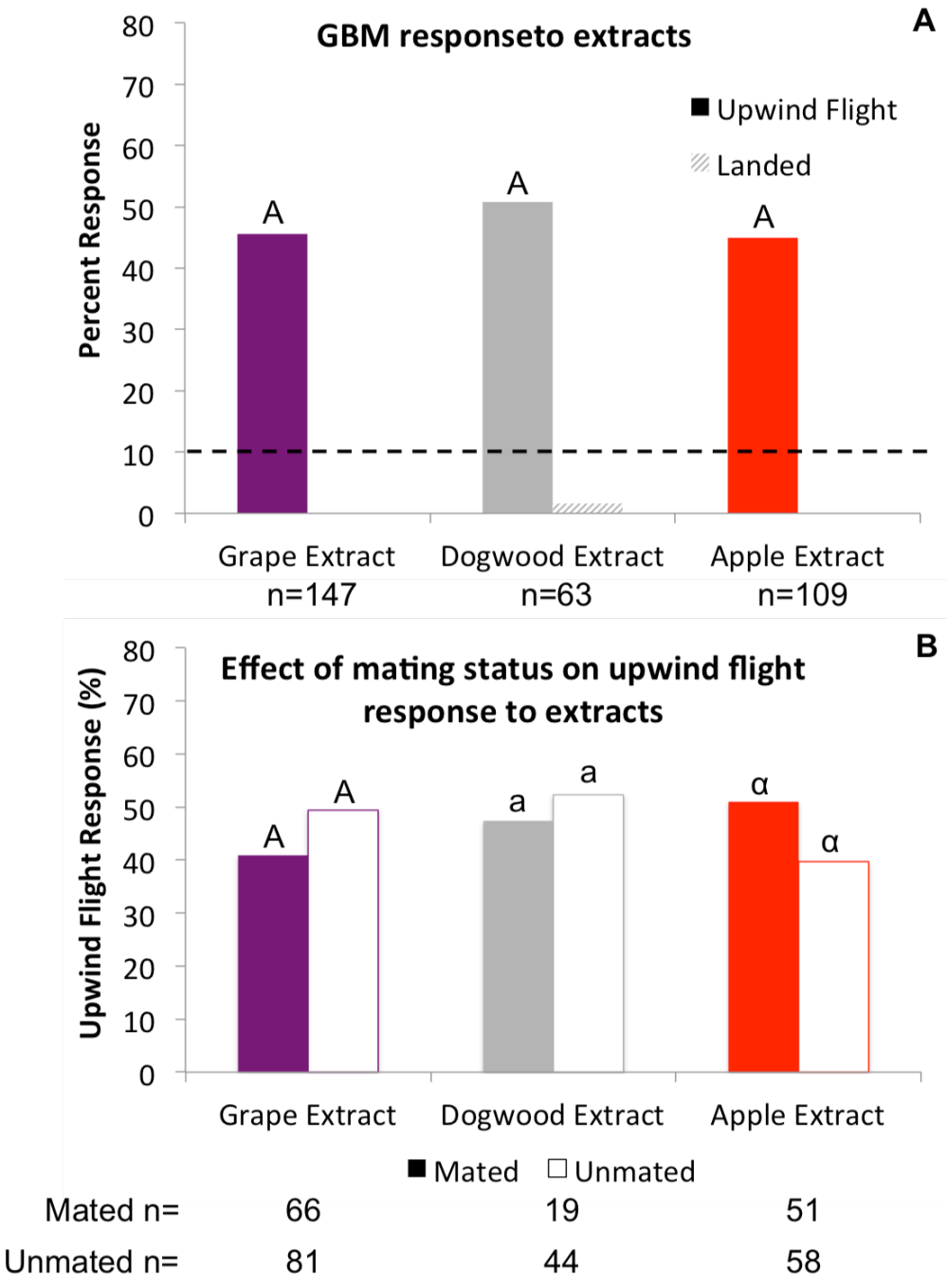



Figure 1.3. GBM response to extracts. Flight tunnel response (%) of GBM females to host and non-host extracts (Panel A). The same letter indicates similar responses ($P < 0.05$; Fisher's exact test). GBM females displayed similar upwind flight responses to all extracts. The non-host extracts elicited a higher percentage of upwind flight and landing than we expected (dotted line) ($P < 0.05$; G-test). Upwind flight of mated and unmated females to extracts (Panel B). The same letter (capital for grape extract, lower case for dogwood extract, Greek for apple extract) indicates similar responses ($P < 0.05$; Fisher's exact test).

*GBM response to synthetic blends*

Female GBM (Figure 1.4A; mated and unmated) flew upwind 51.6% of the time in response to the grape synthetic blend (n = 42), which was similar to the upwind flight to the dogwood (n = 87, 52.9%, Fisher's exact test $P = 1$), and the apple synthetic blends (n = 99, 48.5%, Fisher's exact test $P = 0.84$). The upwind flight response to the dogwood synthetic blend was not significantly different from the response to the apple blend (Fisher's exact test, $P = 0.56$). Upwind flight response levels for all synthetic blends were significantly higher than the expected value of 10% for broad response individuals (G-test $P < 0.001$).

As was observed with the adsorbent extracts, females did not land in response to the three synthetic blends. When considered separately mated and unmated moths flew upwind a similar percentage of the time to the grape synthetic blend (mated n = 24, 50.0%; unmated n = 7, 57.1%; Fisher's exact test $P = 1$), the dogwood synthetic blend (mated n = 44, 61.4%; unmated n = 43, 44.2%; Fisher's exact test $P = 0.20$), and the apple synthetic blend (n = 53, 50.1%; unmated n = 46, 45.7%; Fisher's exact test $P = 0.69$).

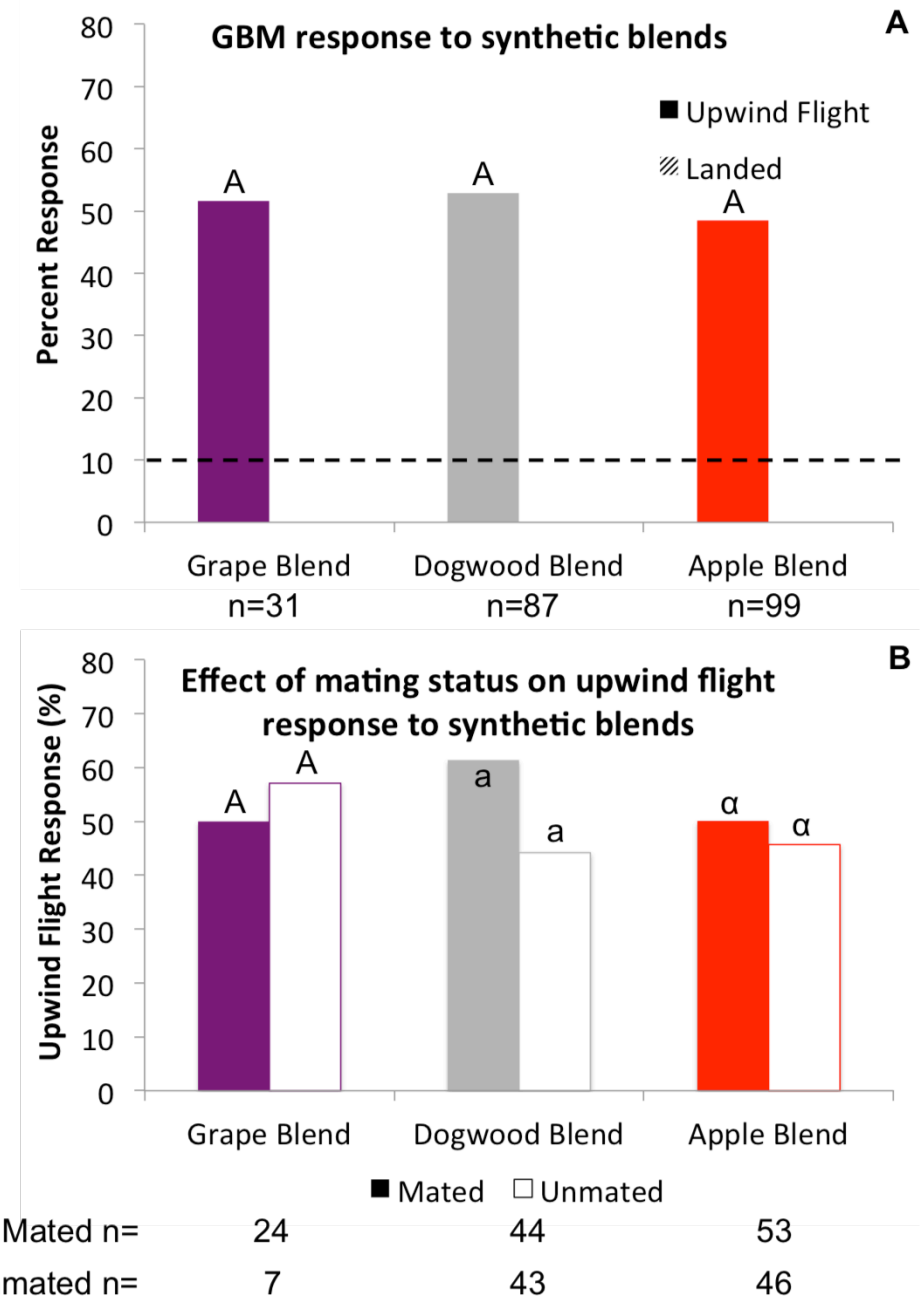



Figure 1.4. GBM response to synthetic blends. Flight tunnel response (%) of GBM females to host and non-host synthetic blends (Panel A). The same letter indicates similar responses ($P < 0.05$; Fisher's exact test). GBM females displayed similar upwind flight responses to all synthetic blends. The non-host extracts elicited a higher percentage of upwind flight and landing than we expected as indicated by dotted line ($P < 0.05$; G-test). Upwind flight of mated and unmated females to synthetic blends (Panel B). The same letter (capital for grape blend, lower case for dogwood blend, Greek for apple blend) indicates similar responses ($P < 0.05$; Fisher's exact test).

*Analysis of GC-EAD active compounds*

All of the previously identified EAD active compounds in the grape volatile profile were also found in the dogwood and apple volatile profiles (Table 1.2; Figure 1.5). The volatile E-β-Ocimene was not previously identified in the EAD active grape volatile blend, but was EAD active and present in all three volatile blends in this study. Z-3-Hexan-1-ol and 1-Methylcyclohexanol were EAD active and only found in the apple volatile blend.

Table 1.2. EAD active compounds. Peaks that showed consistent EAD activity (Figure 6) were identified via GCMS. Synthetic blends were prepared using relative ratios observed in the corresponding extract.

| | | | Relative Ratio | | |
|---|---|---|---|---|---|
| Compound # | Retention Time (min) | Compound Name | Grape | Dogwood | Apple |
| 1 | 6.7 | Z-3-Hexan-1-ol | - | - | 2 |
| 2 | 9.1 | 1-Methylcyclohexanol | - | - | 3 |
| 3 | 9.6 | E-β-Ocimene | 1 | 2 | 4 |
| 4 | 10.0 | Linalool | 3 | 2 | 1 |
| 5 | 10.7 | Z-3-hexen-1-yl acetate | 2 | 15 | 15 |
| 6 | 10.8 | E-4,8-dimethyl-1,3,7-nonatriene | 64 | 23 | 15 |
| 7 | 11.1 | Nonanal | 3 | 2 | 2 |
| 8 | 11.7 | Decanal | 2 | 1 | 1 |
| 9 | 12.4 | Methyl Salicylate | 5 | 2 | 11 |
| 10 | 14.1 | β-Caryophyllene | 8 | 7 | 2 |
| 11 | 15.1 | Germacrene-D | 14 | 2 | 5 |
| 12 | 15.3 | α-Farnesene | 36 | 19 | 41 |

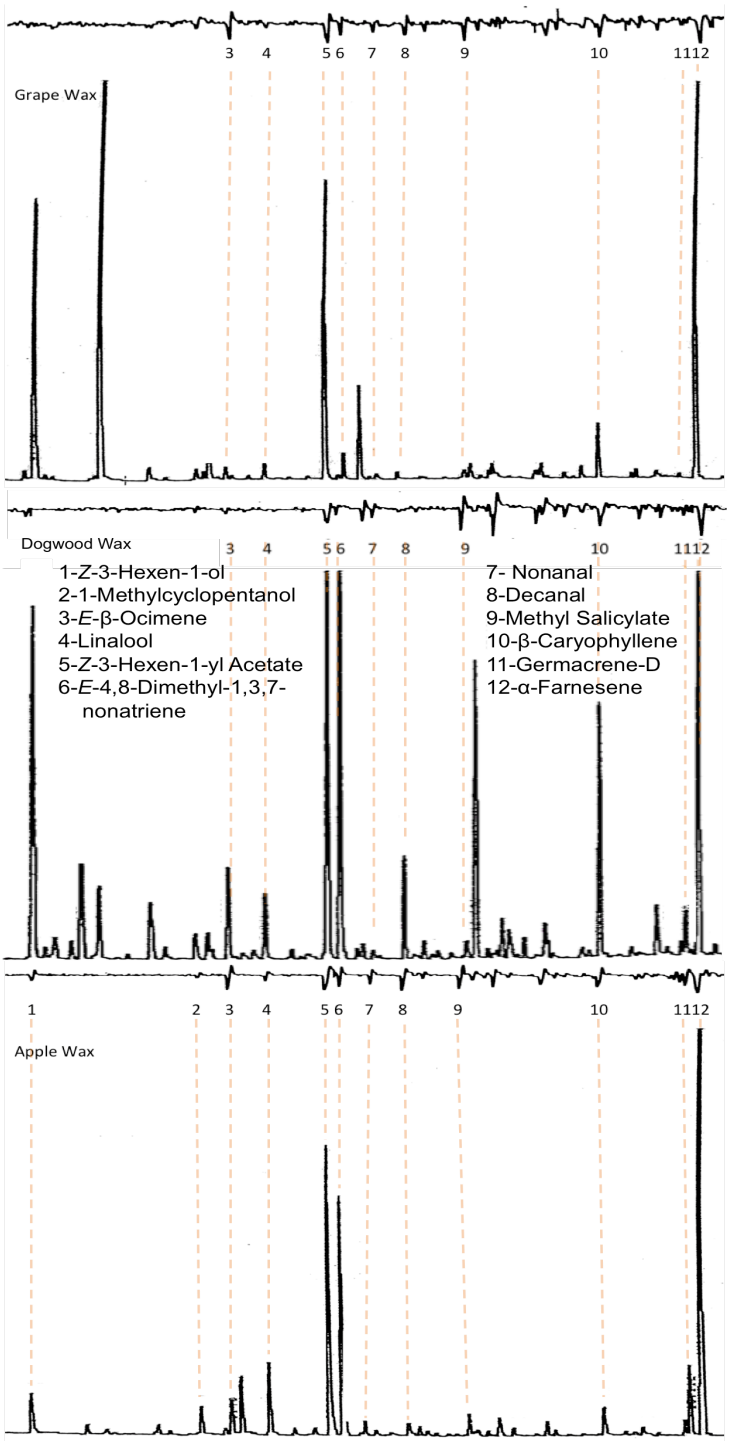



Figure 1.5. Representative GC-EAD responses of GBM female antennae to grape (A), dogwood (B), and apple (C) extracts on a DB-WAX column. EAD recordings are shown above the corresponding chromatograms. Numbered spikes displayed consistent EAD activity and were identified via GCMS (Table 1.2).

***Discussion***

Host plant location by phytophagous insects involves a cascade of behaviors including upwind oriented flight, landing, post-landing assessments of the plant, and host acceptance ultimately culminating with feeding, oviposition or calling (Figure 1.1; (Visser 1986, 1988, Landolt and Phillips 1997). This study focused on the chemically-mediated long-distance behaviors involved in host plant location, and post-landing behaviors were beyond the scope of this study. We used the GBM-grape plant complex as a model for understanding the proximate olfactory mechanisms for host plant location by a specialist phytophagous insect from a distance. GBM females displayed higher levels of upwind flight to the non-host odor sources (Figures 1.2-4B) than we expected from the hypothesis (Bruce et al. 2005) that a specialist species, such as GBM, would detect non-host antagonist compounds as an adaptive mechanism for host plant discrimination (Nojima et al. 2003, Linn et al. 2003, Powell et al. 2012). The similar levels of upwind oriented flight to host grape and non-host gray dogwood support the conclusion that the moths are not discriminating between host and non-host plants at a distance. Mated and unmated females oriented at similar levels to all odor sources (Figures 1.2-4B), further suggesting that plant volatiles are not being specifically used as a long range ovipositional cue.

Phytophagous insects have diverse uses for their host plants. Much of the literature has focused on host plant location for the purpose of feeding or oviposition (Finch and Collier 2000, Bruce et al. 2005, Bruce and Pickett 2011, Webster and Cardé 2016), and have therefore focused

on mated female moths. However, unmated moths may already be on a host plant before releasing sex pheromone, making host location by mated moths less relevant (Shorey 1974). In that case, unmated female moths may display oriented upwind flight to their host plants for courtship/mating purposes, and should therefore be considered in behavioral assays to understand mechanisms for host plant location. In addition to stimulating upwind flight, host plant volatiles also stimulate unmated female ermine moths, *Yponomeuta* spp., corn earworm moths, *Helicoverpa zea*, and cabbage looper moths, *Trichoplusia ni*, to release pheromone (Hendrikse and Vos-Bünnemeyer 1987, Raina et al. 1992, Landolt et al. 1994). Mated and unmated GBM females displayed similar responses to host plant volatiles, suggesting unmated moths could use the host plant as a courtship/mating site in addition to an oviposition site.

In our initial assays with plant shoots, female GBM displayed higher levels of upwind flight and landing to non-host apple shoots than expected (Figure 1.2A), but did so at a lower percentage compared to grape, suggesting that, based only on responses to plant material, antagonist compounds in apple might have been present. However, this difference can also be explained by a difference in concentration or release rate between the plants. The length of the plant shoots used in flight tunnel assays was controlled between plant species, but each species might be releasing volatiles at different rates, which could result in small (~15%) differences in behavior. The moths displayed similar percentages of upwind flight when the concentration of the volatiles was controlled (in the extracts and synthetic blends; Figures 1.3A and 1.4A), supporting the

idea that the difference in behavior could be mediated by differences in relative release rates between grape and apple shoots.

All of the previously identified EAD active compounds in the grape volatile profile were also found in the dogwood and apple volatile profiles (Table 1.2; Figure 1.5). The non-host plants contained volatile compounds not previously identified in the grape blend, but these compounds were not antagonistic as the moths flew upwind a significantly higher percentage of the time to synthetic blends containing these compounds than expected. The redundancy of the compounds in all three volatile profiles supports the observed flight tunnel behavior, and the conclusion that female GBM are not using blends of ubiquitous volatiles to discriminate between host and non-host plants from a distance. Further, compounds in the three blends were present in different relative ratios, but the differences did not affect moth behavior.

*Theories for chemically-mediated host plant location at a distance*

The results of this study do not support the token stimulus (Fraenkel 1959) or the 'ratio specific blends' theories (Figure 1.1A; (Bruce et al. 2005). Bruce et al. (Bruce et al. 2005) suggested that in general, insects use blends of ubiquitous plant volatile compounds rather than species-specific compounds, as Fraenkel had suggested (1959). Furthermore, Bruce et al. (Bruce et al. 2005) argued that insects would be tuned to specific ratios of ubiquitous compounds that comprise an appropriate volatile blend (Bruce et al. 2005, Bruce and Pickett 2011). In previous work, GBM females displayed lower levels of upwind flight

when certain key EAD active volatiles were removed from the complete blend (Cha et al. 2008b). Additionally, GBM females also displayed lower levels of upwind flight when the ratios of key EAD active volatiles were individually doubled, or adjusted to match the ratios emitted by grape plants damaged by Japanese beetles, *Popillia japonica*, (Cha et al. 2011). The results of these studies indicate GBM females are sensitive to the specific composition of the blends. However, the studies also reported higher levels of upwind flight than would be expected (>10%) if these ratios were the result of an adaptive mechanism for host plant discrimination (antagonism).

Our results do support a fourth theory for host plant location (Figure 1.1) by suggesting that host volatiles provide a cue to a suitable habitat where a specific plant can be selected. Furthermore, the theory suggests that insects can use a number of nonspecific habitat cues such as common volatile compounds, $CO_2$ and/or humidity gradients, as well as visual cues, to maximize the likelihood of encountering specific host plant cues (Figure 1.1; Webster and Cardé 2016). Habitat cues differ from host cues in that they are generally not species-specific, are released in large quantities, can be detected at long distances, and are associated with host-specific cues (Webster and Cardé 2016). Habitat cues can attract insects to an area associated with the host (habitat), and once in the habitat the insects can use additional, species-specific cues to locate the host. For example, European grapevine moths, *Lobesia botrana*, have a wild host, *Vitis vinifera,* and a recently colonized host, *Daphne gnidium* (Thiéry and Moreau 2005). Gravid females flew upwind a low percentage of the time

to synthetic blends of EAD active green leaf volatile compounds (GLVs) specific for each plant, as well as a blend of only the common GLVs (Tasin et al. 2009). However, the upwind flight behavior was recovered when the GLVs common to both blends were added to each species-specific blend, suggesting both common and host-specific GLVs are used to locate a host.

Hawkmoth pollinators can use floral $CO_2$ and humidity gradients to select an appropriate nectar source (Thom et al. 2004, von Arx et al. 2012, Contreras et al. 2013). White-lined sphinx moths, *Hyles lineatea*, consistently approached and probed flowers with elevated humidity more than those at ambient humidity, suggesting the moths can use small differences in relative humidity to select a host. Furthermore, tobacco hornworm moths, *Manduca sexta*, displayed high levels of upwind flight in response to small differences in relative humidity (Wolfin et al. in prep.). Additionally, hawkmoths spent more time on the side of the flight tunnel with elevated humidity compared to the side of the tunnel with ambient relative humidity, suggesting humidity could be an important orientation cue.

Using habitat odors to locate a host would be particularly effective in the GBM-grape plant complex given the life histories of the GBM and *V. riparia*. *Vitis riparia* is native to North America from Canada to Texas, and the Rocky Mountains to the Atlantic Ocean (Keller 2015). It is a woody plant that climbs on trees and shrubs along riverbanks (Keller 2015). Female GBM could use habitat cues from either riverbanks (humidity) or surrounding flora to increase the probability of detecting

specific host plant cues and locating a host. Grapevines share range and phenology with wild apple trees and gray dogwood shrubs, and may climb on them in wild habitats. This association between the host and non-host plants may explain the observed orientation and landing behavior of GBM to the non-host plants in this study, and supports the use of habitat cues to locate a host plant (Webster and Cardé 2016).

*Landing response*

Female GBM displayed a higher percentage of landing on the non-host plants than we expected from the hypothesis that this specialist species would be detecting antagonist non-host compounds as an adaptive mechanism for host plant discrimination (Nojima et al. 2003, Linn et al. 2003, Powell et al. 2012). The fact that the moths landed on non-host plants is further evidence that GBM females are not using a 'token stimulus' (Fraenkel 1959), or specific blends of volatile compounds (Bruce et al. 2005) to locate a host. However, the results do support the 'appropriate/inappropriate landings' theory (Finch and Collier 2000). This theory suggests a flying insect is stimulated to land through the detection of nonspecific plant volatiles (Figure 1.1, green). Upon landing, the insect performs multiple post-landing assessments of the plants to discriminate between host and non-host plants. Post-landing behaviors were beyond the scope of the current study, and would require additional behavioral assays.

Habitat cues also may be necessary to elicit GBM landing behavior (Finch and Collier 2000). The 'appropriate/inappropriate landings theory'

(Figure 1.1, green; (Finch and Collier 2000) suggested that nonspecific plant cues such as common GLVs stimulate insects to land on a nearby green surface, and host plant discrimination and acceptance is mediated by post-landing behaviors. For example, cabbage root flies, *Delia radicum*, landed a higher percentage of the time on non-host substrates than expected when presented with host plants paired with non-host plants, and host plants paired with green paper in laboratory bioassays (Kostal and Finch 1994). Additionally, *D. radicum* did not display an ovipositional preference between artificial plants baited with host odors compared with unbaited artificial plants (Prokopy et al. 1983). These studies suggest the volatiles are a nonspecific landing cue, and host plant discrimination occurs post-landing.

Finch and Collier (Finch and Collier 2000) suggested that insects use contact chemoreceptors on their tarsi to assess the host plant. The small cabbage white butterfly, *Pieris rapae*, use tarsal chemoreceptors to detect glucosinolates and cardenolides that act as deterrents or stimulants for oviposition (Roessingh et al. 1992, Stadler et al. 1995). Blaney and Simmonds (1990) observed behavioral and electrophysiological responses of tarsal chemoreceptors in *Spodoptera littoralis, Spodoptera frugiperda, H. virescens, and Helicoverpa armigera.* All four moth species could detect sugars, amino acids, and allelochemicals (azadirachtin) using tarsal receptors, and had varying levels of sensitivities to each chemical stimulus. These sugars, amino acids, and allelochemicals could stimulate important behaviors such as feeding and oviposition (Roessingh et al. 1992, Stadler et al. 1995, Henderson et al. 2004, Zhang et al. 2010).

Because GBM females landed on host-and non-host plants, contact chemoreception might be involved in the host plant selection process.

In the current study female GBM did not land on the rubber septum source in response to the extracts or synthetic blends of the host and non-host plants. If all of the necessary host plant cues were present, we expected the moths to land on the septa a similar percentage of the time as they did on the corresponding plant shoots. However, the lack of landing on any extract or synthetic blend (host or non-host) indicates the moths may require additional cues to land. Cabbage moths, *Mamestra brassicae*, readily flew upwind to extracts of host plant volatiles in a flight tunnel (Rojas and Wyatt 1999). However, the moths did not land on the odor source unless a visual cue was also present. The observed landing response here is consistent with previous studies on GBM females (Cha et al. 2008b). Carbon dioxide or humidity gradients in the presence of olfactory cues may also affect landing behavior (*see* Chapter 2; Thom et al. 2004, Guerenstein and Hildebrand 2008, von Arx et al. 2012, Contreras et al. 2013). Additional flight tunnel experiments were done to determine the role of habitat cues on landing behavior (*see* Chapter 2).

***Conclusions***

Many of the previous studies on insect host location from a distance have focused on oriented upwind flight as a key discriminatory behavior (Bruce et al. 2005, Bruce and Pickett 2011). In the present study, grape berry moth females flew upwind a similar percent of the time to host sources and non-host sources suggesting discrimination is not occurring at

a distance. This result supports the fact that phytophagous insects may fly upwind to locate a favorable habitat rather than host plant, and that discrimination may occur within the habitat, or even post-landing (Finch and Collier 2000, Webster and Cardé 2016). The moths did not land on synthetic odor sources in this study, and a future study will explore the cues that stimulate GBM females to land.

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# CHAPTER 2

# HABITAT CUES SYNERGIZE TO ELICIT CHEMICALLY-MEDIATED LANDING BEHAVIOR IN A SPECIALIST PHYTOPHAGOUS INSECT, THE GRAPE BERRY MOTH, *PARALOBESIA VITEANA*


***Abstract***

Many phytophagous insects locate their host plant using mixtures of volatile compounds produced by the plant (Bruce et al 2005). A key step in host location is landing on (or near) the odor source. In a previous study rubber septa emitting a synthetic blend of volatiles extracted from young shoots of grape plants, *Vitus spp.* elicited equivalent levels of oriented upwind flight by female grape berry moths (GBM; *Paralobesia viteana*) as did actual (control) grape shoots (Cha et al. 2008). However, in contrast to the shoots, females did not land on the odor source when presented with the synthetic blend alone. The present study used flight tunnel assays to investigate and disentangle the interactions between plant volatiles, visual, and moisture cues contributing to the landing response of GBM females in response to host plant odors. We found that individual and paired stimuli did not elicit landing on the odor source. Olfactory cues paired with moisture elicited ~5% landing on the source. Grape berry moth females required olfactory cues, visual cues and moisture to display equivalent levels of landing on artificial sources compared to control grape shoots. Additionally, GBM females displayed low levels of oriented upwind flight to wet cotton strips, and landed a low percentage of the time when the wet cotton strips were paired with the host odor blend.

These results suggest the cues have a synergistic effect, and that landing behavior requires complex sensory processing using multiple sensory inputs. Furthermore, these cues are relatively nonspecific, indicating they could provide a signal for an appropriate habitat rather than specific host plants.

***Introduction***

Many phytophagous insects locate their host plant from a distance using complex mixtures of volatile compounds produced by the plant (Bruce et al. 2005, Bruce and Pickett 2011). A major challenge in the laboratory is to replicate the environmental conditions (photoperiod, temperature, relative humidity, etc.) in which the behaviors occur to study the sequence of behaviors leading to host location and landing. The flight tunnel is among the most sensitive assays to observe in-flight insect behavioral responses to airborne olfactory stimuli because plume structure (Willis and Baker 1984) and environmental conditions (Cha et al. 2008a) found in nature can be controlled and replicated. The flight tunnel has been used to study the response specificity of many insect species to sex pheromone and plant volatile blends (Farkas and Shorey 1972, Visser 1976, Roelofs and Cardé 1977, Visser and Nielsen 1977, Miller and Roelofs 1978, Linn and Roelofs 1989, Allison and Cardé 2016). A key mechanism involved in locating an odor source is optomotor anemotaxis (Kennedy and Marsh 1974, Visser 1976, Roelofs and Cardé 1977). Optomotor anemotaxis involves complex sensory processing, as the insect must detect a favorable odor plume, and detect image flow below (or above) while flying (Visser 1988, Baker and Hansson 2016). Optomotor anemotaxis is oriented upwind flight towards an odor source that requires complex sensory processing, as the insect must detect a favorable odor plume, and detect image flow below (or above) while flying (Visser 1988, Baker and Hansson 2016).

Another key step in host location is the process of landing on (or

near) the odor source potentially leading to critical activities such as mating or oviposition (Yamamoto et al. 1969, Miller and Roelofs 1978, Aker and Udovic 1981, Ramaswamy 1988). Male moths responding to a female-produced sex pheromone may require minimal landing cues (Table 2.1; Miller and Roelofs 1978, Glover et al. 1987). For example, a plastic or metal platform (15 $cm^2$) and a substrate loaded with sex pheromone are sufficient stimuli to elicit landing by male codling moths, *Cydia pomonella*, oriental fruit moths (OFM; *Grapholita molesta*), and tobacco budworm moths, *Heliothis virescens,* (Baker and Cardé 1979, Castrovillo and Cardé 1980, Vickers et al. 1991). Males of the corn earworm, *Helicoverpa zea,* and *Heliothis subflexa* moths land on filter paper loaded with sex pheromone (Quero and Baker 1999, Vickers 2002). Male European corn borer moths, *Ostrinia nubilalis*, prefer vertical objects to horizontal surfaces (Foster and Frérot 1994), and will readily land on the copper tube holding a rubber septum pheromone source (Glover et al. 1987, Linn et al. 1996). Cabbage looper males, *Trichoplusia ni*, also readily approach and land on the copper tubing holding a rubber septum pheromone source more readily than if the source is on a horizontal platform (Linn et al. 1996).

Female moths responding to host plant odors may also land on artificial sources (Table 2.1). Female European grapevine moths, *Lobesia botrana* landed on a vibrating capillary tube releasing host plant volatiles as often as they landed on green grape clusters (Tasin et al. 2006). Increasing in signal complexity, female yellow peach moths, *Conogethes punctiferalis* land on a metal mesh ball wrapped in medical gauze in the

presence of host odors (Luo and Honda 2015). Female cabbage moths, *Mamestra brassicae*, prefer to land on artificial square targets with side lengths of 5 cm or 10 cm significantly more often than on artificial square targets with a side length of 15 cm (Rojas and Wyatt 1999) when combined with host plant volatiles. Female tobacco hawkmoths, *Manduca sexta*, land on surrogate leaves in response to host plant odors (Sparks and Cheatham 1970, Späthe et al. 2013). However, recent studies suggest females may require additional cues to land on an odor source (*see Chapter 1*).

| Insect | Sex | Olfactory cue | Additional Landing Cue | Reference |
|---|---|---|---|---|
| *C. pomonella* | Male | Sex pheromone | 15 x 15 metal platform | Castrovillo and Cardé 1980 |
| *G. molesta* | Male | Sex pheromone | 15 x 15 metal platform | Baker and Cardé 1979 |
| *H. virescens* | Male | Sex pheromone | 15 x 15 metal platform | Vickers et al. 1991 |
| *H. zea* | Male | Sex pheromone | Filter paper | Quero and Baker 1999, Vickers 2002 |
| *H. subflexa* | Male | Sex pheromone | Filter paper | Quero and Baker 1999, Vickers 2002 |
| *O. nubilalis* | Male | Sex pheromone | Copper tube | Glover et al. 1987, Linn et al. 1996 |
| *T. ni* | Male | Sex pheromone | Copper tube | Linn et al. 1996 |
| *L. botrana* | Female | Host plant odor | Vibrating capillary tube | Tasin et al. 2006 |
| *C. punctiferalis* | Female | Host plant odor | Metal mesh ball wrapped in medical gauze | Luo and Honda 2015 |
| *M. brassicae* | Female | Host plant odor | Paper circles | Rojas and Wyatt 1999 |
| *M. sexta* | Female | Host plant odor | Light green tissue paper | Späthe et al. 2013 |
| *P. viteana* | Female | Host plant odor | Unknown | Chapter 2 |

Table 2.1. Summary of landing cues in previous flight tunnel studies.

Upon reaching the odor source, the insect may require additional stimuli to continue the cascade of behaviors and ultimately interact with the odor source (mating, egg laying, releasing pheromone, etc.). For example, after locating its host plant, female tobacco budworm moths, *H. virescens*, land on the plant and continue to assess it by bending their abdomen and/or dragging their ovipositor on the substrate of the plant. If the plant is accepted, the moths eventually lay an egg or release pheromone (Ramaswamy 1988). However, not all of these behaviors are necessary to achieve the desired result (in the case of *H. virescens*, egg laying or calling) when host plants are assayed (thus eliminating visual and contact cues). The insects that landed on the screen surrounding the plant also performed each of the post-landing and host acceptance behaviors listed above except for dragging the ovipositor, suggesting the screen lacked the necessary cues to elicit ovipositor dragging. Thus ovipositor dragging is not an essential behavior to assess the host plant for the purpose of egg laying and calling, and not all post-landing behaviors are necessary for host plant acceptance (Mechaber et al. 2002).

The present study uses flight tunnel assays to investigate and disentangle the role of plant volatiles, visual cues, and moisture contributing to the landing response of GBM females in response to host plant odors. Previous work showed that GBM females displayed optomotor anemotaxis to odors from plants, chemical extracts of plant volatiles, and synthetic blends of EAD active plant volatiles in a flight tunnel (*see Chapter 1*). Surprisingly, the moths only landed on the plants in flight tunnel assays, suggesting additional cues are necessary to elicit

landing near rubber septum odor sources. We report here the results of flight tunnel assays to recover the landing behavior and determine the necessary landing cues. The behavior of female grape berry moths was recorded in response to olfactory stimuli, visual stimuli, and water vapor individually, paired, and combined.

## *Methods*

### *Insects*

GBM were reared in cages placed in walk-in environmental chambers at 26°C and 60% RH under a 16:8 L:D photoperiod. Adults were allowed to oviposit on seedless grapes (*Vitis vinifera*, red flame variety). First and second instars were transferred to a diet cup (30 mL, WinCup Inc.) and reared on semi-synthetic diet (Nagarkatti et al. 2000) that consisted of grapes, pinto beans, and commercially available tobacco hornworm diet (Bio-Serve). For behavioral assays, unmated female moths were taken from cohorts set up by placing 10-15 female pupae (near eclosion) in a Plexiglass mating cage (30 cm H×30 cm W×30 cm D) and provided with a 50% honey and water solution. Twenty male moth pupae were added to additional mating cages loaded with 10-15 female pupae to assay mated females.

### *Plants*

*Vitis riparia,* a native host species of the GBM in northeastern USA, was used for all assays. All plants were maintained in a greenhouse as in Cha et al. (2008a) with temperatures maintained between 21-26 ˚C. Supplemental light was provided to extend the day length to 16 h.

*Flight Tunnel*

The flight tunnel was 2 m in length by 0.6 m in width and 0.6 m in height, with a fan installed at the upwind end to create a steady airflow into the tunnel and an exhaust hood at the downwind end to evacuate odor from the flight tunnel (Cha, et al. 2008ab). Wind speed was set at 0.25 m/s at the wire stand where the moths were introduced into the wind tunnel. A pattern made of dark green paper circles (10 cm diameter) was randomly presented both on a white background glass floor and on the glass ceiling below the light source. During the experiments, the average temperature in the flight tunnel was 23.8 ± 0.07 °C, and the relative humidity ranged averaged 68.0 ± 0.62 %. Female moths were placed in the flight tunnel room 1 h prior to scotophase. Light intensity was reduced to 25 lx 30 min before dark, and remained at that intensity for behavioral assays. Behavioral assays began 15 minutes prior to scotophase.

The odor source was placed 30 cm from the upwind end of the tunnel. Four- to five-day old females were used in all flight tunnel assays, and were discarded after being tested. Female moths were placed in the flight tunnel individually in a metal screen release cage on a wire stand 1.5 m downwind of the source, and their behavior was observed for 5 min. We recorded whether the insect flew out of the release cage, flew upwind (more than 10 cm of oriented flight towards the source), and landed on the source (made contact with the source). Fisher's exact test ($P < 0.05$) was used to compare the percent response of the GBM females to the different

odor sources.

*Treatments*

Treatments are summarized in Table 2.2, and can be seen in Figure 2.1. Two grape shoots (15 cm) were cut, placed in a water pick, and immediately transported to the flight tunnel for behavioral assays as a positive control (Figure 2.1A). Grape shoots were discarded after each session. Chemical odor sources consisted of rubber septa loaded with 300 µL of grape synthetic blend as prepared in Cha et al. (2008b, *see chapter 1*), held by a copper pipe (Figure 2.1B). The synthetic grape blend consisted of (*E*)-4,8-dimethyl-1,3-(*E*)-,7-nonatriene, (*Z*)-3-hexen-1-yl acetate, nonanal, decanal, linalool, β-caryophyllene, α-farnesene, germacrene-D, and methyl salicylate in a 14:11:6:6:6:3:36:12:6 ratio in dichloromethane at a concentration of 0.1 mg/mL. Artificial grape shoots were purchased from Michael's Craft Store (Canandaigua, NY, USA), washed in 95% ethanol, and cut into 15 cm strips. Artificial shoots were stored in a fume hood for at least 48 h when not in use. Cotton sheets (U.S. Cotton #79410016) were cut in 2.5 cm x 2.5 cm x 1 cm strips and stapled to the back of the artificial grape leaves (Figure 2.1D). Ten mL of distilled $H_2O$ was added to each cotton strip in the wet treatments. For the wet cotton alone treatment, cotton sheets were cut 2.5 cm x 25 cm x 1 cm, and 100 mL distilled $H_2O$ was added to each strip (Figure 2.1C). Artificial grape shoots and the moist cotton strips were placed within 10 cm of the copper stand holding the rubber septa (Figure 2.1E). All wet sources were placed in a fume hood for 10 min before being placed in the flight tunnel. A Fisher's exact test was used to analyze the GBM response

to different sources, and a $P$ value $< 0.05$ was considered significantly different.

| **Treatments (number of flights)** | Odor Source | |
|---|---|---|
| Additional cue | No Odor | Blend |
| No visual | 38 | 46 |
| Artificial Grape Shoots | 30 | - |
| Artificial Grape Shoots + Cotton (Dry) | 38 | 30 |
| **Sum of Non- Odor Sources** | 106 | 76 |
| Grape Shoots | 89 | - |
| Wet Cotton | 26 | 54 |
| Artificial Grape Shoots + Cotton (Wet) | 33 | 37 |

Table 2.2. Summary of flight tunnel treatments. Treatments in the grey box were combined to form the "Non- odor sources" treatment. Numbers represent the number of insects flown to each source.

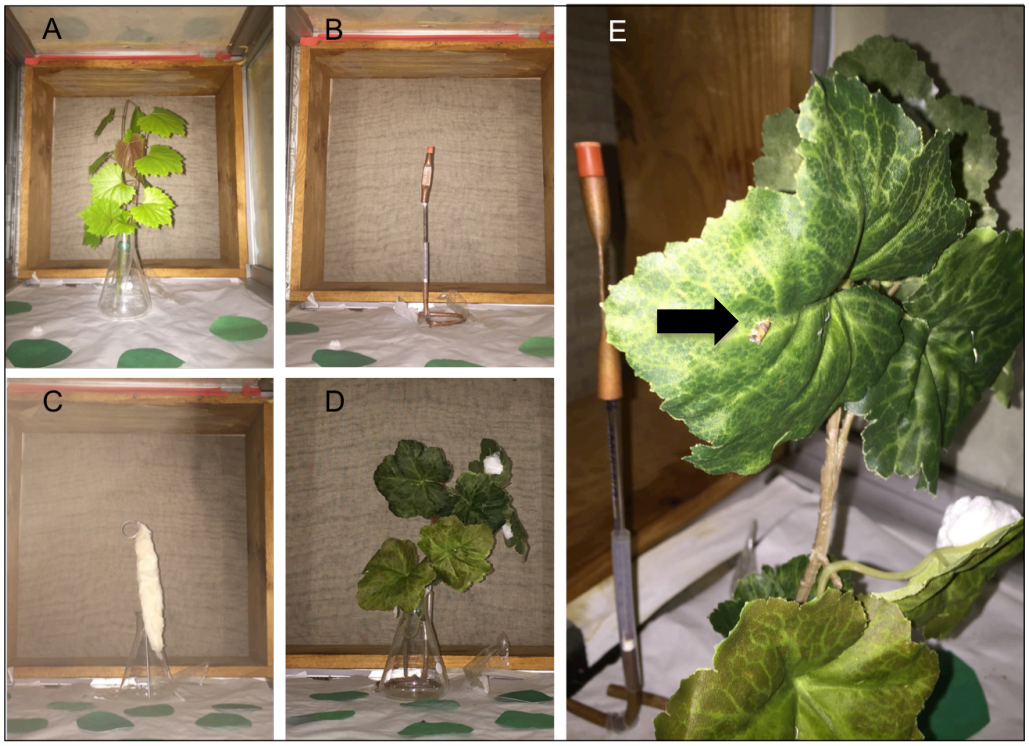


Figure 2.1. Visual stimuli associated with different sources. A) Control grape shoots, B) Copper stand holding a rubber septum, C) Wet cotton strips alone, D) Artificial grape shoots + dry cotton, E) Zoomed in view of the complete model. Arrow indicates the moth on the artificial leaf in Panel E.

*Chemicals*

(Z)-3-hexen-1-yl acetate, ethyl hexanoate, nonanal, linalool, methyl salicylate, decanal, β-caryophyllene, α-farnesene, and 6-pentyl-α-pyrone were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA), Alfa Aesar (Ward Hill, MA, USA), Fluka (Buchs, Switzerland) or TCI America (Portland, OR, USA). All, except α- farnesene (a mixture of various isomers) were greater than 97% purity. The 4,8-dimethyl-1,3(E),7-nonatriene was provided by the Chong lab (University of Waterloo, Ontario, CA). Germacrene-D was isolated from golden rod as 91% germacrene-D and 9% β-caryophyllene (by USDA-ARS Chemistry Research Unit, Gainesville, FL, USA).

## ***Results***

*Response to Controls*

Grape berry moth females took flight 79.8%, flew upwind 69.7%, and landed 25.8 % of the time in response to live grape shoots as positive control (n = 89, Figure 2.2). Three different non-odor sources were tested and the similar levels of response allowed us to group them as controls. The first non-odor source, the blank, consisted of no structure at all at the upwind end of the tunnel. With this treatment females took flight 65.8% of the time, but did not fly upwind (0.0%), or land (0.0%, n = 38). The moths behaved similarly to the second non-odor source, consisting of artificial grape shoots alone (n = 30), taking flight 66.7% (Fisher's exact test, $P = 0.60$) of the time, and also did not fly upwind (0.0%) or land (0.0%). Female response to the third non-odor source, consisting of artificial grape + dry cotton also was similar to the response to the blank

(Figure 2.2; n = 38; took flight = 65.8; Fisher's exact test, $P = 1$; upwind flight = 0.0%; land = 0.0%).

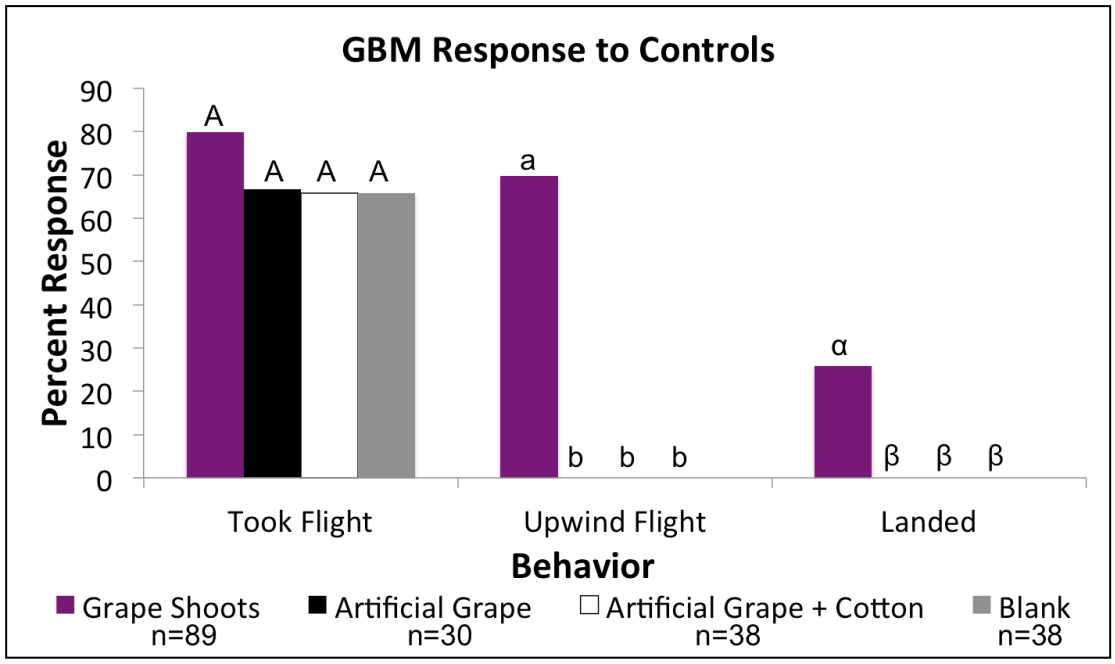


Figure 2.2. GBM Response to Controls. Flight tunnel response (%) of GBM females to control plant shoots and three non- odor sources. Different letters (capital for took flight response, lower case for upwind flight response, Greek for landing response) indicate significant differences ($P < 0.05$; Fisher's exact test).

*Response to individual stimuli: synthetic blend alone*

Grape berry moth females took flight (76.0%) and flew upwind (60.9%) a similar percentage of the time to rubber septa loaded with synthetic blend (n = 46) as they did to the plant (Figure 2.3; Fisher's exact test, took flight $P = 0.39$, upwind flight $P = 0.85$). However, the moths did not land on the rubber septum loaded with synthetic blend (0.0%).

*Response to individual stimuli: wet cotton strips alone*

When wet cotton strips alone were the source (Figure 2.3; n = 26),

GBM females took flight (53.8%) and did not land. However, the moths displayed 15% oriented upwind flight toward the wet cotton, which was significantly higher than for the non-odor sources (Fisher's exact test, $P$ = 0.009), and significantly lower than for the plant (Fisher's exact test, $P$ = 0.001). The moths also took flight and landed a significantly lower percent of the time to the wet cotton than they did to the plant (Fisher's exact test, took flight $P$ = 0.012; landed P < 0.0001).

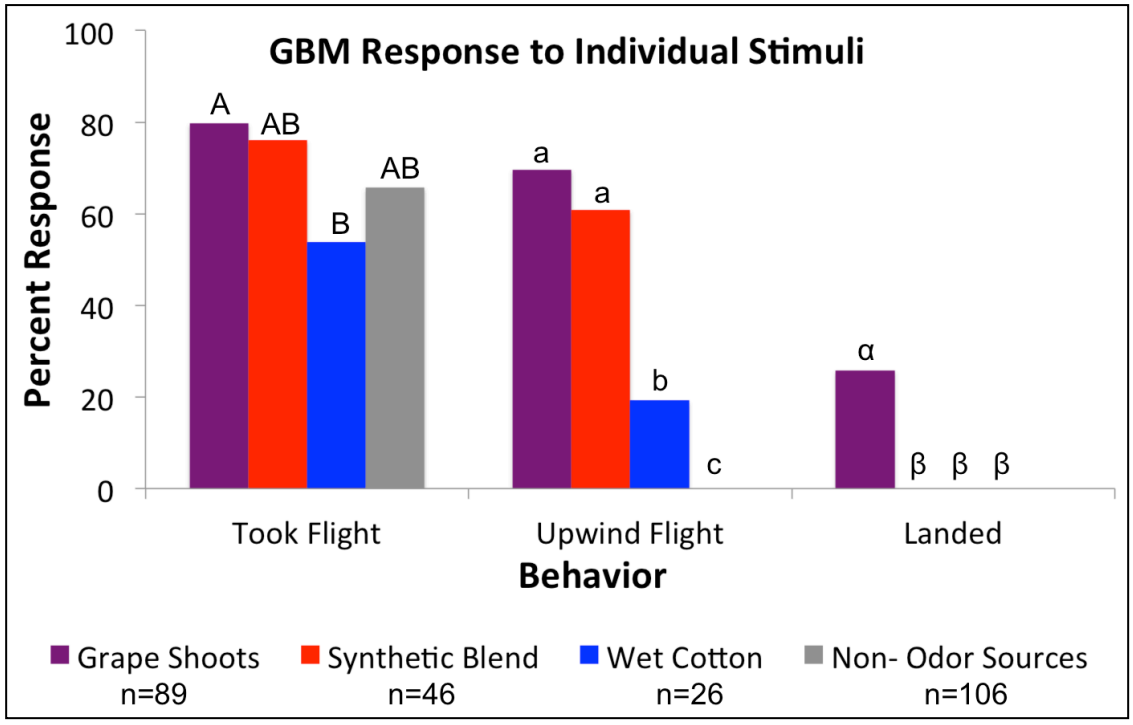


Figure 2.3. GBM Response to Individual Stimuli. Flight tunnel response (%) of GBM females to different individual sources. Different letters (capital for took flight response, lower case for upwind flight response, Greek for landing response) indicate significant differences ($P$ < 0.05; Fisher's exact test).

*Response to paired stimuli: 1. Artificial grape shoots + grape synthetic blend*

Moths took flight and flew upwind a similar percent of the time to

the artificial grape shoots + blend (n = 30) as they did to the grape shoots, taking flight 93.3% of the time (Figure 2.4; Fisher's exact test, $P = 0.09$), flying upwind 66.6% of the time (n = 30; Fisher's exact test, $P = 0.82$). However, the moths did not land on the artificial grape shoots + blend (0%).

*Response to paired stimuli: 2. Wet cotton + grape synthetic blend*

Moths took flight (79.5%), and flew upwind (53.7%) a statistically similar percent of the time to the wet cotton + blend source (Figure 2.4; n = 54) as they did to the plant (Fisher's exact test, took flight $P = 0.16$, upwind flight $P = 0.07$). The moths landed on the wet cotton + blend 5.6% of the time, which was a statistically higher percentage than on the non-odor sources, which did not elicit landing ($P = 0.034$), but a statistically lower percentage than on the plant ($P = 0.002$)

*Response to paired stimuli: 3. Wet cotton + artificial grape shoots*

Moths took flight 60.6% of the time to the wet artificial grape shoots (Figure 2.4; n = 33), which was similar to the response to live grape shoots (Fisher's exact test, $P = 0.38$). The moths flew upwind 15% of the time to the wet artificial grape shoots, which was lower than with the grape shoots (Fisher's exact test, $P < 0.001$), and a higher percentage than with the non-odor sources (Fisher's exact test, $P = 0.018$). This response was statistically similar to the wet cotton strips alone (Fisher's exact test, took flight $P = 0.79$, flew upwind $P = 0.74$, landed $P = 1$).

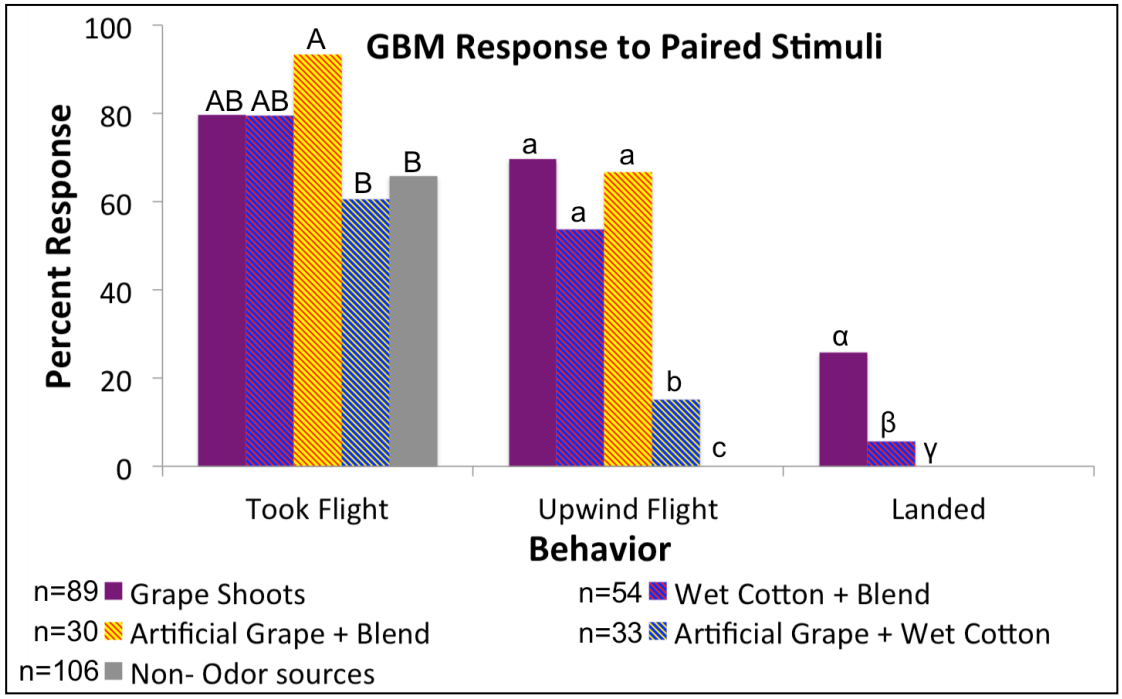


Figure 2.4. GBM Response to Paired Stimuli. Flight tunnel response (%) of GBM females to different paired sources. Different letters (capital for took flight response, lower case for upwind flight response, Greek for landing response) indicate significant differences ($P < 0.05$; Fisher's exact test).

*Response to Complete Model: wet cotton + artificial grape shoots + grape synthetic blend*

Moths responded to the wet model + blend (n = 37) at similar levels as they did to the grape shoots at all behaviors, taking flight 89.2% of the time (Figure 2.5; Fisher's exact test, $P = 0.30$), flying upwind 75.7% of the time (Fisher's exact test, $P = 0.67$), and landing on the source 43.3% of the time (Fisher's exact test, $P = 0.06$).

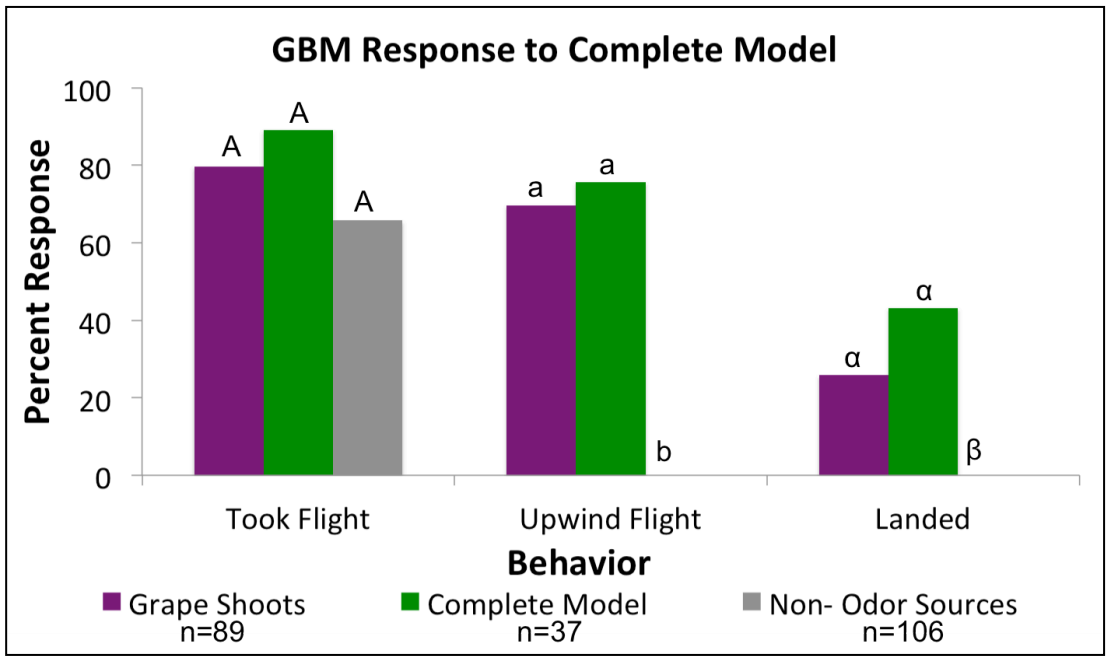


Figure 2.5. GBM Response to Complete Model. Flight tunnel response (%) of GBM females to the complete model (artificial fake shoots + wet cotton strips + synthetic grape blend). Different letters (capital for took flight response, lower case for upwind flight response, Greek for landing response) indicate significant differences ($P < 0.05$; Fisher's exact test).

### *Discussion*

In a previous study, rubber septum sources containing a synthetic blend of grape shoot volatiles elicited equivalent levels of oriented upwind flight by GBM females as grape shoots (Cha et al. 2008b). In contrast to the grape shoots, however, females did not land on the odor source when presented with the synthetic blend alone (*see Chapter 1*; Figure 2.3). This result called into question whether the identified GC-EAD active compounds making up the synthetic blend represented the complete volatile mix involved in long distance host location, or the insect required additional cues to land. Here we show that landing requires not only an olfactory stimulus, but also a visual stimulus, and moisture

(Figure 2.5). Interestingly, whereas the artificial grape visual stimulus alone did not elicit oriented flight, wet cotton strips alone and paired with artificial grape shoots elicited a low level (~15%) of oriented upwind flight, and moths landed on the septum a low percentage of the time when a wet cotton strip was paired with the synthetic blend (5.6%). Landing behavior equivalent to the plant was only recovered when wet cotton strips were added to artificial grape shoots and the septum releasing the synthetic blend.

The fact that GBM females did not land on the copper tube holding the rubber septum was a surprising result given the number of previous studies where male moths landed on that source responding to sex pheromone in the flight tunnel, specifically with European corn borer moths in the same flight tunnel used here (Glover et al. 1987, Linn et al. 1996). The odor sources used in many pheromone studies were designed to optimize the insect's oriented upwind flight behavior by providing consistent plume structure and release rate of volatile compounds (Miller and Roelofs 1978, Castrovillo and Cardé 1980, Glover et al. 1987), with less attention paid to details of the landing response. However, our results show GBM female moths required a visual cue and moisture, in addition to host plant volatiles to land, which suggests that landing might be a more complex process (Piñero et al. 2008). For example, Lu et al. (2012, 2015) found sexual differences in both electrophysiological and behavioral responses of OFM males and females to host volatiles. Thirteen host plant volatiles were found to be antenally active for OFM females, and only 8 of which were antenally active for OFM males (Lu et

al. 2015). The 13-component blend was more attractive to OFM females, and the 8-component blend was more attractive to OFM males. Interestingly, only the OFM males landed on the odor source (Lu et al. 2012, 2015), suggesting females require additional landing cues. Further behavioral studies are necessary to determine sexual differences in GBM response to host plant volatiles.

The observed synergistic landing response by GBM females required both specific (plant odor blend) and nonspecific cues (water vapor, artificial leaves). Behaviorally active plant volatile blends are often comprised of a specific subset of antenally active volatile compounds collected from the headspace of the host plant (Ave and Visser 1978, Honda 1995, Natale et al. 2003, Bruce et al. 2005, Piñero et al. 2008, Cha et al. 2008b). Although all these volatiles are common green leaf volatiles (Bruce et al. 2005), the behavioral response is dependent upon specific blends of these volatiles, and the presence or absence of key compounds greatly affects moth behavior (Cha et al. 2008b).

Water vapor, on the other hand, is passively released into the environment by plant vegetative tissue through the process of evapotranspiration (Monteith 1965, Saugier and Katerji 1991). Nonspecific plant cues such as relative humidity and $CO_2$ can mediate interactions between insects and their host plants. Carbon dioxide is released by plants through respiration, and moths may use it to locate their host plants (Eyer and Medler 1940, Stange et al. 1995, Goyret et al. 2008). Female cactus moths (*Cactoblastis cactorum*) use their labial palps to

detect $CO_2$ both in flight and while walking, suggesting it is both an orientation cue and an oviposition cue (Stange et al. 1995). The role of humidity as a foraging cue has been explored in tobacco hornworm moths (*Manduca sexta*) in a foraging context (Wolfin et al. *in prep*). Von arx et al. (2012) showed that the during the first 30 minutes of anthesis, the floral headspace of newly opened *Oenothera cespitosa* flowers produced local humidity levels ~4% higher than ambient conditions, and moths approached flowers with the elevated humidity over those with ambient humidity levels. The increased number of flower visits indicates the moths can detect small differences in relative humidity, and these differences can have an effect on moth in-flight orientation.

The observed consistent level of oriented upwind flight by GBM females to the wet cotton strips (with and without the artificial grape shoots) was a surprising result as there are limited examples of moth oriented flight to wet sources when presented alone in a flight tunnel. Raguso et al. (2005) showed that *M. sexta* moths extend their proboscises in response to a humidified airstream in a flight tunnel. In their study, when the humid airstream was paired with floral odor, an additive effect was observed rather than a synergistic effect. In flight tunnel experiments where a humidity gradient was established within the flight tunnel (Wolfin et al. *in prep*), hawkmoths spent more time on the side of the flight tunnel with elevated humidity compared to the side of the tunnel with ambient relative humidity. However, this effect was nullified when floral odor was introduced into both the ambient and elevated sides of the flight tunnel, suggesting the response is context-dependent rather than

synergistic. In our study, although the addition of wet cotton strips did not add to or synergize with the moth's oriented upwind flight to the synthetic blend, the moths landed on the source when wet cotton strips were paired with synthetic blend (and did not land to either cue individually), indicating a synergistic effect of the two stimuli. The moth's response to all three stimuli simultaneously (complete model; Figure 2.5) was equivalent to the moth's response to the plant, suggesting even stronger synergism.

The visual stimulus (artificial grape shoots) might also be a nonspecific cue. Finch and Collier (2000) suggested that even specialist phytophagous insects indiscriminately land on green objects. In flight cage assays, Kostal and Finch (1994) found that cabbage root flies landed on brown paper a similar percentage of time compared to the host plant, and landed on green paper a higher percentage of time compared to the host plant. Tobacco hornworm moths preferred to contact surrogate leaves supplemented with host plant odors compared to surrogate leaves without supplemented host odors in binary choice wind tunnel assays (Späthe et al. 2013). However, the hawkmoths also contacted the unscented leaves when paired with scented leaves in the choice tests, suggesting that hawkmoths may land indiscriminately over a distance of at least 40 cm (the distance between sources). Furthermore, gravid *M. sexta* females laid an equivalent number of eggs on polyurethane foam wetted with plant extract as they did on the host plant (Sparks 1970). Our study used commercially available artificial grape shoots as a visual cue. Future studies are necessary to explore the specificity of the visual component of

the landing cue for GBM females (size, shape, color, amount of moisture added, etc.). Additionally, future studies could explore whether artificial sources elicit GBM post-landing behaviors necessary for host plant acceptance. The appropriate/inappropriate landings theory (Finch and Collier 2000) suggests flying insects are stimulated to land indiscriminately by plant volatiles, and host plant discrimination and acceptance occurs post landing after multiple assessments of different leaves. We also note however, that while not a specific aim of the flight tunnel experiments, the tunnel floor contained a number of green paper circles that provide a non-directional visual field for moth upwind flight, and in no instance was a female observed to descend from upwind flight and land on one of the circles.

The use of ubiquitous green leaf volatiles coupled with the additional nonspecific cues of relative humidity and a green visual stimulus could serve as habitat cues rather than host cues specific for a particular plant species. Habitat cues differ from host cues in that they are generally not species-specific cues, are released in large quantities, can be detected at long distances, and are associated with host-specific cues (Webster and Cardé 2016). Insects can use these habitat cues to increase the probability of detecting specific host cues and locating a host plant. Webster and Cardé (2016) suggested that habitat cues elicit general upwind movement, localized searching behavior, and enhanced response to host odor cues. Previous work showed that host and non-host sources elicited similar orientation behavior in the flight tunnel (*see Chapter 1*). Non-host plants (apple, *Malus domestica;* gray Dogwood, *Cornus*

*racimosa*) were chosen because of their overlapping range and phenology with the host. This apparent broad orientation response to host and non-host plants, coupled with additional non-specific landing cues, could simply indicate the presence of "vegetation worthy of closer inspection" (Webster and Cardé 2016), rather than a specific host plant, which supports the habitat odor hypothesis and the appropriate/inappropriate landings theory (Finch and Collier 2000). Further behavioral studies are necessary to observe the post-landing behavior of GBM females on both host and non-host plants and understand the host-acceptance process.

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# CHAPTER 3
# PLANTS, MICROORGANISMS, AND ODORANTS INVOLVED IN INSECT HOST PLANT LOCATION: WHO'S MAKING THE MESSAGE?


## Abstract

The grape berry moth (GBM), *Paralobesia viteana*, is a specialist pest insect of cultivated grape, *Vitis spp.*, in the eastern United States. An active blend of volatile compounds has been isolated from plant material that attracts the insect in flight tunnel assays. However, the plant odor space is potentially complicated by the presence of microbial organisms (bacteria and fungi) living on the surface of the plant. Microbial volatile organic compounds can affect insect behavior, and therefore must be considered to fully understand the olfactory mediated behaviors. We report here the efficacy of a technique used to sanitize the surface of plant material, as well as the chemical and behavioral analysis of the volatile profiles produced in both the sanitized and control shoot treatments. The sanitization technique removed 96.4 ± 3.8 % of the surface microorganisms for the duration of the behavioral assays and volatile collections. Overall, the surface microorganisms did not significantly contribute to the volatile profile of the grape shoots, as all of the peaks in the volatile profile of sanitized shoots were found in the profile of control shoots. Female GBM displayed the same level of upwind oriented flight response in the flight tunnel to sanitized shoots (flew upwind 57.4 ± 0.69%, landed 30.9 ± 0.42%) as they did to control shoots (flew upwind= 57.8 ± 0.69%; landed 31.0 ± 0.43%) suggesting

surface microorganisms did not play a significant role in the production of previously identified blend of behaviorally active volatiles.

## *Introduction*

Phytophagous insects rely on their host plants for food (Dethier 1954), and sites for mating (Trona et al. 2010), and oviposition (Hildebrand 1995). However, the number of plant species that insects are able to use is often limited, and it is therefore crucial for females to be able to locate and select an appropriate host plant. In many cases phytophagous insects utilize plant volatiles to locate and select a potential host from a distance (Bruce et al. 2005; de Bruyne & Baker, 2008). In recent years, however, it has been suggested that the compounds in the plant odor space are not all produced by the plant (Raguso 2004, Davis et al. 2013). Microbial organisms may produce key volatiles that insects can use to locate or evaluate their host plant (Honda et al. 1988, Tasin et al. 2012b, Witzgall et al. 2012, Davis et al. 2013).

When ripening fruits serve as host tissues, the idea that microbial metabolism contributes information to host-foraging insects makes intuitive sense. For example, Becher et al. (2012) explored the tritrophic interactions of the fruit fly, *Drosophila melanogaster,* with baker's yeast, *Saccharomyces cerevisiae*, and grapes, *Vitis vinifera*. Lower levels of attraction and oviposition were observed when the fly was presented with yeast-free sources compared to sources that contained yeast. Furthermore, the yeast alone was sufficient to attract flies and induce oviposition. The flies displayed the same response to synthetic blends of fermentation products produced by the yeast as they did to fermenting fruit, further suggesting that the yeast contributes significantly to the odor space and is producing behaviorally active volatiles. Similarly, Witzgall

et al. (2012) showed a close association between the codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae) and yeasts of the genus *Metschnikowia*. Larval feeding in apples enables yeast proliferation within the apple. These yeasts produce volatiles that elicited upwind flight responses from females. Seventeen behaviorally active compounds were identified to be of microbial origin, including butyl acetate, 3-methyl-3-butanol, 3-methyl-3-butan-1-ol, 1-octen-3-ol, nonanol, geranial, all of which are known components of attractive host plant mixtures identified for other insects (Nojima et al. 2003; Cha et al. 2008 ab).

The grape berry moth (GBM; *Paralobesia viteana*) is a tortricid moth native to the eastern United States and an important pest of cultivated grape, *Vitis spp.* (Williamson and Johnson 2005). The moth is an ovipositional specialist, laying its eggs almost exclusively on grape clusters or sometimes grape flower buds (Johnson and Hammer 1912, Clark & Dennehy, 1988), with larvae burrowing into and feeding on the grapes (Tobin et al. 2001). Cha et al. (2008a) showed that GBM females displayed oriented flight toward host plant material in a flight tunnel, and showed that grape shoots (rather than fruit) elicited maximal levels of upwind flight. Additionally, Cha et al. (2008b) identified a blend of eleven antenally active compounds that attracted GBM females in the flight tunnel. Two different 7-component blends were characterized that elicited equivalent behavior under the same conditions (Cha et al. 2008ab). The origin of these volatiles (plant-or microorganism-produced) remains unknown.

Here we examine the origin of the antenally active volatile

compounds in the plant odor profile, asking whether the removal of surface microorganisms affects orientation behavior of adult GBM. We report on the efficacy of a technique used to surface-sanitize plant shoots and the orientation behavior of GBM females flown in a flight tunnel to either control or surface-sanitized grape shoots. Shoots, rather than fruit were used as odor sources in flight tunnel assays due to the higher response levels of shoots compared with fruit in previous studies (Cha et al. 2008a).

## *Methods*

### *Insects*

Grape berry moths were reared in cages placed in walk-in environmental chambers at 26°C and 60% RH under a 16:8 L:D photoperiod. Adults were allowed to oviposit on seedless grapes (*Vitis vinifera*, red flame variety). First and second instar larvae were transferred to a diet cup (30 mL, WinCup Inc.) and reared on semi-synthetic diet (Nagarkatti et al. 2000) that consisted of grapes, pinto beans, and commercially available tobacco hornworm diet (Bio-Serve). For behavioral assays, unmated female moths were taken from cohorts set up by placing 10-15 female pupae (near eclosion) in a Plexiglass mating cage (30 cm H×30 cm W×30 cm D) and provided with a 50% honey and water solution. Twenty male moth pupae were added to additional mating cages loaded with 10-15 female pupae to assay mated females.

### *Plants*

*Vitis riparia,* a native host species of the GBM in northeastern

USA, was used for all assays. All plants were maintained in a greenhouse as in Cha et al. (2008a) with temperatures maintained between 21-26 ˚C. Supplemental light was provided to extend the day length to 16 h.

*Sanitization*

Grape shoots (15 cm in length) were cut, placed in water picks, and immediately transported to a fume hood. Shoots were wiped with lab tissue paper (Kimwipes, TM) soaked in 70% ETOH, dipped in 70% ETOH for 15 seconds, dipped in autoclaved water for 15 seconds, and allowed to dry in the fume hood (Becher et al. 2012). These shoots were considered "sanitized" (rather than sterilized) as the disinfection procedures did not eliminate all forms of microbial life on the shoots (see below). Control shoots were dipped in autoclaved water and allowed to dry in the fume hood (designated below as hydrated grape). Untreated grape shoots were cut, placed in water picks, and immediately transported to the flight tunnel for behavioral assays as a positive control.

*Length of sanitization studies*

To test the effect of sanitization on surface microbes over time, we cut a grape shoot with 8 leaves and transported it to a fume hood where the leaves were removed and individually sanitized using the above procedures. Leaves were pressed (plated) against petri dishes containing a layer of potato dextrose agar (BD Difco brand; http://www.bd.com/ds/productCenter/213200.asp) immediately after drying, 4 h, and 24 h after being sanitized. Control leaves (not sanitized) were also plated immediately after drying, as well as 24 h later. The 4 h

treatment was chosen because volatile collections and behavioral assays would not exceed 4 h. Leaves for 4 h and 24 h post-sanitization treatments were stored in the fume hood before they were plated. Each leaf was plated only once before it was discarded. After plating, petri dishes were sealed using parafilm and incubated at room temperature with no light. Microbial colonies were counted at 96 hours under a microscope. This study was replicated 6 times. A Kruskal-Wallis one-way analysis of variance followed by Dunn *post hoc* multiple comparison test was used to assess the difference in median colony count among all treatments.

*Adsorbent Sampling*

We used a closed collection system to collect headspace volatiles. The system was comprised of four custom-made, glass chambers (11 cm OD, 2.9 L) with two air-in adapters (7 mm ID) on the top and one air-out adapter near the base (7mmID) (Cornell Glass Shop, Ithaca, NY). Air was filtered using a custom-built air filtration system (Norgren Inc. Littleton, CO, USA), followed by a charcoal filter. Air entered a manifold and was split into 4 effluent lines entering individual volatile collection chambers. Airflow into each chamber was 1.2 L/min, and was monitored using flow meters (Cole Parmer Vernon Hills, IL, USA). Air was pushed out of the volatile collection chambers across a charcoal trap (ORBO32 – small, Supelco Inc., Bellefonte, PA, USA), and eluted with 300 µL dichloromethane every 30 minutes for 4 h and stored in a freezer (-20 ˚C) until used for chemical analysis (Becher et al. 2012). ORBO traps were discarded after each 4-h collection period. Collections from the same

source were combined and condensed under a stream of nitrogen until the final volume of the sample was 2 mL.

*Volatile collection from 96 hour plates*

To collect volatiles produced by microorganisms growing on control, unsanitized grape leaves, six leaves were cut from the same shoot, plated, and incubated as above for 96 h. All 6 plates were placed in the same volatile collection unit, and the volatiles were collected as stated above for 4 h. We collected volatiles from six plates containing only PDA using the same procedures.

*Flight Tunnel*

The flight tunnel was 2 m in length by 0.6 m in width and 0.6 m in height, with a fan installed at the upwind end to create a steady airflow into the tunnel and an exhaust hood at the downwind end to evacuate odor from the flight tunnel (Cha, et al. 2008 ab). Wind speed was set at 0.25 m/s at the wire stand where the moths were introduced into the wind tunnel. A pattern made of dark green paper circles (10 cm diameter) was randomly presented both on a white background glass floor and on the glass ceiling below the light source. During the experiments, the average temperature in the flight tunnel was 23.8 ± 0.07 °C, and the relative humidity ranged from 22% to 71%. Female moths were placed in the flight tunnel room 1 h prior to scotophase. Light intensity was reduced to 25 lx 30 min before dark, and remained at this intensity for behavioral assays. Behavioral assays began 15 minutes prior to scotophase.

The odor source was placed 30 cm from the upwind end of the

tunnel. Four- to five-day old females were used in all flight tunnel assays. All insects were discarded after being assayed. Female moths were placed in the flight tunnel individually in a metal screen release cage on a wire stand 1.5 m downwind of the source, and their behavior was observed for 5 min. We recorded whether the insect flew out of the release cage, flew upwind (more than 10 cm of oriented flight towards the source), and landed on the source (made contact with the source). Fisher's exact test ($P < 0.05$) was used to compare the percent response of the GBM females to the different odor sources.

*Chemical analysis*

Chemical extracts were analyzed using an Agilent 5890 gas chromatograph coupled with a 5973n mass selective detector running in EI mode at 70 eV. The GC was equipped with a DB-1 Column (30 m×0.25 mm ID, 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA). Helium was used as the carrier gas at a constant flow of 1.0 mL/min. The oven temperature was programmed to hold at 40°C for 5 min then increase by 15°C/min until the oven reached 250°C, and hold at that temperature for 5 min. Volatile compounds were tentatively identified by mass spectral matches to library spectra and confirmed by retention time and mass spectral matches to available authentic standards. Peaks of sanitized and control shoots were recorded and compared (minimum peak area = 100000).

*Chemicals*

(Z)-3-hexen-1-yl acetate, ethyl hexanoate, nonanal, linalool, methyl

salicylate, decanal, β-caryophyllene, α-farnesene, and 6-pentyl-α-pyrone were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA), Alfa Aesar (Ward Hill, MA, USA), Fluka (Buchs, Switzerland) or TCI America (Portland, OR, USA). All, except α-farnesene (a mixture of various isomers) were greater than 97% purity. The 4,8-dimethyl-1,3(E),7-nonatriene was provided by the Chong lab (University of Waterloo, Ontario, CA). Germacrene-D was isolated from golden rod as 91% germacrene-D and 9% β-caryophyllene (by USDA Chemistry Research Unit, Gainesville, FL, USA).

## *Results*

### *Length of Sanitization*

Sanitization significantly reduced the number of microbial colonies at 96 h (Kruskal-Wallis: $\chi^2 = 24.137$; df = 4; $P < 0.0001$; Figure 3.1). All three sanitized treatments resulted in significantly fewer numbers of colonies than the control leaf treatments (Dunn's test $P < 0.05$). The mean number of colonies in any of the sanitized treatments was not significantly different from each other (Dunn's test, $P > 0.05$). The mean numbers of colonies in the control leaf treatment replicates ($T_0$ and $T_{24}$) were not significantly different ($P > 0.05$).

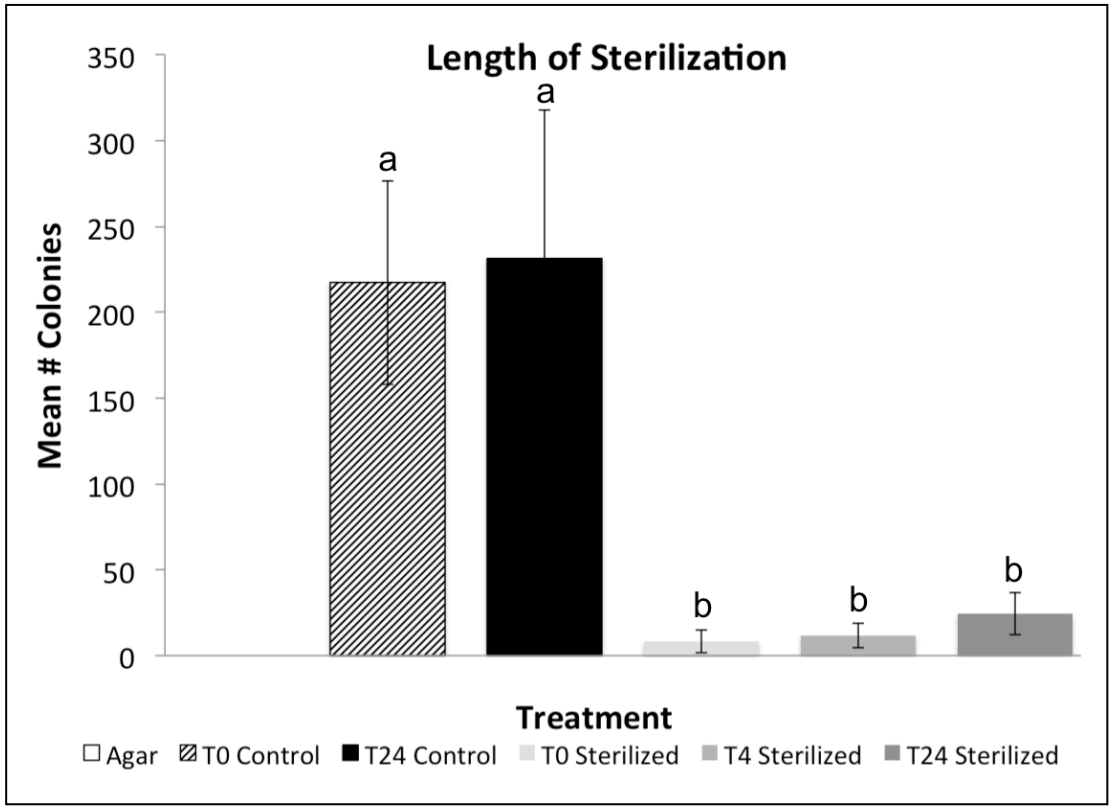


Figure 3.1. Number of colonies on agar plates after 96 hours of incubation. Leaves were plated directly after sanitization (T0 Sanitized), 4 h (T4 Sanitized), and 24 h post sanitization (T24 Sanitized), and control leaves were plated directly after their sanitized compliment. Error bars display 95% confidence interval, and different letters on bars indicate statistically significant differences ($P < 0.05$).

*Behavioral Assays*

We conducted flight tunnel assays to observe GBM female response to sanitized and control plant sources (Figure 3.2). The moths took flight 85.9 ± 0.94% of the time, flew upwind 57.8 ± 0.69% of the time, and landed 31.0 ± 0.43% of the time when presented with untreated grape shoots (n = 71). The moths did not behave in a significantly different way to untreated shoots than they did to sanitized grape (n = 70, took flight = 88.2 ± 0.97%, flew upwind 57.4 ± 0.69%, landed 30.9 ±

0.42%; Fisher's exact test $P > 0.5$, df= 1) or rehydrated shoots (n = 62, took flight = 90.3 ± 0.99%, flew upwind= 54.8 ± 0.69%, landed = 22.6 ± 0.33%; Fisher's exact test $P > 0.5$, df= 1). In addition, the moths did not behave significantly different to the untreated grape shoots than they did to the rehydrated shoots (Fisher's exact test $P > 0.5$, df= 1).

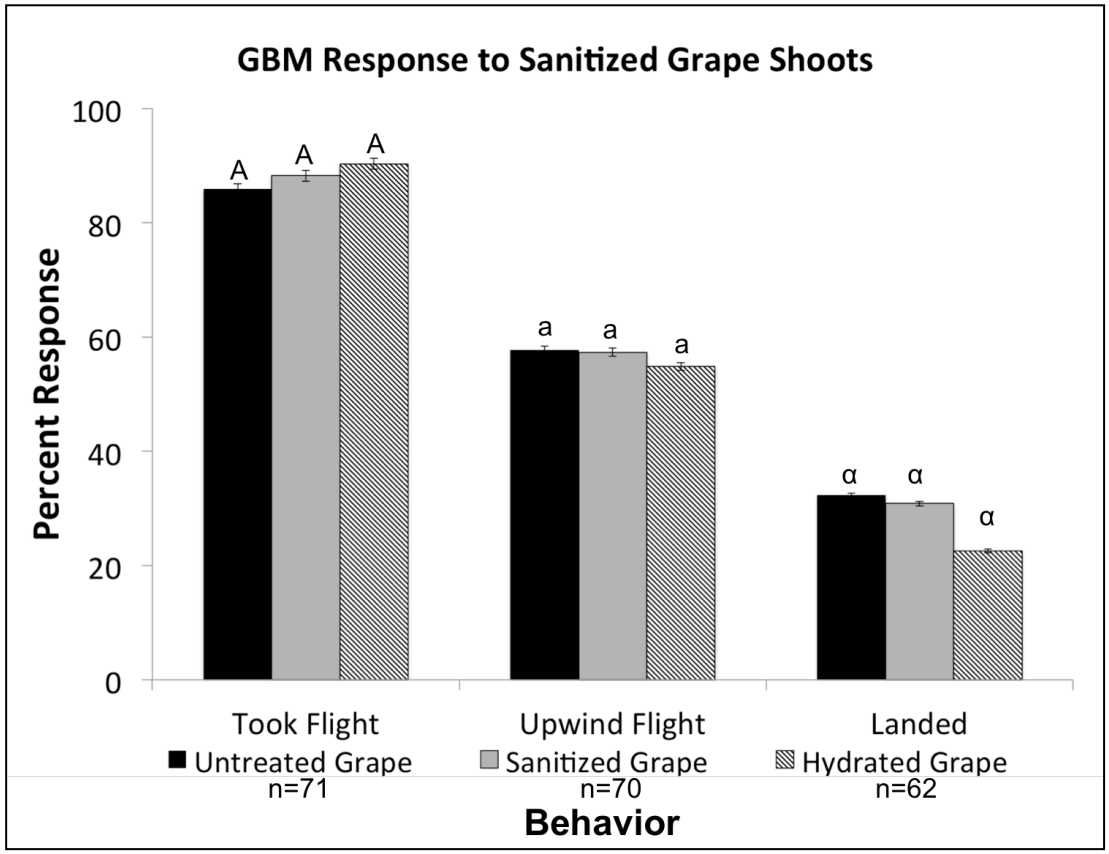


Figure 3.2. Behavioral responses of GBM females to sanitized and control grape shoots in the flight tunnel. Moths took flight, flew upwind, and landed on all sources a statistically similar proportion of the time (Fisher's exact test, $P > 0.05$). Error bars indicate 95% confidence interval. Different letter styles represent different statistical tests (capital letters for took flight response, lower case letters for upwind flight responses, and Greek letters for landing response), and the same letter represents statistical similarities ($P > 0.05$).

*Volatile Profiles*

The volatile profiles of the sanitized and control grape shoots, as

well as the surface microorganisms from the PDA plates and control PDA plates, were analyzed via GC-MS (Figure 3.3, Table 3.1). Sixty-seven total peaks were recorded across all profiles. Forty-six peaks were recorded in the volatile profile of sanitized shoots, and 48 peaks were recorded in the volatile profile of the control shoots (Table 3.1). All 46 of the peaks present in the sanitized profile were present in the control profile. The two peaks found in the control shoots but not in the sanitized shoots (Peaks 53 and 67) have not yet been identified. The sanitized and control grape shoots had the same compounds previously identified to be antenally active for GBM (designated in bold, Table 3.1). The compound Z-3-Hexen-1-yl acetate was 5x more abundant in sanitized shoots compared to control shoots. All other antenally active compounds were present in the same ratio in the sanitized and control profiles. Ethyl hexanoate, one of the non-antennally active volatiles, was 80x more abundant in the sanitized extract than the control extract.

The profile for the microorganisms on the PDA plates (Figure 3.3) contained 19 peaks (Table 3.1), none of which were found in the sanitized or control shoots. Two common microbial volatiles, 6-pentyl-α-pyrone and 3-methyl-1-butanol, were present in the volatile profile of the microorganisms. The profile of the microbial volatiles and the PDA control plate chromatogram were transformed to be shown on the same axes as the sanitized and control shoots (displayed as [counts] – 600000 and [counts] – 625000, respectively).

Table 3.1. Compounds identified from the volatile profiles of the sanitized and control grape shoots, and of the plated microorganisms. Compounds accompanied by an asterisk were identified using retention time matches to synthetic standards and library matches to mass spectra. Compounds 53 and 67 were only present in the profile of control shoots. All other peaks were present in the profiles of both shoots. The profile of the microorganisms did not contain any of the previously identified behaviorally active compounds.

| Peak # | ID | RT | m/z | Ratio (Sanitized:Control) |
|---|---|---|---|---|
| 1 | 3-Penten-2-ol | 8.28 | 86 | Microbe Plate Only |
| 2 | 2-Heptanone | 8.45 | 114 | Microbe Plate Only |
| 3 | 3-Methyl-1-butanol | 8.79 | 88 | Microbe Plate Only |
| 4 | 2-Hexanol | 8.98 | 102 | Microbe Plate Only |
| 5 | Unknown | 9.16 | 138 | Microbe Plate Only |
| 6 | 1,6-Methyl-5-hepten-2-one | 9.17 | 126 | 0.65 |
| 7 | Unknown | 9.25 | 155 | Microbe Plate Only |
| 8 | Ethyl hexanoate | 9.47 | 144 | 81.64 |
| 9 | **Z-3-Hexen-1-yl acetate*** | 9.55 | 142 | 5.14 |
| 10 | Hexyl acetate | 9.65 | 144 | 6.21 |
| 11 | Unknown | 9.76 | 108 | 3.61 |
| 11 | Unknown | 9.90 | 114 | Microbe Plate Only |
| 12 | Unknown | 9.93 | 154 | 4.21 |
| 13 | Unknown | 10.13 | 107 | Microbe Plate Only |
| 14 | 4-Hexen-1-ol acetate | 10.22 | 142 | Microbe Plate Only |
| 15 | (*E*)-β-Ocimene* | 10.24 | 136 | 0.50 |
| 16 | Heptadecane* | 10.55 | 240 | 0.72 |
| 17 | Anisole | 10.56 | 108 | Microbe Plate Only |
| 18 | Unknown | 10.61 | 138 | 0.94 |
| 19 | Unknown | 10.65 | | 1.41 |
| 20 | Unknown | 10.71 | 134 | Microbe Plate Only |
| 21 | **Nonanal*** | 10.83 | 142 | 1.19 |
| 22 | **Linalool*** | 10.83 | 154 | 1.19 |
| 23 | Unknown | 10.95 | 114 | Microbe Plate Only |
| 24 | Unknown | 10.97 | 198 | 1.04 |
| 25 | **(*E*)-4,8-Dimethyl 1,3,7-nonatriene*** | 11.10 | 150 | 0.63 |
| 26 | (Z)-3-Hexenyl butyrate | 11.33 | 170 | 4.12 |
| 27 | Ethyl benzoate | 11.54 | 150 | 9.23 |
| 28 | Unknown | 11.79 | 136 | 2.45 |
| 29 | Unknown | 11.87 | 204 | Microbe Plate Only |
| 30 | Unknown | 11.90 | 136 | 2.63 |

| | | | | |
|---|---|---|---|---|
| 31 | **Decanal*** | 11.98 | 156 | 1.57 |
| 32 | Unknown | 12.02 | 168 | 3.37 |
| 33 | 2,5-Hexanedione | 12.19 | 114 | Microbe Plate Only |
| 34 | 2-Hexenoic acid, 4-hydroxy, ethyl ester | 12.25 | 116 | 4.33 |
| 35 | Unknown | 12.31 | 126 | 3.87 |
| 36 | Unknown | 12.42 | | 1.09 |
| 37 | Unknown | 12.49 | 186 | 1.24 |
| 38 | Unknown | 12.62 | 190 | 1.20 |
| 39 | Unknown | 12.78 | 152 | 1.72 |
| 40 | Unknown | 12.85 | 154 | 1.19 |
| 41 | Tetradecane* | 13.00 | 198 | 0.84 |
| 42 | Unknown | 13.24 | 126 | 1.62 |
| 43 | Unknown | 13.35 | | 1.36 |
| 44 | Nonadecane* | 13.43 | 268 | 1.25 |
| 45 | (Z)-β-Hexenyl caproate | 13.73 | 198 | 2.21 |
| 46 | Hexahydrofarnesol | 13.98 | 228 | 2.04 |
| 47 | 1,3-Dimethoxy benzene | 14.28 | 138 | Microbe Plate Only |
| 48 | **β-Caryophyllene*** | 14.34 | 204 | 1.69 |
| 49 | Unknown | 14.57 | 180 | 1.10 |
| 50 | Unknown | 14.59 | 182 | 1.25 |
| 51 | **α-Farnesene*** | 14.92 | 204 | 0.87 |
| 52 | Unknown | 14.97 | 168 | 1.00 |
| 53 | Unknown | 15.08 | | Control Only |
| 54 | Unknown | 15.33 | | 0.98 |
| 55 | Unknown | 16.13 | 248 | 1.66 |
| 56 | Unknown | 16.32 | 268 | 0.99 |
| 57 | Octacosane* | 16.68 | 394 | 0.77 |
| 58 | Unknown | 16.98 | | 0.83 |
| 59 | Unknown | 17.32 | 244 | 0.56 |
| 60 | Unknown | 17.38 | 298 | 0.73 |
| 61 | Unknown | 17.86 | 242 | Microbe Plate Only |
| 62 | Unknown | 17.93 | 178 | Microbe Plate Only |
| 63 | Verticiol | 18.07 | 290 | Microbe Plate Only |
| 64 | Unknown | 18.20 | 222 | 0.68 |
| 65 | Unknown | 18.46 | 164 | Microbe Plate Only |
| 66 | Unknown | 18.47 | | 1.26 |
| 67 | Unknown | 19.25 | 296 | Control Only |

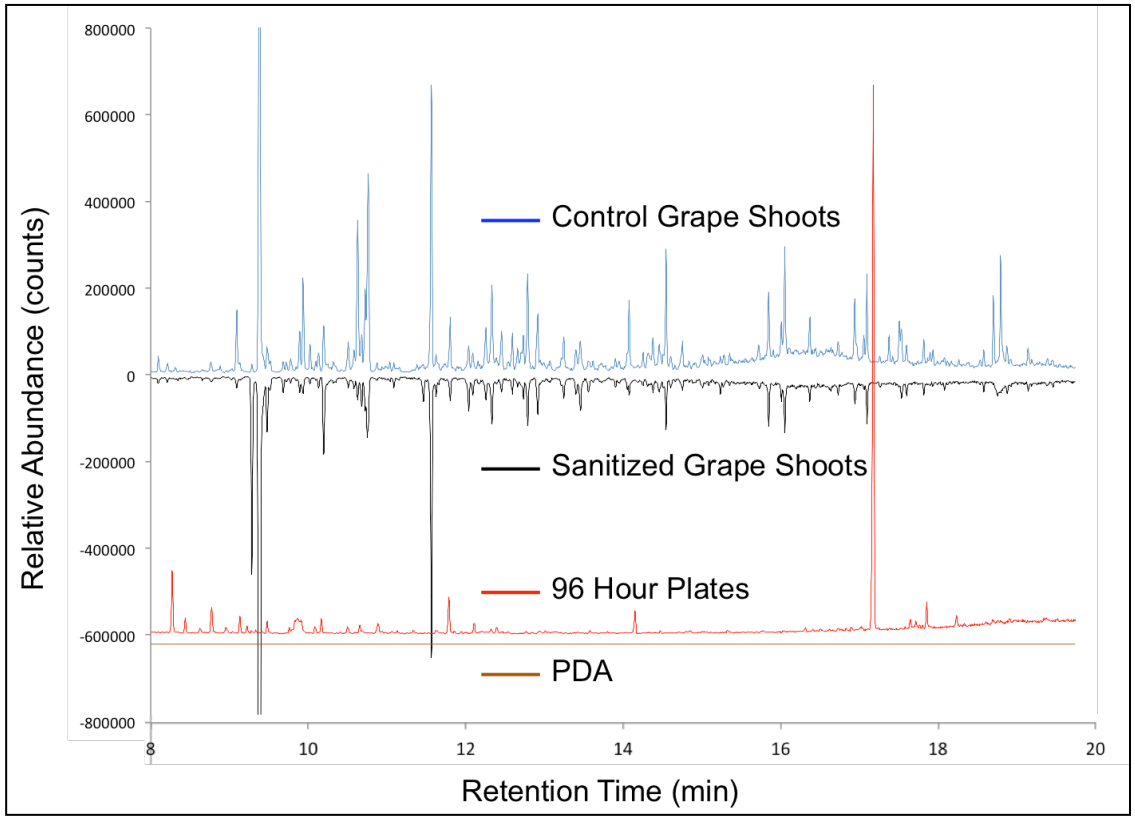


Figure 3.3. Chromatograms of sanitized and control grape shoots, plates inoculated with the surface microorganisms from grape shoots, and potato dextrose agar. See Table 3.1 for details concerning peak identifications and comparisons.

## *Discussion*

Previous studies showed that GBM females displayed the highest levels of oriented upwind flight to the grape shoots compared to other plant tissue (Cha, et al. 2008a). Thus, our study focused on the microorganisms in the phyllosphere of grape shoots. Grape shoots were sanitized using 70% ethanol and plated after 4 h to assess the length and efficacy of the sanitization procedures. The sanitization procedure removed 96.4 ± 3.8 % of surface microorganisms, and remained surface-sanitized for the duration of volatile collections and behavioral assays. These sanitized grape shoots were used as odor sources in flight tunnel assays.

The moths displayed the same level of response in the flight tunnel to sanitized grape shoots as they did to control shoots. These results suggest that surface microorganisms did not produce the previously identified behaviorally active volatiles and that our sanitization technique did not significantly degrade shoot physiology. Further, the volatile profiles of sanitized and control grape shoots were similar. Forty-six of the 48 GC peaks were present in both volatile profiles. None of the peaks unique to either the control or sanitized shoot profile were previously identified to be antennally active for GBM. Both profiles contained antennally active compounds identified in previous work (Cha et al. 2008a). One of these volatiles, (Z)-3-hexen-1-yl acetate was 5x more abundant in sanitized shoots compared to control shoots. Ethyl hexanoate, a non-antennally active volatile, was 80x more abundant in the volatile profile of sanitized grape shoots compared to control grape shoots. These differences could be an artifact of the sanitization process. Carboxylic acids treated with excess alcohol (in this case, ethanol) form esters (Fischer Esterification, Bruice, 2007). Importantly, however, these differences in the volatile profile did not affect the insect's behavioral response to the grape shoots. The compound 6-pentyl-α-pyrone (6PP) is a common fungal volatile (Rocha-Valadez et al. 2005), and was highly abundant in the volatile profile of the surface microorganisms on PDA plates. However, 6PP was not seen in the volatile profiles of either control or sanitized grape shoots, supporting the conclusion that surface microorganisms are not contributing to the volatile profile of the grape shoots.

Other studies have investigated the interactions between insects and microorganisms (Lauzon et al. 1998, Becher et al. 2012, Witzgall et al. 2012, Davis and Landolt 2013, Douglas and Dobson 2013, Davis et al. 2013). Adult female codling moths were attracted to the volatiles produced by yeast, and also laid more eggs on apples inoculated with yeast than on sanitized apples (Witzgall et al. 2012). Similarly, fruit flies, *D. melanogaster*, were attracted to the volatiles produced by yeast, and laid more eggs on sources containing yeast compared to yeast-free sources. It is important for adult females to detect the presence of the yeast as it is an essential part of the larval diet for both species (Anagnostou et al. 2010). Like the codling moth, GBM females lay their eggs on the fruit of the host, and larvae develop within the fruit (Clark and Dennehy 1988). We found no evidence of an association between the GBM and microorganisms living on the phyllosphere of grape plants as GBM females displayed the same level of response to sanitized shoots as they did to control shoots.

Endophytes within the plant tissue were likely not removed using our sanitization technique, and were therefore beyond the scope of this project. However, it is well established that endophytic microorganisms contribute to the "plant" odor space, either by altering the volatiles produced by the plant (Guerrieri et al. 2004), or by producing volatiles themselves (Zhi-Lin et al. 2012). Endophytic fungi of the *Muscodor* genus colonize soapberry shrubs, *Paullinia spp.*, and produce volatiles that modify the behavior of insect pests. Specifically, naphthalene was

identified in the fungal volatile blend and repelled the wheat stem sawfly, *Cephus cinctus*, in Y-tube assays (Daisy et al. 2002). Raguso and Roy (1998) found that endophytic rust fungi in the *Puccinia* complex (Pucciniacae, Uredinales, and Bacidiomycetes) can infest plants of the genus *Brassicaceae* and produce common floral odors to enhance pollinator visitation.

Insects can use volatiles to gain valuable information on host quality (Tasin et al. 2012a). The plants used in our flight tunnel assays were grown under greenhouse conditions, and were likely under different biotic and abiotic stresses compared to plants in nature (Mccormick 2016). Stressed plants can produce different volatile profiles, depending on whether the stress is due to microbial or pathogen infestation or herbivory, which can alter host quality (Becher et al. 2010, Tasin et al. 2012b, Zakir et al. 2013). The European grapevine moth, *Lobesia botrana*, can avoid hosts infested with the phytopathogenic fungus *Botrytis cinerea* by detecting 3-methyl-1-butanol, a volatile produced by the fungus (Tasin et al. 2012b). *Drosophila melanogaster* has a conserved dedicated neural circuit for detecting geosmin, a volatile produced by fungi and bacteria (Stensmyr et al. 2012). Flies displayed avoidance behavior to synthetic geosmin alone at concentrations 1,000x more dilute than other known repellants under laboratory conditions.

With respect to herbivory, insect pests can play a major role in the production of induced volatiles that can influence insect behavior (Kessler 2015, Dicke 2016). Tobacco hornworm larvae, *Manduca sexta*, feeding

on sacred Datura, *Datura wrightii*, induce isomeric changes in green leaf volatiles. These isomeric shifts can be detected by gravid *M. sexta* females, and reduce oviposition on the plant (Allmann et al. 2013). Japanese beetles, *Popillia japonica*, feeding on grape plants, *V. riparia*, induce changes in the ratio and concentration of the volatiles leading to reduced upwind flight response by *P. viteana* (Cha et al. 2011). Our study focused on the GBM response to uninfested, or "control" plant material. Further studies using plant material from nature, and artificially infested plant material are necessary to observe insect responses to altered volatile profiles that result from microbial infestations and/or herbivory.

The results of our study suggest that surface microorganisms did not contribute to the behaviorally active odor profiles of grape shoots as used in our study system. Future studies should not ignore the possible contribution of microbes to the odor space of a plant, especially given the range of damage and stress (biotic or abiotic) that can occur in nature. The influence of microbial compounds can have positive or negative effects on potential herbivores and thus is an important consideration in development of pest management techniques using plant volatiles, such as push-pull systems, which depends on our complete understanding of the olfactory environment in which the insects are active (Wallingford et al. 2017).

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