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Chemically Mediated Interactions Between Hosts, Parasitic Plants and Insect Herbivores

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CHEMICALLY MEDIATED INTERACTIONS BETWEEN HOSTS, PARASITIC PLANTS AND INSECT HERBIVORES

A Dissertation Presented

by

MUVARI C. TJIURUTUE

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

MAY 2016

Plant Biology Graduate Program
CHEMICALLY MEDIATED INTERACTIONS BETWEEN HOSTS, PARASITIC PLANTS AND INSECT HERBIVORES

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MUVAI C. TJIURUTUE

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To my loving parents Konstancia Tjimoma Ngutjinazo & Rudolf Kapee Ngutjinazo and grandparents Simon Tjiurutue & Naomi Uandjua Tjiurutue.
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A special thanks goes to my advisor Lynn Adler for her support and patience throughout all these years. Thank you for never giving up on me and for always saving the day. I would also like to thank my committee members for their support and understanding. Special thanks to Anne Averill for all the encouragement and kind words, Hilary Sandler for her never-ending help and patience, and Peter Alpert for attending all my poster presentations and providing good advice for the future.

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ABSTRACT

CHEMICALLY MEDIATED INTERACTIONS BETWEEN HOSTS, PARASITIC PLANTS AND HERBIVORES

MAY 2016

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Directed by: Professor Lynn S. Adler

Species interactions, by changing phenotypic traits, can alter the outcome of subsequent interactions. Plant-mediated responses to herbivores have been extensively studied, but little is known about plant-mediated responses involving parasitic plants within a broader community context that also includes herbivores. Because parasitic plants are important components of many ecosystems and can shape community structure, it is important to understand how host-mediated interactions influence parasite preference and success. The goal of this thesis is to examine interactions between hosts, parasitic plants and herbivores mediated by chemical traits. We first examined the effects of dodder (*Cuscuta* sp.) parasitism on induced defenses in cranberry, and asked how cranberry chemistry affected dodder preference and performance. We found dodder preference for some cultivars, and dodder parasitism induced many changes in cranberry chemistry, which could influence other interactions with cranberry hosts. We next examined the effects of gypsy moth herbivory on cranberry chemistry, and how plant-mediated changes affected subsequent dodder preference. Herbivory delayed and reduced the number of dodder plants that attached to cranberry hosts. Herbivory also induced
changes in cranberry phenolic acids and phytohormones, which could mediate defenses against dodder parasitism. We also assessed the effects of previous herbivory (by tobacco hornworm or mechanical) and previous dodder parasitism on subsequent dodder preference on tomato hosts. Previous attachment followed by removal of dodder slowed subsequent dodder attachment on tomato hosts, but prior herbivory did not affect subsequent dodder attachment. Lastly, we asked whether damage to host induced changes in the host, and if attached parasites assimilated host defenses in response to host damage. Damage to host plants induces higher jasmonic acid in both hosts and attached parasites, and herbivores fed on leaves from parasites attached to damaged hosts ate more than herbivores fed on leaves attached to undamaged parasites. In summary, these studies demonstrate that parasites can induce changes in host responses that can potentially shape other interactions with the same hosts. Similarly, both herbivores and host responses can influence parasite preference, which could alter behavior of herbivores and pollinators, shaping community dynamics.
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CHAPTER 1

CRANBERRY RESISTANCE TO DODDER PARASITISM: INDUCED CHEMICAL DEFENSES AND BEHAVIOR OF A PARASITIC PLANT


1.1 Abstract

Parasitic plants are common in many ecosystems, where they can structure community interactions and cause major economic damage. For example, parasitic dodder (Cuscuta spp.) can cause up to 80-100% yield loss in heavily infested cranberry (Vaccinium macrocarpon) patches. In spite of their ecological and economic importance, remarkably little is known about how parasitic plants affect, or are affected by, host chemistry. To examine chemically-mediated interactions between dodder and its cranberry host, we conducted a greenhouse experiment asking whether: (1) dodder performance varies with cranberry cultivar, (2) cultivars differ in levels of phytohormones, volatiles or phenolics, and whether such variation correlates with dodder parasitism, (3) dodder parasitism induced changes in phytohormones, volatiles or phenolics, and whether the level of inducible response varied among cultivars. We used five cranberry cultivars to assess
host attractiveness to dodder and dodder performance. Dodder performance did not differ across cultivars, but there were marginally significant differences in host attractiveness to dodder, with fewer dodder attaching to Early Black than to any other cultivar. Dodder parasitism induced higher levels of salicylic acid (SA) across cultivars. Cultivars differed in overall levels of flavonols and volatile profiles, but not phenolic acids or proanthocyanidins, and dodder attachment induced changes in several flavonols and volatiles. While cultivars differed slightly in resistance to dodder attachment, we did not find evidence of chemical defenses mediating these interactions. However, induction of several defenses indicates that parasitism alters traits that could influence subsequent interactions with other species, shaping community dynamics.

**Key Words**-Parasitism, plant-plant interactions, induced responses, phytohormones, volatiles, flavonols

**1.2 Introduction**

Plants face a wide range of antagonistic interactions, including competition from other plants and consumption by herbivores. Although plants usually interact with other plants as competitors, many ecosystems also include parasitic plants that may play a key role in structuring community interactions (Pennings and Callaway 2002). In managed ecosystems, parasitic plants can cause major economic damage (Smith et al. 2013). In spite of the importance of parasitic plants in both natural and agricultural settings, the role of plant chemical defenses mediating resistance to parasitism and the effect of parasitic plants on induced host responses are still unexplored.
The effect of different types of herbivory on induction of phytohormones is well established, but the extent to which plant-parasitic plant interactions are similar to those of plant-herbivore interactions is largely unknown. In general, piercing and sucking herbivores (e.g., aphids and leafhoppers) induce salicylic acid (SA) mediated responses similar to those induced by biotrophic pathogens (Glazebrook 2005; Walling 2000), while chewing insects and necrotrophic pathogens generally induce jasmonic acid (JA) mediated defense responses (Glazebrook 2005; Walling 2000). However, both JA and SA have been shown to crosstalk, and the classification of JA as an herbivore defense response and SA as a pathogen defense response is not mutually exclusive (Thaler et al. 2012).

Several previous studies have examined induced chemical defenses in response to parasitic plants. The stem parasite, *Cuscuta pentagona*, had little effect on JA and SA accumulation upon first attachment to 10-day old tomato plants (*Lycopersicon esculentum*), and did not induce a hypersensitive-like response (Runyon et al. 2010). However, older tomato plants responded to a second dodder attachment by activating both JA- and SA- signaling pathways and inducing a strong hypersensitive-like response (Runyon et al. 2010). The hypersensitive-like response has been reported in dodder-resistant tomato cultivars in response to attachment by *C. reflexa* (Sahm et al. 1995), suggesting it may play an important role in host plant resistance to dodder. In the *Striga* system, which has been extensively studied, parasitism by *S. hermonthica* induced genes involved in SA defense responses in the most resistant sorghum cultivar, suggesting that SA-induction may mediate interactions between the host and parasite (Smith et al. 2009). Additionally, in non-host species, resistance to *Striga asiatica* typically involves
browning and necrosis of the root cortical cells of the host accompanied by cell wall thickening (Hood et al. 1998). However, more work should be done in other systems to determine the generality of host induced responses and mechanisms of resistance to parasitic plants.

*Cuscuta* species may exhibit ‘foraging behavior,’ and discriminate between hosts based on quality. *Cuscuta pentagona* seedlings showed directed growth towards tomato seedlings compared to artificial tomato plants, and toward extracted tomato plant volatiles in the absence of other cues (Runyon et al. 2006), suggesting that dodder uses volatiles to find host plants. *Cuscuta europaea* also exhibited directed growth towards hawthorn (*Crataegus monoguna*) hosts with high nutritional content, and grew away from hosts with low nutritional content (Kelly 1992), but the mechanism by which parasites distinguished between hosts was unknown. Understanding the mechanism of dodder preference and host resistance will broaden our understanding of cues used in plant foraging, and the roles that plant defenses play in mediating interactions with a range of antagonists.

Cranberry (Ericaceae: *Vaccinium macrocarpon*) is a temperate, perennial vine common in North American wetlands (Rodriguez-Saona et al. 2011) and native in Massachusetts. With sale values of $99.8 million in 2012, cranberry production was the second largest of all agricultural commodities in Massachusetts (National Agricultural Statistics Service 2011). Cultivated cranberry is genetically similar to native wild genotypes, making research with agricultural cultivars very relevant to understanding ecological interactions in native systems (Rodriguez-Saona et al. 2011). There is some evidence for differences in host resistance across cultivars; for example, chemical
defenses and gypsy moth (*Lymantria dispar*) performance differed across cranberry cultivars (Rodriguez-Saona et al. 2011). Gypsy moth performed best on the highest yielding variety, NJS98-23, and on its parental variety, Ben Lear. The NJS98-23 cultivar had lower concentrations of JA and of induced volatile sesquiterpenes compared to ancestral cultivars, suggesting that high yielding cultivars may be susceptible to herbivore damage due to reduced chemical defenses (Rodriguez-Saona et al. 2011).

Dodder (*Cuscuta* sp.) is a generalist host stem parasite that infests and causes extensive damage each year to a wide range of agricultural crops including tomato (*Solanum lycopersicum*), alfalfa (*Medicago sativa*), potato (*Solanum tuberosum*), soybean (*Glycine max*), and onion (*Allium cepa*) (Runyon et al. 2008). Dodder can cause up to 80-100% yield loss in heavily infested cranberry patches (Devlin and Deubert 1980). Dodder management is extremely difficult because seeds can remain dormant for several years underground, and the close association of dodder and its host necessitates highly specific pesticides that will target the parasite without killing the crop (Goldwasser et al. 2012). Currently, effective management of dodder requires integrating various methods, including killing current plants with herbicides, preventing seed production and restraining the growth of new seedlings (Sandler and Ghantous 2014). Given the economic costs of dodder as a cranberry pest, it is important to assess variation in cultivar resistance to dodder and evaluate the potential role of chemical defenses and induced responses mediating resistance. Such information could be used to target traits for developing resistant cranberry cultivars, offering producers an alternative management strategy for dodder control (Sandler 2010).
To examine chemically mediated interactions between dodder and its cranberry host, we conducted greenhouse experiments to ask the following questions:  
1. Does host attractiveness to dodder and dodder performance vary with cranberry cultivar? 2. Do cultivars vary in levels of phytohormones, volatiles or phenolics, and does such variation correlate with host attractiveness to dodder and dodder performance? 3. Does dodder parasitism induce chemical changes in phytohormones, volatiles or phenolics, and does the level of inducible response vary among cultivars?

1.3 Methods and materials

1.3.1 Cranberry cultivars and propagation

We used five cranberry (V. macrocarpon, Ericaceae) cultivars: Crimson Queen, Mullica Queen, Stevens, Howes and Early Black. Early Black and Howes are wild cultivars that represent more than 50% of MA acreage and were parental cultivars used for the development of some new cultivars (Caruso 2008). Stevens was a result of the first USDA breeding program, a hybrid from a cross between McFarlin and Potter, and was bred for its high productivity, fair coloring and good fruit rot resistance (Caruso 2008). Mullica Queen and Crimson Queen were both released in 1996 as new cultivars by Rutgers University (Vorsa 2010). Crimson Queen resulted from a cross between Stevens and Ben Lear, and was bred for its high yield, good anthocyanin production, high stolon vigor and early fruit ripening (Caruso 2008, Vorsa 2010). Mullica Queen resulted from a cross between LeMunyon and #35 (one of the original 40 cultivars in the USDA breeding program), and was bred for its high anthocyanin production, high stolon vigor and fruit rot resistance comparable to Stevens or better (Caruso 2008, Vorsa 2010).
Cranberry vines were collected from the University of Massachusetts Cranberry Station in East Wareham, MA, over a period of two days in early October 2010. Cranberry vines were cut into 7.6 cm sections and were sown in 72 plug trays filled with a 3:1 sand: peat soil mixture. Cultivar identities were confirmed via DNA finger printing using SCAR markers (Rodriguez-Saona et al. 2011). Roots were well established by November 2010 and cuttings were moved in December into cold storage at 5°C and 78% humidity. The cuttings were taken out of cold storage in mid-March 2011 after experiencing more than 2500 dark chilling hours, transported to University of Massachusetts Amherst, and placed in the greenhouse with natural lighting. One week later, upright cuttings were repotted into 10 cm plastic round pots in 3:1 sand: peat moss mixture. Plants were watered twice daily by hand. Approximately 1.5 g of 14-14-14 Osmocote fertilizer (Scotts-Sierra Horticultural Products Company, Marysville, OH, USA) was added to each pot on 16 April 2011.

1.3.2 Experimental design

We conducted two parallel experiments simultaneously in the same greenhouse. Both experiments used 4 uprights of the same cultivar per pot, and pot was considered the unit of replication. Blocks in each experiment, containing one pot per cultivar per treatment, were randomly rotated regularly to reduce variation due to greenhouse lighting. The first experiment assessed host attractiveness to dodder and dodder performance on each cranberry variety. This experiment used 20 replicates per 5 cultivars, all with germinated dodder seeds, for a total of 100 replicate pots in 20 blocks. The second experiment measured traits related to chemical defense (phytohormones, volatiles and phenolics) in
each cultivar, and asked whether dodder parasitism induces changes in these compounds. The induction experiment involved 20 replicate pots per 5 cultivars x 2 treatments (with or without dodder) = 200 pots total. Twenty blocks, each containing one pot of each cultivar-by-treatment combination, were established by grouping plants by height.

1.3.3 Dodder treatments

Dodder seeds, *Cuscuta spp.* (Convolvulaceae) were collected on 28 September 2008 from Swan Holt, a commercial cranberry bog in Carver, MA. Identification of *Cuscuta* species can be challenging; PCR of DNA from dodder collected from several sites in this region indicated that plants were mostly *C. gronovii*, but with some *C. campestris* and possibly *C. compacta* co-occurring (K Ghantous, University of Massachusetts Cranberry Experiment Station, pers. comm.) (Ghantous et al. 2012). Seeds were scarified in batches of 100 (0.01 g) in a 2 ml microcentrifuge tube for approximately 3 minutes using a small dremel tool (Ghantous and Sandler 2012). Seeds were then placed on a fine mesh strainer, rinsed, and placed in Petri dishes lined with 90 mm moistened filter paper and sealed with Parafilm. Petri dishes were placed in an incubator at 23°C until the seed germinated, approximately 2 days later. Over a period of 3 weeks as seeds germinated, each pot received one seedling per upright for both the performance and induction experiments. Dodder was added to all pots receiving dodder within a block on the same day. Seedlings were placed about 1 cm away from the base of each upright (vertical stem) using fine tweezers. We measured the length of each cranberry upright on the day dodder was added for potential use as a covariate in analyses. Uprights were monitored daily and first attachment (coiling around stems) of dodder was recorded for each pot to determine dates to measure induced responses.
1.3.4. Host attractiveness and dodder performance experiment

To measure host attractiveness to dodder, days to first attachment and the total number of attached dodder per pot was recorded. After at least 3 weeks of dodder attachment, we measured dodder performance as the number of coils, haustorial attachments and mass of dodder. First, the total number of coils per pot was determined. Following this, dodder was removed from uprights using tweezers and total number of haustorial attachments was counted. Dodder vines were dried at 45°C for a week and weighed to assess total dry mass of dodder per pot. Number of coils, number of haustoria and dodder weight per pot were divided by the number of dodder attached per pot to obtain a mean value per dodder per pot for each response.

1.3.5 Induction experiment: chemical responses

1.3.5.1 Phytohormones

We measured leaf JA, SA and abscisic acid (ABA) phytohormones from parasitized and non-parasitized cranberry cultivars. We used one experiment to measure phenolics and volatiles (below) that was conducted simultaneously with the host attractiveness and dodder performance experiment. Due to freezer failure and loss of original samples, a separate experiment with identical design was used to measure phytohormones in April 2012. Phytohormone analysis was performed on a randomly selected subsample of 80 pots (8 pots per cultivar per treatment x 5 cultivars x 2 treatments) from the original 100 pots. Leaves of parasitized plants were collected 1-2 days after attachment with the corresponding control plant in that block, placed in separate 5 ml cryovials and immediately frozen in liquid nitrogen before storage at -80°C.
Phytohormone extraction and analysis were based on Thaler et al. (2010). Briefly, 200-300 mg of frozen leaf tissue was transferred into a 2 ml screw cap tube containing pre-weighed 0.9 g Silica beads (BioSpec, Bartelsville, OK, USA) and leaves were crushed into small particles inside the tubes. We added 100 µl of d4-SA and d5-JA (800 pg ml\(^{-1}\) each) as internal standards (CDN Isotopes, Point-Claire, Canada) with 1 ml extraction buffer (iso-propanol:water:hydrochloric acid 2:1:0.005 by volume) and homogenized the tissue in a FastPrep homogenizer (MP Biomedicals, Solon, Ohio, USA) at 6 m/s for 45 seconds. We centrifuged the samples at 4 °C for 20 min at 20,800 x g (14,000 rpm). We then carefully transferred the supernatant of each sample into a fresh 2 ml tube, added 1 ml of dichloromethane and vortexed for 30 min. We centrifuged the samples at 4 °C for 20 min at 12,000 x g for 2 min for phase separation. We then removed the aqueous (top) and middle layer completely and discarded before evaporation of samples overnight under a fume hood. Samples were dissolved in 200 ml methanol and filtered through a 0.45 mm syringe filter (13 mm diameter) into 2 ml HPLC vial with insert and 15 µl of the remaining solvent was analyzed on a triple-quadrupole LC-MS/MS system (Quantum Access; Thermo Scientific, Waltham, Massachusetts, USA). A C18 reversed-phase HPLC column (Gemini-NX, 3µ, 150 x 2.00 mm; Phenomenex, Torrance, California, USA) was used to separate compounds using a solution of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) at a flow rate of 300 µl/min. Separation of compounds were performed using a gradient of increasing solvent B content. The initial gradient of solvent B was maintained at 10% for 2 min and then increased linearly to 100% at 20 min. Phytohormones were analyzed using negative electrospray ionization (spray voltage: 3.5 kV; sheath gas: 15; auxiliary gas: 15; capillary
temperature: 350°C), collision-induced dissociation (argon CID gas pressure 1.3 mTorr [1.3 micron Hg], CID energy 16 V) and selected reaction monitoring (SRM) of compound-specific parent/product ion transitions: SA 137→93; d4-SA 141→97; JA 209→59; d5-JA 214→62 (Thaler et al. 2010).

1.3.5.2 Volatiles

We sampled volatiles over a period of two weeks in June, sampling each replicate 1-3 days after dodder’s first attachment. We chose 1-3 days post-attachment with the intention of covering peak induction, since gypsy moth damage induced chemical changes after two days of damage (Rogriquez-Saona et al. 2011). Parasitized and non-parasitized pots of each cultivar within a block were sampled on the same day. We sampled 200 pots in total (20 pots per cultivar per treatment x 5 cultivars x 2 treatments). We collected volatiles using dynamic headspace sampling for 4 hr between 11:15 and 15:15 each day spanning a period of 2 weeks as dodder attached to plants. One or more uprights were enclosed in polyethylene bags (Toppits, Cofresco Frischhalteprodukte GmbH & Co. Kg, Minden, Germany). Only parasitized uprights were sampled in treatment pots, and we excluded dodder tissue by sampling new upright growth above parasitism sites. All living uprights with new growth were sampled in control pots. Thus, we sampled between one and four uprights together per pot, and considered pot as the unit of replication. A cartridge packed with 100 mg Porapak (Waters Corporation, Milford, MA USA) was carefully inserted in the top opening of the polyethylene bag, and ambient air was pulled by vacuum pump at a flow rate of ca. 200 ml/min (Air Check 52 or Air Check 2000 diaphragm pump, SKC, Eighty Four, Pennsylvania). A small inlet hole at the bottom of the bags allowed airflow. We collected ambient air at each sampling
date for subtraction purposes. Cranberry flower fragrance and dodder fragrance were also collected for subtraction purposes, and we recorded the number of flowers on each upright if present. After sampling, cartridges were wrapped in aluminum foil, placed in a cooler, and then eluted with 3 ml n-hexane into 4 ml vials and stored in a refrigerator at -20 °C. Cartridges were cleaned with 10 ml acetone followed with 5 ml n-hexane (Fisher Scientific brand, Fair Lawn, New Jersey, USA), and stored in aluminum foil in polyethylene bags in the refrigerator between uses.

An internal standard (IS) of 3 µl of anisole was added to the samples, which were then dried to 75 µl under a constant flow of nitrogen gas. We analyzed the samples using combined capillary gas chromatography-mass spectrometry (GC-MS), with an Agilent GC 6890 equipped with a Mass Selective Detector 5973 (Agilent Technologies, Santa Clara, California, USA). The GC was injected with 1 µl of each sample onto a non-polar column (ZB-5ms, 30 m x 0.25mm x 0.25 µm; Zebron, Phenomenex), at an initial temperature of 50° C held for 2 min and then increased 10° C per min until temperature reached 275° C and held there for 3.5 min. Compounds were identified by matching GC retention times to previously used standards and to the Wiley Mass Spectral Library (Theis et al. 2009). Compounds were quantified by dividing the peak area of the mass ion of each scent compound by the peak area of the mass of ion of the internal standard and by the product of both mass of the internal standard and a coefficient that corrected for the response of the GC-MS to the specific scent compound (Theis et al. 2009). Compound identity was determined by running standards, mass spectral libraries and published Kovats indices.
1.3.5.3 Phenolics

Here and after, we will use the term ‘phenolics’ to include the sub-categories of flavonols (quercetin glycosides), phenolic acids (total chlorogenic acids) and proanthocyanidins (total individual oligomers and polymers). We measured leaf phenolics from parasitized and non-parasitized cranberry plants for each cultivar. This analysis was performed on a randomly selected subsample of 60 pots from the original experiment (6 samples per cultivar per treatment x 5 cultivars x 2 treatments) after volatile sampling. Leaves of both parasitized and non-parasitized control plants were placed in separate 5 ml cryovials (Fisher Scientific, cat. No.12-567-502, Fair Lawn, NJ, USA) and immediately frozen in liquid nitrogen before storage at -80°C. For extraction, leaves were crushed with liquid nitrogen using a mortar and pestle. Extraction and purification of leaf samples were carried out as described in Vvedenskaya et al. (2004). Briefly, approximately 0.25 g of leaf powder was placed into a 2 ml centrifuge tube and 0.7 ml of a mixture of 80% HPLC acetone, 0.1% HPLC acetic acid and 19.9% distilled water (by volume) was added to the tube. Samples were vortexed for 2 min, sonicated for 15 min, and then centrifuged at 12000 rpm for 15 min at 4°C. The supernatant from each sample was then transferred into a new centrifuge tube and the extraction was repeated using 0.5 ml of the acetone-acid-water solution. The supernatants were combined and filtered using Spin-X microcentrifugal filters at 5000 rpm for 0.5 min, and the filtered samples were dried using a speed vacuum concentrator. Each sample was mixed with 1.2 ml of solvent B (20% water adjusted to pH 3.5 using formic acid, 20% methanol and 60% acetonitrile) and vortexed until the pellet was completely broken, followed by sonication for 20 min, centrifugation at 12000 rpm for 1 min, and filtration using spin filters as
described above. Complete analytical detection of phenolic acids and flavonol glycosides was achieved using HPLC (Waters, Milford, MA) with a C18 Luna column (4.6 X 150 mm; particle size 5 µm; Phenomenex, Torrance, CA) (Wilson et al. 2008). Forty µl of each filtered extract was injected and compound separation was achieved using binary solvent system of solvent A (10% methanol in water adjusted to pH 3.5 using formic acid) and solvent B (20% water adjusted to pH 3.5 using formic acid, 20% methanol and 60% acetonitrile) with a linear gradient of 0% B to 27% B from 0-5 min; 27% B to 40% B from 5-27 min; isocratic elution of 40% B from 27-30 min; linear gradient of 40% B to 50% B from 30-35 min; 50% B to 90% B from 35-40 min; 90% B to 0% B from 40-45 min and isocratic elution of 0% B from 45-55 min at a flow rate of 1 ml/min for a final run time of 55 min. Equilibrium at 100% A was performed for 5 min before and after each injection. Phenolic acids and flavonol glycosides were detected at 320 nm and 366 nm respectively in a photodiode array (PDA) detector. Identification of phenolic acids and flavonols was achieved by comparing their retention times and absorbance spectra of previously published data and authentic standards (Ranger et al. 2007; Vvedenskaya et al. 2004; Wilson et al. 2008).

Identification of individual oligomeric proanthocyanidins was obtained using a Dionex (Sunnyvale, CA) HPLC apparatus equipped with a G-40 gradient pump, model 100 PDA detector, model AS50 autosampler/thermal compartment, and model ED50 detector. Separation of compounds was obtained by injecting 20 µl of each filtered sample onto A Develosil® diol column (250 X 4.6 mm internal diameter; particle size 5 µm; Phenomenex, Torrance, CA) at 25º C with a binary solvent system of solvent A (acetonitrile:acetic acid:10 mM ammonium acetate, 98:1:1 by volume) and solvent B
(methanol:10 mM ammonium acetate:acetic acid, 95:3:2 by volume) with linear gradient of 0% B to 10% B from 0-5 min; 10% B to 12% B from 5-8 min; 12% B to 13% B from 8-10 min; 13% B to 20% B from 10-15 min; 20% B to 40% B from 15-35 min; isocratic elution of 40% B from 35-40 min; linear gradient of 40% B to 0% B from 40-45 min and isocratic elution of 0% B from 45-50 min at a flow rate of 1 ml for a total run time of 50 min. Proanthocyanidins were detected at 280 nm in PDA detector and identified based on peak retention times and absorbance spectra (Wilson et al. 2008).

1.3.6 Statistical analysis

1.3.6.1 Host attractiveness and performance experiment

We used R. Studio (version 0.98.507, RStudio, Inc.) to carry out all statistical analyses. We analyzed host attractiveness as days to first attachment using analysis of variance (ANOVA) and analyzed the total number of dodder attached per pot using generalized linear mixed models (GLMMs) with a quasibinomial distribution and logit link function to correct for overdispersion. We measured dodder performance as mean number of coils per dodder, mean number of haustoria per dodder, and mean dry weight per dodder, all log-transformed to improve normality. We tested for effects of cultivar on these three measures of performance using MANOVA. For all analyses, the model included cultivar as a fixed factor and block as a random factor, using linear mixed effects models (LME) where appropriate. Host height was initially included as a covariate but dropped because it was not significant. Significant MANOVA results were followed with separate ANOVAs for each response variable. We used Tukey’s Studentized Range test ($\alpha = 0.05$) for post-hoc tests of differences between cultivars.
1.3.6.2 Induction experiment: General approach

Models (GLMMs) for all chemical responses included dodder treatment, cultivar, and their interactions as fixed effects and block as a random variable. In all analyses host height was initially included as a covariate, but removed because it was never significant. All chemical responses were tested for normality and log-transformed when appropriate. All significant MANOVAs were followed by separate ANOVAs, and we used Tukey’s Studentized Range test ($\alpha = 0.05$) for post-hoc comparisons between cultivars and treatments.

To determine whether induced responses were stronger with more dodder plants attached and whether cultivars differed in the strength of induction in response to multiple attachments, we ran a separate ANCOVA or MANCOVA for each chemical response category using only dodder treated plants, including cultivar as a fixed effect, block as random factor, the number of dodder seedlings attached as a covariate, and the number of dodder seedlings attached x cultivar interaction. We also included the number of days between dodder attachment and sampling as a covariate. However, there was never a significant relationship between number of dodger attached and the strength of induction, and so for simplicity we did not report these analyses.

1.3.6.3 Phytohormones

Independent ANOVAs were used for SA, JA, and ABA; each response was log-transformed prior to analysis. We did not use MANOVA because 7 strong outliers that violated normality assumptions (all 3 SD above the mean) were deleted for JA (3 dodder with Howes, one Early Black and one Crimson Queen; 2 control Howes and one control
Mullica Queen), of which a subset of three were also outliers for ABA (one control Mullica Queen; one control Howes and one parasitized Early Black). Including all phytohormones in one MANOVA would have removed those 7 replicates from all analyses.

1.3.6.4 Volatiles

All volatile emissions were calculated as an hourly emission rate scaled by the wet mass of the sample, with the units ng/g wet mass/hour. We log-transformed all volatile classes to improve normality. Volatiles were grouped based on their biosynthetic origin into sesquiterpenoids, homoterpenoids, monoterpenoids, esters, fatty acids, alkanes, and unknowns that were analyzed as responses with MANOVA. We also analyzed individual volatiles as volatile composition with a separate MANOVA, and analyzed total terpenoids (sum of sesquiterpenoids, homoterpenoids and monoterpenoids) and total volatile emissions using separate ANOVAs. We deleted two outliers from the analysis of alkanes (one Mullica Queen control plant and one Early Black dodder treatment, both 3 SD above the mean) and one outlier from unknowns (the same deleted for alkanes from Mullica Queen) because they violated normality assumptions.

We also used a permutational multivariate analysis of variance (PERMANOVA), a non-parametric test that is more robust to violations of normality assumptions than MANOVA (Anderson 2001) to test for differences in volatile composition, including cultivar and dodder treatment as independent variables, block as a random factor, height as a covariate and all individual volatiles or group of volatiles described earlier as responses. However, this analysis gave results that were very similar to the MANOVA.
and so for simplicity we did not report it. Finally, we calculated volatile diversity for all individual compounds, and calculated diversity within volatile groups and then averaged across groups for each replicate, using the Shannon-Weiner diversity index and evenness using Evar (Smith and Wilson 1996). Separate ANOVAs were employed to test measures of volatile diversity and evenness, including dodder treatment and cultivar as independent variables.

1.3.6.5 Phenolics

We analyzed phenolics in 3 major groups consisting of flavonols, total phenolic acids, and proanthocyanidins. Flavonols included quercetin-3-galactoside, quercetin-3-xyloside, quercetin-3-arabinopyranoside, quercetin-3-arabinofuranoside, quercetin-3-rhamnoside and quercetin aglycone, and were analyzed using MANOVA. Total flavonols were analyzed separately with ANOVA. Phenolic acids were calculated from total chlorogenic acids and total proanthocyanidins were calculated from individual proanthocyanidin oligomers and polymers. Phenolic acids and total proanthocyanidins each comprised single categories and were analyzed using separate ANOVAs. We log-transformed proanthocyanidins and all individual flavonols to improve normality; total flavonols and phenolic acids were untransformed.

1.4 Results

1.4.1 Host attractiveness and performance experiment

Host attractiveness, measured as the number of attached dodder stems per pot, marginally differed across cultivars ($t_{99} = 1.98$, $P = 0.051$). Although the overall cultivar
effect was only marginally significant, in Tukey’s post-hoc contrasts Early Black was least preferred by dodder, with significantly fewer attachments per upright than other cultivars (Figure 1.1). Days to first attachment did not differ across cultivars ($F_{4, 76} = 0.44, P = 0.78$). No other measure of dodder performance (number of haustoria, number of coils and weight per dodder) was affected by cultivar ($F_{4, 18} < 1.3, P > 0.3$ for all).

1.4.2 Induction experiment

1.4.2.1 Phytohormones

Dodder increased SA concentrations by approximately 50%, but this effect was only marginally significant (Table 1; Figure 1.2A). There was no dodder treatment effect on JA and ABA and no dodder-by-cultivar interaction for any phytohormone (Table 1.1). All three phytohormones (SA, JA and ABA) differed with cultivar (Table 1). Post-hoc tests showed that SA concentrations were highest in Stevens and lowest in Mullica Queen (Figure 1.3A). JA was highest in Crimson Queen and Howes, and lowest in Stevens (Figure 1.3B). Stevens had significantly higher levels of ABA than Mullica Queen, Crimson Queen and Howes, with Early Black intermediate (Figure 3C; see Figures S1: 1 and S1: 2 for phytohormones means across all cultivar and dodder treatments).

1.4.2.2 Volatiles

Dodder parasitism did not induce changes in volatile groups (dodder treatment: Pillai’s trace = 0.038, $F_{8, 164} = 0.81, P = 0.59$) and cultivars did not differ in their response to parasitism (cultivar x dodder interaction: Pillai’s trace = 0.13, $F_{32, 668} = 0.69, P = 0.91$). However, cultivars differed in volatile groups (MANOVA; cultivar: Pillai’s trace = 0.66, $F_{32, 668} = 4.13, P < 0.001$), and total volatile emissions ($F_{4, 15} = 3.89, P = 0.0049$; Figure
1.4A). Subsequent ANOVAs (Table 1.1) showed that cultivars differed in sesquiterpenoids (Figure 1.4B), homoterpenoids (Figure 1.4C), alkanes, fatty acids and unknowns. Cultivars did not differ in emissions of monoterpenoids, terpenoids, aromatics or esters. Similarly, when analyzing individual compounds rather than groups, dodder parasitism did not induce changes in volatile composition (dodder treatment: Pillai’s trace = 0.23, $F_{47,125} = 0.81$, $P = 0.79$) and cultivars did not differ in their response to parasitism (cultivar x dodder interaction: Pillai’s trace = 0.84, $F_{188,512} = 0.73$, $P = 0.99$). However, cultivars did differ in their volatile composition (MANOVA; cultivar: Pillai’s trace = 2.14, $F_{188,512} = 3.12$, $P < 0.0001$). Separate ANOVAs showed that cultivars differed in many individual compounds (see Table S1: 1).

Volatile diversity did not differ with cultivar or treatment using the Shannon-Weiner diversity index ($F < 1.4$, $P > 0.1$ for both). When calculating diversity first within volatile groups and then averaging across groups, volatile diversity differed with cultivars across groups ($F_{4,4} = 6.41$, $P = 0.05$) and marginally with dodder treatment ($F_{4,4} = 5.56$, $P = 0.08$). Post-hoc Tukey’s HSD student test showed that Howes was more diverse and significantly different than Stevens. Diversity did not differ with cultivar or treatment for within groups ($F < 0.04$, $P > 0.86$ for all) and evenness did not differ with cultivar or treatment for total or grouped volatiles ($F < 1.30$, $P > 0.40$ for all).

1.4.2.3 Phenolics

Overall, both cultivar and dodder treatment affected flavonol levels (MANOVA; cultivar: Pillai’s trace = 0.86, $F_{28,188} = 1.83$, $P = 0.0097$; dodder treatment: Pillai’s trace = 0.40, $F_{7,44} = 4.20$, $P = 0.0013$) but the cultivar-by-dodder interaction did not (Pillai’s trace = 0.56, $F_{28,188} = 1.09$, $P = 0.35$). Dodder parasitism increased the levels of two
flavonols, quercetin-3-galactoside (Table 1.1; Figure 1.2B) and quercetin-3-xyloside (mean ± SE: dodder treatment = 2.168 ± 0.396; control = 1.652 ± 0.302) by at least 250% compared to unparasitized plants, but reduced quercetin-3-rhamnoside concentrations by approximately 38% compared to controls (Figure 1.2C). Dodder parasitism increased phenolic acid concentrations by 37% compared to controls (Figure 1.2D).

Cultivars differed significantly in five flavonols: quercetin-3-galactoside, quercetin-3-xyloside, quercetin-3-rhamnoside, quercetin-3-arabinopyranoside, and quercetin-3-arabinofuranoside (Table 1.1, Figure 1.5). Mullica Queen had generally higher concentrations of flavonols compared to other cultivars, while Howes and Crimson Queen tended to have the lowest concentrations. Cultivars did not differ in concentrations of the flavonol quercetin aglycone, or in total proanthocyanidins or phenolic acids (Table 1.1).

1.5 Discussion

1.5.1 Does host attractiveness to dodder and dodder performance vary with cranberry cultivar?

We hypothesized that dodder attachment or performance would differ with cultivar, and that such differences would correspond with variation in chemical defense. Although marginally significant, we found that dodder distinguished between cultivars, with greater than 50% decrease in the number of attachments to Early Black than any other cultivar. Thus, cranberry joins a small list of other crops with varieties that differ in dodder resistance (Goldwasser et al. 2001; Goldwasser et al. 2012), although host traits responsible for resistance were not examined in their studies. In our study, although dodder attachment differed between cultivars, dodder performance post-attachment did
not. This suggests that the best approaches for managing dodder may involve breeding for traits that influence attractiveness, thus preventing dodder attachment rather than traits that affect dodder performance after attachment.

1.5.2 Do cultivars vary in levels of phytohormones, volatiles, or phenolics and does such variation correlate with host attractiveness to dodder and dodder performance?

Cultivars differed in a wide range of chemical traits. Although closely related to wild progenitors, cranberries have been subjected to selective breeding under domestication for favorable plant traits such as high yield, vigorous growth, early-season fruit ripening, fruit color and size, which may or may not be correlated with plant defensive traits (Rodriguez-Saona et al. 2011). Selection for these traits may have resulted in tradeoffs with plant defense, as plants allocate more resources to fruit production and rapid growth. However, selection of some traits, such as high levels of anthocyanins favored for color intensity and antioxidant properties that can benefit human health (Blumberg et al. 2013), may have enhanced plant defense (Rodriguez-Saona et al. 2011). Thus, it might be reasonable to expect that some cranberry hybrids may have reduced plant defense as a result of selective breeding compared to their parental counterparts, and others may have enhanced defenses. We found that Crimson Queen, a hybrid resulting from a cross between Stevens and Ben Lear, had low overall levels of flavonols but higher levels of JA compared to its parental cultivar Stevens (Figure 1.3 and 1.5). On the other hand, Mullica Queen, another recent hybrid cross, had high levels of overall flavonols but lower levels of SA compared to other cultivars (Figure 1.3 and 1.5). Thus, recent breeding efforts have produced new hybrid cultivars that differ widely in levels of phytohormones and defensive compounds. These changes
in plant defenses may affect the outcome of interactions not only with parasites, but also with herbivores and natural enemies.

Although cultivars differed in levels of phenolics and phytohormones, we have no evidence to implicate any particular compound in dodder resistance. Early Black had lower dodder attachment than any other cultivar (Figure 1.1), but no phenolic compound or phytohormone stood out as being noticeably higher or lower in Early Black compared to other cultivars (Figure 1.3 and 1.5; data not shown for others). However, we only used leaf tissue for chemical analysis. It is possible that bark flavonoids and other secondary defenses could play a role in dodder resistance. For example, Kelly (1990) reported that the dodder *C. subinclusa* can recognize host species when foraging and may respond to the presence of flavonoid compounds from the bark of its host plant, *Malosma laurina*. Thus, it is possible that host bark traits specifically, rather than leaf chemical traits, affect host attractiveness to dodder and attachment. Future studies should assess the role of cranberry bark host chemistry in dodder resistance.

Cultivars differed widely in both the amount and composition of volatile emissions. Runyon et al. (2006) showed that dodder searches for hosts and chooses between preferred hosts (tomato) and non-preferred hosts (wheat) based on volatile cues. Furthermore, experiments with individual compounds from tomato blends showed that dodder grew towards the monoterpenes β-phellandrene, β-myrcene, and α-pinene, and one compound (((Z)-3- hexenyl acetate) caused a significant negative growth response. These results suggest that individual volatile compounds can attract or deter dodder from particular hosts. As with the phenolics, Early Black was not notably different from other cultivars in total or any particular category of volatile emissions. It is also possible that
blends of volatile compounds, rather than single compounds or compound classes, mediate dodder responses to cranberry (Snoeren et al. 2010). Although volatile diversity differed with cultivar, Howes rather than Early Black had the highest diversity, suggesting that diversity per se does not explain differences in dodder preference. Exploring plant parasite responses to different cranberry host volatile cues as whole blends rather than individual compounds or compound classes may yield more insights into the mechanisms of dodder resistance.

1.5.3 Does dodder parasitism induce chemical changes in phytohormones, volatiles or phenolics, and does the level of inducible response vary among cultivars?

Dodder parasitism did not affect the concentrations of JA and ABA, but increased SA concentrations. SA is involved in defense responses induced by pathogens (Brading et al. 2000), while JA is usually involved in mediating responses to chewing herbivores (Thaler et al. 2001). However, the classification of SA as a pathogen induced-signaling pathway and JA as a chewing herbivore induced-signal pathway is not always mutually exclusive and cross-talk can occur between the two pathways (Thaler et al. 2010). Our results suggest that dodder may induce a defense response that is similar to that induced by pathogens. The only other study to examine phytohormone induction in response to dodder parasitism found that a second dodder attachment to a 20-day-tomato plant induced both JA and SA responses, which appeared to reduce growth of the parasite (Runyon et al. 2010). In that study, both JA and SA pathways were induced with different time courses, suggesting that the host may recruit both pathways as a defense mechanism (Runyon et al. 2010).
Dodder parasitism induced many changes in phytohormone, volatile and flavonol levels in cranberry. However, we found no evidence suggesting that any of these compounds influenced dodder parasitism, which did not differ across cultivars, or preference. Cranberries may have a general wound response against parasitism instead of a specific defense mechanism against plant parasites.

Despite the important roles that parasitic plants play in communities (Pennings and Callaway 2002), little is known about plant defenses against parasitic plants and how these defenses affect other host-plant interactions. Runyon et al. (2008) found that parasitized tomato plants by *C. pentagona* produced one-third lower JA levels in response to insect feeding by the beet armyworm (*Spodoptera exigua*, Noctuidae: Lepidoptera) compared to unparasitized tomato plants. Additionally, parasitized tomato plants did not produce herbivore-induced volatiles after 3 days of insect feeding and growth of the beet armyworm was slower on parasitized compared to unparasitized plants. Thus, understanding induced defenses in response to parasitic plants is important not only for understanding ecological dynamics, but also to explore the manipulation of defense pathways to control parasitic pests in agriculture. Our work and one previous study (Runyon et al. 2008) both indicate that dodder may induce a range of chemical changes in host plants that have the potential to affect herbivore preferences and shape subsequent herbivore communities, with likely consequences for crop yield.

### 1.6 Acknowledgements

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1.7 Notes


Hood ME, Condon JM, Timko MP, Riopel JL (1998) Primary haustorial development of *Striga asiatica* on host and nonhost species. Phytopathology 88:70-75


Table 1.1: ANOVA results for effects of dodder on cranberry chemistry (continued onto next few pages)

F values from mixed model ANOVAs testing effects of dodder treatment and cultivar on cranberry chemistry, with block as a random factor. For all analyses, the numerator df is 4 for cultivar, 1 for dodder treatment and 4 for their interaction; error df is listed for each analysis. * P < 0.08, * P < 0.05, ** P < 0.01, *** P < 0.001). Bold values indicate significant effects at P < 0.05

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**Phenolic acids**

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**Proanthocyanidins**

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Figure 1.1: Dodder host attractiveness on cranberry cultivars

Host attractiveness measured as the total number of dodder stems attached, across cranberry cultivars. Different letters above bars indicate significantly different means using Tukey’s Post-Hoc test ($P<0.05$). Bars are mean ±1SE.
Figure 1.2: Effects of dodder parasitism on cranberry induced defenses

Effects of dodder presence using ANOVA on (A) salicylic acid (SA), and the flavonols (B) quercetin-3-galactoside and (C) quercetin-3-rhamnoside, as examples of induced increases and decreases following dodder parasitism, and (D) phenolic acid concentrations. Bars are mean ±1SE.
Figure 1.3: Phytohormone differences between cranberry cultivars

Phytohormone differences for overall levels between cranberry cultivars. (A) salicylic acid (SA), (B) jasmonic acid (JA), and (C) abscisic acid (ABA). Different letters above bars indicate significantly different means using Tukey’s Post-Hoc test ($P<0.05$). Bars are mean ±1SE.
Figure 1.4: Volatile differences between cranberry cultivars

Differences between cranberry cultivars for overall (A) total volatile emissions, (B) sesquiterpenes and (C) homoterpenes. Different letters above bars indicate significantly different means using Tukey’s Post-Hoc test ($P < 0.05$). Bars are mean ±1SE.
Figure 1.5: Differences in phenolics between cranberry cultivars

Differences between cranberry cultivars for the flavonols (A) quercetin-3-galactoside, (B) quercetin-3-arabinopyranoside, (C) quercetin-3-rhamnoside, and (D) quercetin-3-xyloside. Different letters above bars indicate significantly different means using Tukey’s Post-Hoc test ($P<0.05$). Bars are mean ±1SE.
Table S1.1: Detailed information on analysis of individual volatile compounds (continued onto next few pages)

F values from mixed model ANOVAs testing effects of dodder treatment and cultivar on cranberry volatile composition, with block as random factor. For all analyses, numerator df is 4 for cultivar, 1 for dodder treatment and 4 for their interaction; error df is 152 for all. \(^a P < 0.08, \,* P < 0.05, \,** P < 0.01, \,*** P < 0.001\). Bold values indicate significant effects at \(P < 0.05\).

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Note. A control collected from pots with sand but without cranberry plants had significant amounts of undecane, tridecane, pentadecane and decanal.
Figure S1.1: Effects of dodder parasitism and cultivar on cranberry phytohormones

(A) salicylic acid (SA), (B) jasmonic acid (JA), and (C) abscisic acid (ABA). Error bars represent standard error.
Figure S1.2: Effects of dodder parasitism and cultivar on volatiles

(A) Total volatile emissions, (B) sesquiterpenoids and (C) homoterpenoids. Error bars represent standard error.
CHAPTER 2

HEBIVORY DELAYS PARASITE ATTACHMENT TO CRANBERRY HOSTS

2.1 Abstract

Interactions between species can have cascading effects that shape subsequent interactions. For example, herbivory can induce plant defenses that affect subsequent interactions with herbivores, pathogens, mycorrhizae and pollinators. Parasitic plants are present in most ecosystems, and play important roles in structuring communities. However, the effects of host herbivory on parasitic plants, and the potential mechanisms underlying such effects, are not well known. We conducted a greenhouse study to ask whether gypsy moth (*Lymantria dispar*) damage, cranberry cultivar, and their interaction affected preference of the stem parasite dodder (*Cuscuta* spp.) on cranberry hosts (*Vaccinium macrocarpum*), and assessed induced changes in phytohormones and secondary compounds that could underlie such effects. When dodder plants were added one week after damage, gypsy moth damage reduced attachment by more than 50%. When dodder were added two days after damage, damage delayed attachment by ~ 0.3 days Gypsy moth damage significantly increased jasmonic acid (JA) levels and significantly increased the flavonol quercetin aglycone, suggesting a possible mechanism of cranberry defense against dodder parasitism. Dodder preference also differed between cranberry cultivars, with the highest attachment on the cultivar that had significantly lower levels of total phenolic acids, suggesting that phenolics may mediate dodder preference. Our results indicate that herbivory can have cascading effects on subsequent
interactions with a highly damaging parasitic plant, demonstrating the potential importance of early interactions for shaping subsequent community dynamics.

**Key words:** chemical defense, parasitism, parasitic plants, phenolics, phytohormones, species interactions

### 2.2 Introduction

As sessile organisms, plant use cues from their environment to dynamically respond to interactions (Karban and others 1999). By inducing changes in plant phenotypes, interactions that occur early in the growing season have the potential for cascading effects on subsequent interactions at different trophic levels. For example, wild radish plants (*Raphanus sativus*) previously damaged by specialist caterpillars had reduced subsequent growth of a generalist herbivore and decreased subsequent herbivory by grasshoppers (Agrawal 1999). In milkweeds (*Asclepias syriaca*), the identity of herbivores causing early-season damage can have large impacts on performance of subsequent herbivores, indicating that there are community-level consequences of early-season interactions (Van Zandt and Agrawal 2004). In addition to affecting subsequent herbivores, early leaf damage can also alter interactions with pollinators, root feeders, and mycorrhizae (Erb and others 2011; Gehring and Bennett 2009; Gilbert and Johnson 2015; Lucas-Barbosa 2016; Lucas-Barbosa and others 2011). Despite all the evidence that herbivory can shape subsequent interactions through plant-mediated changes, little is known about how herbivory affects subsequent interactions with parasitic plant.

Parasitic plants are present in most ecosystems, where they structure community
composition (Pennings and Callaway 1996) of both animal and plant species across multiple trophic levels. For example, a parasitic plant can change the outcome of competition between plant species, altering community composition (Pennings and Callaway 2002). More recently, Hartley and others (2015) manipulated densities of the hemiparasite *Rhinanthus minor* and compared the plant and invertebrate communities in plots with the parasite removed, present at natural densities, or augmented to higher densities. Plots with augmented *R. minor* had reduced plant biomass but twice the abundance of invertebrates, with effects evident in herbivores, predators, and detritivores. Thus, parasitic plants, through their effects on host plants, can also structure animal communities. Although the effect of parasitic plants on plant communities via altering competitive dominance has been demonstrated in several systems (Pennings and Callaway 2002), we know remarkably little about how herbivore-mediated changes in host chemistry affects parasite preference.

Parasite preference and performance is largely dependent on host quality. For example, parasite performance is often better on legume hosts, suggesting that nitrogen is important to performance (Matthies 1996; Matthies 1998; Seel and others 1993), although this is not always the case (Pennings and Simpson 2008; Ren and others 2010; Rowntree and others 2014). Host defenses can also affect parasite performance. For example, dodder species grew larger on hosts deficient in salicylic acid (SA) production or insensitive to jasmonic acid (JA) (Runyon and others 2010); these phytohormones mediate plant responses to pathogens and herbivores, but their role mediating interactions with parasitic plants is largely unknown. Although several studies have shown that host species and quality affect parasite performance, only a handful of studies have assessed
parasite preference. The parasite dodder (*Cuscuta pentagona*) could differentiate between preferred tomato (*Lycopersicon esculentum*) and non-preferred wheat (*Triticum aestivum*) hosts using volatiles cues released by the host plants (Runyon and others 2006). Dodder (*Cuscuta* spp.) seedlings given choices between the same host species with varying nutritional content, grew towards and coiled around hosts of higher nutritional content and grew away from hosts with lower nutritional content (Kelly 1992). Both these studies indicate that dodder is capable of making foraging decisions and suggest that dodder chooses higher-quality hosts based on volatile cues, but we currently do not know whether prior species interactions can influence parasite choice.

Herbivory can induce changes in host chemistry, including volatiles, and plant quality (Karban and Baldwin 1997; Ohgushi 2005; Stam and others 2014). Inducing volatile release could attract parasites if they use the volatiles for host finding, or deter parasites if the volatiles indicate a poor-quality host due to damage (Kelly 1992; Runyon and others 2006). Furthermore, herbivory may activate both the salicylic acid (SA) and jasmonic acid (JA) signaling pathways, which have been reported to affect parasite performance (Runyon and others 2010). Induced chemical defenses could be costly to parasites if the compounds are toxic to the parasite, but benefit parasites if secondary metabolites provide protection. For example, the hemiparasite *Castilla indivisa* experienced reduced herbivory when attached to a high alkaloid compared to low-alkaloid host, which resulted in higher parasite seed set (Adler and others 2001). These changes may alter how dodder perceives its host, and thus affect parasite preference.

To our knowledge, no study has examined how insect herbivory affects subsequent parasitic plant preference on hosts. We hypothesize that herbivory preceded
dodder may cause changes in host chemistry that reduce dodder preference. We used the generalist gypsy moth (*Lymantria dispar*), a destructive cranberry pest (Franklin 1950), to assess the effects of herbivory on plant chemistry and dodder preference. Gypsy moth appears on cranberry bogs before dodder seedling emergence (H. Sandler, personal observation), making this question both ecologically and economically relevant. To examine effects of herbivory on host parasitism, we conducted a greenhouse study to ask how herbivory affects subsequent host preference, and assessed the role of phytohormones and phenolic induction as underlying mechanisms.

### 2.3 Methods and materials

#### 2.3.1 Study system

Wild cranberry bogs provide foraging habitats for many species including birds, fish, amphibians and several invertebrates (Jorgensen and Nauman 1993). Cranberry cultivars are genetically similar to their wild progenitors, making work with cultivars relevant to understanding ecological interactions in native systems (Rodriguez-Saona and others 2011). Cranberry cultivars vary in their secondary chemistry, including phenolic and volatile profiles (Rodriguez-Saona and others 2011; Tjiurutue and others 2016). Some newly-bred cranberry cultivars seem to be more susceptible to dodder parasitism (Tjiurutue and others 2016) or to damage by gypsy moth larvae (*Lymantria dispar*, L.), suggesting that there may be compromised defense chemistry as a result of breeding for traits such as higher anthocyanin production, bigger berries and high yields (Rodriguez-Saona and others 2011). Given this, it is expected that cultivars might chemically respond differently to herbivory, potentially affecting future interactions with other species.
Gypsy moth is a destructive pest of Northeastern forests that was accidently introduced in the late 1800’s in the US (Elkinton and Liebhold 1990), and is present in both wild and cultivated cranberry. Dodder (Cuscuta spp.) is another serious of pest of cranberries (Devlin and Deubert 1980) and a native component of cranberry bogs (Dawson and others 1984). Dodder species typically exhibit broad host ranges and can be damaging to a variety of economically important crops and wild plants (Dawson and others 1984; Press and Graves 1995).

2.3.2 Cranberry cultivars and plant propagation

We used three cranberry cultivars, Howes, Stevens and Mullica Queen. Howes is a parental cultivar and thus more closely related to wild-type genotypes than Stevens and Mullica Queen. Stevens is an older hybrid that was selected for its high productivity, fair coloring and good fruit rot resistance (Caruso 2008; Dana 1983), while Mullica Queen is a newer hybrid bred for its high anthocyanin production, high stolon vigor and fruit rot resistance (Caruso 2008; Vorsa 2010).

Cranberry stem cuttings were collected from the University of Massachusetts Cranberry Research Station in East Wareham, Massachusetts in 23 June 2011. Stem cuttings of ~ 8 cm sections were sown in 72-plug trays in a 3:1 sand: peat soil mixture on 27 June 2011. Once the stem cuttings were well established, they were moved into cold storage at 4°C and 78% relative humidity. Established stems were repotted in November 2011 into 10 cm pots each containing four stems. Approximately 1.5 g of 14-14-14 Osmocote fertilizer (Scotts-Sierra Horticultural Products Company, Marysville, OH, USA) was added to each pot in November 2011.
Dodder seeds (Cuscuta spp.) were collected on 28 September 2008 from a commercial cranberry bog in Carver, Massachusetts and stored in a glass vial. Identification of Cuscuta species can be challenging; PCR of DNA from dodder collected from several sites in this region indicated that plants were mostly C. gronovii, but with some C. campestris and possibly C. compacta co-occurring (Ghantous and others 2012).

Seeds were scarified in batches of 100 (0.01 g) in a 2 mL microcentrifuge tube for approximately 3 minutes using a small dremel tool (Ghantous and Sandler 2012). The seeds were then rinsed with water using a fine mesh strainer, placed in Petri dishes lined with 90 mm moistened filter paper, and sealed with Parafilm. Petri dishes were then placed in an incubator at 23°C until seed germination approximately 2 days later.

Gypsy moth larvae were obtained from U.S. Department of Agriculture (USDA), APHIS, Otis, Buzzards Bay, MA, USA and were reared on an artificial wheat germ diet at 24-25 °C, 70-75% RH, and 16:8 hour light-dark cycles (Bell and others 1981). Larvae were kept at 25 °C in the laboratory until use.

2.3.3 Experimental design

We conducted two greenhouse experiments in parallel, manipulating gypsy moth damage on three cranberry cultivars. In the Preference experiment, we damaged cranberry plants with gypsy moth and then measured subsequent dodder preference as the response. In the Induction experiment, we assessed induced effects of herbivory on cranberry chemistry by measuring plant phytohormones and phenolics. These responses were measured in separate experiments to avoid influencing dodder responses through destructive sampling to measure chemical defenses.
To measure dodder preference, we had 25 replicate pots x 3 cultivars x 2 treatments (gypsy moth damaged or control) for a total of 150 pots. To assess effects of gypsy moth damage on plant chemistry, we had an additional 25 replicate pots x 3 cultivars x 2 treatments (damaged and control), for a total of 150 pots. The two experiments were carried out at the same time, and pots were placed into blocks with one replicate of each treatment in each block, for total of 25 blocks for each experiment. Each pot contained four uprights of each cultivar, and pot was the unit of replication for both experiments. Plants in the damage treatment received eight third instar gypsy moth caterpillars in a single mesh bag surrounding all four uprights. Caterpillars were left to feed on the plants for two days before removal; control plants had mesh bags without caterpillars that were added and removed at the same time as for damage treatments. Caterpillars were not put on all plants on the same day; treatments were spaced out over 6 days (14-19 December 2011), but all plants in the same block received treatments (bags with or without caterpillars) on the same day. In both experiments, we measured cranberry damage as the proportion of leaves damaged. Treatment damage did not differ between cranberry cultivars ($F < 2.5, P > 0.09$), indicating that differences in cultivar response to damage are not due to different amounts of damage between cultivars.

### 2.3.4 Dodder preference experiment

After two days of caterpillar damage, which is sufficient to induce chemical responses in cranberries (Rodriguez-Saona and others 2011), all bags were removed. Two days later, 96 of the plants (16 blocks; each control and corresponding damaged treatment pot within a block) received dodder seedlings, with one seedling added per upright, to assess short-term effects of gypsy moth damage on parasite preference. The other 54 plants (9 blocks)
received dodder seeds one week later to assess longer-term effects. We measured the length of each cranberry stem on the day dodder was added. Seedlings were placed approximately 1 cm from each stem using tweezers. We monitored uprights daily and recorded the day of first attachment for each stem over a period of two weeks. Dodder preference was measured as the total number of dodder that attached per pot and average days to attachment.

2.3.5 Induction experiment

2.3.5.1 Phytohormones

We measured phytohormones from control and caterpillar-damaged plants for each cultivar on a subsample of 60 pots (10 samples per treatment x 3 cultivars x 2 treatments). Leaves of both control plants and damaged replicates were placed in separate 5 mL cryovials (Fisher Scientific, Fair Lawn, NJ, USA) and immediately frozen in liquid nitrogen before storage at -80°C.

About 200-300 mg of frozen leaf tissue was transferred into 2 mL screw cap tubes containing pre-weighed 0.9 g silica beads (BioSpec, Bartelsville, OK, USA), and leaves were crushed into small particles inside the tubes. We added 100 µl of d4-SA and d5-JA (800pg ml⁻¹ each) as internal standards (CDN Isotopes, Point-Claire, Canada) with 1 ml extraction buffer (iso-propanol:water:hydrochloric acid 2:1:0.005 by volume) and homogenized samples in a FastPrep homogenizer (MP Biomedicals, Solon, Ohio, USA) at 6 m/s for 45 seconds. Samples were then centrifuged at 4 °C for 20 min at 20,800 x g (14,000 rpm) before careful transfer of the supernatant of each sample into a fresh 2 ml tube. Following this, we added 1 ml of dichloromethane and vortexed for 30 min. We
then centrifuged the samples at 4 °C for 20 min at 12,000 x g for 2 min for phase separation and removed the aqueous (top) and middle layer completely before evaporation of samples overnight under a fume hood. Finally, samples were dissolved in 200 ml methanol and filtered through a 0.45 mm syringe filter (13 mm diameter) into 2 ml HPLC vial with insert and 15 µl of the remaining solvent was analyzed on a triple-quadrupole LC-MS/MS system (Quantum Access; Thermo Scientific, Waltham, Massachusetts, USA). A C18 reversed-phase HPLC column (Gemini-NX, 3µ, 150 x 2.00 mm; Phenomenex, Torrance, California, USA) was used to separate compounds using a solution of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) at a flow rate of 300 µl/min. Separation of compounds was achieved by using a linear gradient of increasing solvent B content. The initial gradient of solvent B was maintained at 10% for 2 min and then increased linearly to 100% at 20 min. Phytohormones (abscisic acid, jasmonic acid and salicylic acid) were analyzed using negative electrospray ionization (spray voltage: 3.5 kV; sheath gas: 15; auxiliary gas: 15; capillary temperature: 350°C), collision-induced dissociation (argon CID gas pressure 1.3 mTorr [1.3 micron Hg], CID energy 16 V) and selected reaction monitoring (SRM) of compound-specific parent/product ion transitions: SA 137→93; d4-SA 141→97; JA 209→59; d5-JA 214→62 (Thaler and others 2010).

2.3.5.2 Phenolics

Hereafter, we use the term 'phenolics’ to include the sub-categories of flavonols (quercetin glycosides), phenolic acids (total chlorogenic acids), and proanthocyanidins (total individual oligomers and polymers). We measured leaf flavonols, proanthocyanidins and phenolic acids from control and caterpillar-damaged
replicates for each cultivar. This analysis was performed on a subsample of 30 pots (5 samples per cultivar x 3 cultivars x 2 treatments) that were not used for phytohormone analysis. Leaves of both control plants and damaged plants were placed in separate 5 mL cryovials (Fisher Scientific, Fair Lawn, NJ, USA) and immediately frozen in liquid nitrogen before storage at -80°C. Detailed method of extraction and purification of leaf samples can be found in Vvedenskaya and others (2004). Briefly, approximately 0.25 g of leaf powder was placed into a 2 ml centrifuge tube and 0.7 ml of a mixture of 80% HPLC acetone, 0.1% HPLC acetic acid and 19.9% distilled water (by volume) was added to the tube. Samples were vortexed for 2 min, sonicated for 15 min, and then centrifuged at 12000 rpm for 15 min at 4°C. The supernatant from each sample was then transferred into a new centrifuge tube and remaining tissue (supernatants) were dissolved using 0.5 ml of the acetone-acid-water solution. The supernatants were combined and filtered using Spin-X microcentrifugal filters at 5000 rpm for 0.5 min and dried using a speed vacuum concentrator. We added 1.2 ml of solvent B (20% water adjusted to pH 3.5 using formic acid, 20% methanol and 60% acetonitrile) and vortexed samples until the pellet was completely broken, followed by sonication for 20 min, centrifugation at 12000 rpm for 1 min, and filtration using spin filters as described above. Phenolic acids and flavonol glycosides analytical detection was achieved by using an HPLC (Waters, Milford, MA) with a C18 Luna column (4.6 X 150 mm; particle size 5 µm; Phenomenex, Torrance, CA) (Wilson and others 2008). Forty µl of each filtered extract was injected and compound separation was attained using binary solvent system of solvent A (10% methanol in water adjusted to pH 3.5 using formic acid) and solvent B (20% water adjusted to pH 3.5 using formic acid, 20% methanol and 60% acetonitrile) with a linear
gradient of 0% B to 27% B from 0-5 min; 27% B to 40% B from 5-27 min; isocratic elution of 40% B from 27-30 min; linear gradient of 40% B to 50% B from 30-35 min; 50% B to 90% B from 35-40 min; 90% B to 0% B from 40-45 min and isocratic elution of 0% B from 45-55 min at a flow rate of 1 ml/min for a final run time of 55 min.

Equilibrium at 100% A was performed for 5 min before and after each injection. Phenolic acids and flavonol glycosides were detected at 320 nm and 366 nm respectively in a photodiode array (PDA) detector. Phenolic acids and flavonols were identified by comparing their retention times and absorbance spectra of previously published data and authentic standards (Ranger and others 2007; Vvedenskaya and others 2004; Wilson and others 2008).

Identification of individual oligomeric proanthocyanidins was obtained using a Dionex (Sunnyvale, CA) HPLC apparatus equipped with a G-40 gradient pump, model 100 PDA detector, model AS50 autosampler/thermal compartment, and model ED50 detector. Compounds were separated by injecting 20 µl of each filtered sample onto a Develosil® diol column (250 X 4.6 mm internal diameter; particle size 5 µm; Phenomenex, Torrance, CA) at 25°C with a binary solvent system of solvent A (acetonitrile:acetic acid:10 mM ammonium acetate, 98:1:1 by volume) and solvent B (methanol:10 mM ammonium acetate:acetic acid, 95:3:2 by volume) with linear gradient of 0% B to 10% B from 0-5 min; 10% B to 12% B from 5-8 min; 12% B to 13% B from 8-10 min; 13% B to 20% B from 10-15 min; 20% B to 40% B from 15-35 min; isocratic elution of 40% B from 35-40 min; linear gradient of 40% B to 0% B from 40-45 min and isocratic elution of 0% B from 45-50 min at a flow rate of 1 ml for a total run time of 50
min. Proanthocyanidins were detected at 280 nm in PDA detector and identified based on peak retention times and absorbance spectra (Wilson and others 2008).

2.3.6 Statistical methods

2.3.7 General approach

We used R version 3.2.1 for Mac (R Core Team 2014) to carry out all statistical analysis. We tested all responses using MANCOVA unless otherwise stated. All models included damage treatment, cultivar and their interactions as fixed effects, block as a random effect and plant height as a covariate. Significant MANCOVAs were followed by one-way mixed model ANCOVAs (with LME function). All responses for phytohormones and phenolics were tested for normality prior to running models. We used Tukey’s Studentized Range test ($\alpha = 0.05$) for post-hoc tests of differences between cultivars.

2.3.7.1 Dodder preference

To test for effects of damage treatment and cultivar on the number of dodder that attached and average days to attachment to cranberry hosts, we used separate ANCOVAs including number of dodder attached and days to attachment as responses, and whether dodder was added 2 or 7 days after damage (referred to as ‘days to dodder application’ hereafter). Upon finding a significant damage by days to dodder application interaction (see Results), we analyzed preference separately for measurements 2 or 7 days post-damage and we only report these results.

2.3.8 Induction experiment

2.3.8.1 Phytohormones
We did not use MANCOVA to analyze phytohormones because 5 outliers were deleted that violated assumptions of MANCOVA. Deleting these outliers for one response would have removed them from the entire analysis because MANOVA excludes replicates with any missing responses. The outliers included two for SA (both damage/Stevens, 4SD and 3SD above mean) and three for JA (control/Stevens (3SD above mean), control/Howes (5SD above mean) and damage/Stevens (4SD above mean)). We ran separate mixed model ANCOVAs with SA, JA and ABA as dependent responses. We initially included difference in days to sample collection as a fixed factor and plant height as a covariate, but removed them from both analyses because they were not statistically significant.

2.3.8.2 Phenolics

We analyzed phenolics as 3 major groups: flavonols, phenolic acids and proanthocyanidins. Responses for the MANOVA included the following flavonols: quercetin-3-galactoside, quercetin-3-xyloside, quercetin-3-rhamnoside, quercetin-3-arabinopyranoside, quercetin-3-arabinofuranoside and quercetin aglycone. We ran mixed model ANCOVAs (with LME function) with each of the flavonols plus total flavonols (all flavonols combined) as separate dependent variables. Because proanthocyanidins (total individual oligomers and polymers) and phenolic acids (total chlorogenic acids) were single responses, we ran separate ANCOVAs for each. Plant height and difference in days to collection were initially included but removed from the models because they were not statistically significantly. We also correlated total flavonols, phenolic acids and proanthocyanidins using the cor function in R with the Spearman method.
2.4 Results

2.4.1 Dodder preference

There was a significant interaction between days to dodder application and damage treatment for both number of dodder attached and average days to attachment \((P < 0.030\) for both). Therefore, we analyzed preference separately for dodder applied 2 or 7 days post-damage.

2.4.1.1 Two days post-damage

Cultivars differed in the number of dodder that attached \((F_{2, 75} = 3.88, P = 0.025)\), but there was no significant effect of damage treatment or the damage treatment by cultivar interaction for the number of dodder that attached \((F < 0.38, P > 0.68\) for both). Stevens had significantly more dodder attached than Mullica Queen and Howes (Figure 2.1). However, gypsy moth damage delayed attachment by around 0.3 days \((F_{1, 50} = 12.66, P = 0.0014; \text{Figure 2.2A})\), but there was no effect of cultivar and the damage by cultivar interaction \((F_{2, 50} < 1.96, P > 0.15\) for both).

2.4.1.2 Seven days post-damage

Damage reduced the number of dodder that attached by \(~ 50\%\) \((F_{1, 39} = 8.59, P = 0.0056; \text{Figure 2.2B})\), but cultivar and the damage by cultivar interaction did not affect attachment \((F < 0.69, P > 0.51\) for both). There was no effect of damage, cultivar or their interaction on days to attachment \((F < 0.69, P > 0.15\) for all).

2.4.2 Induction Experiment

2.4.2.1 Phytohormones

Caterpillar damage increased JA levels by approximately 45\% compared to the controls (Table 2.1; Figure 2.3A), but there was no effect of cultivar or the damage by
cultivar interaction (Table 2.1). For SA, there was no main effect of damage treatment or cultivar (Table 2.1), but the effect of damage differed with cultivar (Table 2.1). Further analysis within cultivar showed that gypsy moth damage doubled SA levels compared to controls in Howes (mean ± SE: control = 19.23 ng/g ± 3.47; damage = 38.16 ng/g ± 5.91) but had no effect in the other cultivars. ABA was not affected by any factor (Table 2.1).

2.4.2.2 Phenolics

Flavonols differed across cultivars (MANOVA; cultivar: Pillai’s trace = 1.38, $F_{10, 42} = 9.36, P < 0.0001$) but were not affected by damage or the cultivar by damage interaction (MANOVA; Pillai’s trace < 0.15, $F < 0.72, P > 0.60$ for both). In univariate ANOVAs, all 6 flavonols and total flavonols differed across cultivars (Table 2.1; Figure 2.4A, B and C). We note that damage significantly increased levels of quercetin aglycone (Table 2.1; Figure 2.3B) and marginally decreased levels of quercetin-3-xyloside (Table 2.1; Figure 2.3C), even though the MANOVA found no main effects of damage.

Phenolic acids differed between cultivars (Figure 2.4D), but were not affected by damage or the damage by cultivar interaction (Table 2.1). Proanthocyanidins were not affected by cultivar, damage or the damage by cultivar interaction (Table 2.1). Total flavonols, proanthocyanidins and phenolic acids were not correlated ($r < 0.27, P > 0.15, n = 23$ for all).

2.5 Discussion

We hypothesized that gypsy moth herbivory to cranberry hosts would reduce dodder preference due to changes in host chemistry. We found that gypsy moth damage delayed and reduced attachment to cranberry hosts. However, the effects of damage were
dependent on the length of time between damage and when dodder encountered the host. When dodder was added two days after gypsy moth damage, damage delayed attachment time by \( \sim 0.3 \) days, but the number of dodder attached was not affected. When dodder was added a week after damage, the number of dodder that attached was reduced by about 50%, but the time to attachment was not affected. This suggests a longer induction period that not only delayed attachment initially, but subsequently reduced dodder attachment even at one week following damage. We speculate that gypsy moth attack changes cranberry host scent, which may interfere with dodder foraging and delay attachment. Dodder species can differentiate between hosts and choose preferred host based on smell and nutritional quality (Kelly 1992; Runyon and others 2006). It is possible that damage by gypsy moth induced changes in host scent that delayed and reduced subsequent dodder attachment.

Herbivory can induce changes in host chemistry, potentially changing host quality and thus influencing parasite performance and preference. For example, leaf defoliation of the host plant *Poa annua* reduced subsequent performance of the hemiparasite *Rhinanthus serotinus* (Puustinen and Salonen 1999). We found that gypsy moth induced JA, which may be the mechanism underlying differences in dodder preference. Specifically, we note that Stevens had the highest number of dodder attached (Figure 2.1) and although not statistically different, also had the lowest amounts of JA, suggesting that JA could activate defense pathways downstream that may confer resistance to dodder parasitism. The role of the JA-signaling pathway in mediating plant defenses against chewing insect herbivores has been well established (Walling 2000), but is less clear in parasitic plants. *C. pentagona* grew larger on both JA-insensitive and SA-deficient
tomato hosts, suggesting that both SA and JA responses may be effective against parasitism (Runyon and others 2010). Furthermore, both JA and SA have been shown to be involved in the hypersensitive response (HR), which is an effective defense against *C. reflexa* (Goldwasser and others 2001; Ihl and others 1988; Runyon and others 2010). Our results suggest that JA signaling can also deter parasitic plants, with important potential fitness benefits given that dodder can completely kill hosts (Devlin and Deubert 1980).

Gypsy moth damage induced higher levels of the flavonol quercetin aglycone and marginally lower levels of quercetin-3-xyloside (Table 2.1, Figure 2.3), even though the overall MANOVA for flavonols was not significant. Quercetin glycosides have been implicated in mediating plant defenses against chewing herbivores (Beninger and AbouZaid 1997; Rodriguez-Saona and others 2011). However, these compounds cannot be the only mechanism mediating resistance, since Howes and Mullica Queen, which had the fewest dodder attached, had variable amounts of flavonols (Figure 2.1A and Figure 2.4A, B&C) compared to Stevens. Similarly, in a previous study, although dodder generally induced many changes in flavonols, we did not find any evidence to implicate flavonols as the mechanism of resistance to dodder parasitism because the least preferred cultivar was not notably different from other cultivars in any of these compounds (Tjiurutue and others 2016). These findings suggest that induced flavonol responses in cranberries evolved for other functions, such as defenses against insect herbivores, rather than against dodder parasitism.

Cultivars also differed in phenolic acids that could mediate interactions with dodder and other species. Stevens, which had the most dodder attached after two days of
damage, also had significantly lower concentrations of phenolic acids compared to Howes and Mullica Queen. This suggests that phenolic acids could be a defense mechanism against dodder in cranberries. The role of phenolic acids against herbivore defense is well established, and has been previously implicated in parasitic plant defense. Host resistance against parasite establishment in the cortex and endodermis of host has been linked to deposition of callose, suberin, crosslinking of host cell proteins and accumulation of phenolic acids, which could be toxic to the parasite (Pérez-de-Luque and others 2008; Yoder and Scholes 2010). Additionally, phenolic compounds may act as deterrents, reduce digestibility or palatability of leaf tissues, reduce insect growth and be toxic to insect herbivores (Lattanzio and others 2006). Thus, it is plausible that phenolic acids could mediate defenses against dodder in cranberry.

It is interesting that cultivars generally did not differ in their induced responses to gypsy moth damage. Plant genotypes often respond differently to same herbivores (Gouinguene and others 2001; Uesugi and others 2013). By responding differently to the same herbivore, genotypes may support different species, leading to more unpredictable species composition (Stam and others 2014). Consistent with this study, in previous work we found that cranberry cultivars did not respond differently to dodder parasitism (Tjiurutue and others 2016)

In conclusion, we found that damage to cranberry hosts delayed and reduced parasite attachment, which could provide an important subsequent benefit of early leaf herbivory given the highly damaging effects of this parasite. Parasitic plants play important roles in many ecosystems, but little is known about chemically-mediated interactions between hosts, parasites and other interacting species. Understanding how
parasites and other species interact through a shared host will help gain a clearer understanding of the underlying mechanisms that influence parasite preference and performance. Our results suggest that herbivore damage could have indirect benefits by reducing attachment of a highly damaging parasitic plant, consistent with cascading effects of early damage on a wide range of other interactions (Poelman and others 2010; Van Zandt and Agrawal 2004).

2.6 Acknowledgements

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2.7 Notes


Table 2.1: ANOVA results for effects of damage on cranberry chemistry (continued onto next few pages)

F values from mixed model ANOVAs testing effects of herbivore damage and cultivar on cranberry chemistry, with block as a random factor. For all analyses, the numerator df is 4 for cultivar, 1 for dodder treatment and 4 for their interaction; error df is listed for each analysis. a P < 0.08, * P < 0.05, ** P < 0.01, *** P < 0.001. Bold values indicate significant effects at P < 0.05.

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<th>Cultivar</th>
<th>Cultivar x Damage</th>
<th>Error df</th>
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Figure 2.1: Effects of gypsy moth damage on subsequent dodder preference across cranberry cultivars

Preference measured as the total number of dodder stems attached for dodder applied 2 days and 7 days post damage respectively. Stevens had more dodder plants attached compared to Howes and Mullica Queen at 2 days post damage. Different letters above bars indicate significantly different means using Tukey’s Post-Hoc test ($P < 0.05$); means comparisons were only conducted within plants that had dodder applied either 2 or 7 days post-damage, indicated by the use of lower and capital letters. Error bars represent standard error.
Figure 2.2: Effects of gypsy moth damage on subsequent dodder preference

Preference measured as the (A) average days to attachment and (B) total number of dodder stems attached for the 2 and 7 day to dodder application post damage respectively. Damage delayed attachment to plants 2 days post damage and reduced dodder plants that attached 7 days post damage. *Indicates $P < 0.05$ and error bars represent standard error.
Figure 2.3: Effects of gypsy moth damage on cranberry induced defenses

(A) JA, and the flavonols (B) quercetin aglycone and (C) quercetin-3-xyloside. Damage increased JA and quercetin aglycone levels. Error bars represent standard error.
Figure 2.4: Differences in cranberry chemistry between cultivars

(A) total flavonols, and two example flavonols, (B) quercetin aglycone and (C) quercetin-3-xyloside, and (D) total phenolic acids. Different letters above bars indicate significantly different means using Tukey’s Post-Hoc test ($P < 0.05$) and error bars represent standard error.
CHAPTER 3
DODDER (CUSCUTA) REMOVAL DETERS FUTURE PARASITE ATTACHMENT ON TOMATO (SOLANUM Lycopersicum)

3.1 Abstract

Plants face many antagonistic interactions that occur sequentially. Often, plants employ defense strategies in response to the initial damage that are highly specific, and can affect interactions with subsequent antagonists. In addition to herbivores and pathogens, plants face attacks by parasitic plants, but we know little about how prior herbivory compared to prior parasite attachment affects subsequent host interactions. If host plants can respond adaptively to these different damage types, we might predict that prior parasitism would have a greater deterrent effect on subsequent parasites than would prior herbivory. To test the effects of prior parasitism and prior herbivory on subsequent parasitic dodder (Cuscuta spp.) preference, we conducted two separate greenhouse studies with tomato hosts (Solanum lycopersicum). In the first experiment, we tested the effects of previous dodder attachment on subsequent dodder preference on tomato hosts. In the second experiment, we tested the effects of previous caterpillar damage (Spodoptera exigua) and mechanical damage on future dodder attachment on tomato hosts. Dodder attached most slowly to tomato hosts that had dodder plants previously attached and then removed, compared to control plants or plants with continuous dodder attachment. In contrast, herbivory did not affect subsequent dodder attachment rate. These results indicate that dodder preference depended on the identity and the outcome of
the initial attack, suggesting that early-season interactions have the potential for profound impacts on subsequent community dynamics.

**Key Words:** *Cuscuta*, herbivory, induced defenses, parasitism, preference, sequential-attack, species interactions

### 3.2 Introduction

Throughout their life cycles, plants face many antagonistic interactions that often occur sequentially. Studies using herbivores have established two themes describing how prior antagonistic interactions affect subsequent interactions via changes in plant phenotypes: specificity of elicitation, in which a plant’s phenotype depends on the identity of the initial attacker (Karban and Baldwin 1997, Van Zandt and Agrawal 2004); and specificity of effect, in which subsequent antagonists respond differently to a given plant phenotype (Karban and Baldwin 1997, Agrawal 2011). For example, damage to milkweed plants (*Asclepias syriaca*) by milkweed beetles (*Labidomera clivicollis*) increased latex production compared to controls, but damage by monarchs (*Danaus plexippus*) did not, indicating specificity of elicitation (Van Zandt and Agrawal 2004). In the same study, beetles were not affected by previous damage from either conspecifics or monarchs, but monarchs were smaller on plants that had been damaged by either herbivore, indicating specificity of effect (Van Zandt and Agrawal 2004). In another system, feeding by flea beetles (*Psylliodes affinis*) and tortoise beetles (*Plagiometriona clavata*) on *Solanum dulcamara* induced different phytochemical responses, and reduced oviposition compared to plants damaged by conspecifics or mechanically damaged
Plants may also respond to closely related herbivores by releasing different volatile blends that affect herbivore preference and attract natural enemies specific to each herbivore species (De Moraes et al. 1998).

Changes that occur in plant traits can depend on the identity of the plant as well as identity of the attacker (Ali and Agrawal 2012, Thaler et al. 2012, Stam et al. 2014). Plant genotypes can vary in resistance, quality, or induced responses (Underwood 2009, Schaedler et al. 2010, Uesugi et al. 2013), and therefore can affect subsequent interactions differently. For example, the effects of previous feeding by meadow spittlebugs (*Philaenus spumarius*) on stem galler performance (*Eurosta solidaginis*) on goldenrod (*Solidago altissima*) could be positive, negative or neutral depending on plant genotype (Cronin and Abrahamson 1999). In another study, performance and density of Mexican bean beetles (*Epilachna varivestis*) differed among four soybean genotypes (*Glycine max*), suggesting that these genotypes vary in some aspect of their quality as hosts for the beetles (Underwood et al. 2000). Genotypic variation among plants, and specificity of effect and elicitation can therefore have significant impacts on the composition and population density of herbivores.

Studies documenting the effects of prior herbivory on subsequent herbivores and pathogens have established the importance of specificity and genetic variation in responses (Thaler et al. 2001, Thaler et al. 2010, Agrawal 2011, Uesugi et al. 2013, Stam et al. 2014). However, plants also face attacks by other plants. Parasitic plants can severely impact growth and reproduction of their hosts (Press and Phoenix 2005), and play critical roles in structuring communities at multiple trophic levels (Pennings and
Some of the world’s most economically devastating agricultural pests are parasitic plants (Press and Graves 1995). For example, dodder, *Cuscuta* spp., cause significant damage to a wide variety of agricultural crops including alfalfa, clover, potato, carrot, sugar beets, chickpea, onion, cranberry, blueberry, citrus, and tomato (Dawson et al. 1984, Runyon et al. 2009). Despite the critical ecological and economic roles played by parasitic plants, little is known about how previous host interactions affect parasitic plant attachment.

Host quality and host defenses have been shown to influence parasite performance. Host quality can have both negative and positive impacts on parasite preference and performance (Pennings and Callaway 2002, Press and Phoenix 2005). Parasite performance can also be affected by host defenses (Press and Phoenix 2005, Irving and Cameron 2009). For example, some parasites may benefit from taking up host secondary metabolites due to reduced herbivory, indirectly increasing pollinator visitation and fruit set of the parasite (Adler 2000). Other studies report that higher host secondary metabolites may deter parasites (Press and Phoenix 2005). Since parasitic plant performance depends on host quality, previous parasitism or herbivory could affect parasite preference. However, we know very little about how comparable the effects of parasite attachment and herbivory are in affecting subsequent interactions with parasites themselves.

Very few studies have demonstrated the effects of herbivory on subsequent interactions with parasitic plants. For example, host clipping early in the season reduced biomass of the hemiparasite *Rhinathus minor* (Puustinen and Salonen 1999). Although this study provides a glimpse into the effects of prior herbivory on subsequent plant-plant
interactions, more studies involving other parasites and host plants are necessary to establish clear patterns.

We tested the effects of prior dodder (*Cuscuta* spp.) parasitism and herbivory on subsequent dodder preference using tomato host plants in separate greenhouse studies. We hypothesized that previous stimuli will result in specific adaptive changes, such that host plants with prior parasite attachment would become more resistant to parasites, and that prior parasite attachment should deter subsequent dodder more than herbivory would. This study is unique in that, to our knowledge, no studies have examined the effects or prior parasitism on future attachment of parasitic plants.

### 3.3 Methods and materials

#### 3.3.1 Study system

Tomato (*Solanum lycopersicon*) is consumed worldwide and touted for its health benefits (Tedeschi et al. 2011). A wide range of tomato cultivars and hybrids are available commercially with varying chemical and nutritional characteristics, including high antioxidant content (Tedeschi et al. 2011). We used six different commercial Heinz hybrid cultivars (Heinz Seed Company, Tomato Hybrid seeds, Modesto, CA, USA) with varying known resistance to dodder. Heinz cultivars ‘9492’, ‘9553’, and ‘9992’ are considered relatively resistant to dodder parasitism due the parasite’s inability to form viable haustorial connections with the host stems (Goldwasser et al. 2001), but resistance of tomato cultivars ‘3402’, ‘5608’ and ‘8504’ has not been demonstrated to our knowledge.

Dodder is serious of pest of many agricultural crops including tomato, making it one of the most economically important agricultural pests (Runyon et al. 2009). The
parasite lacks chlorophyll and relies solely on its hosts for survival. Dodder forms close connections with its host using specialized organs called haustoria to uptake water and nutrients, and this intimate connection between the parasite and host makes dodder especially hard to control using conventional methods (Sandler 2008). Dodder seeds were collected on 28 September 2008 from a commercial cranberry bog in Carver, MA. Identification of Cuscuta species can be challenging; polymerase chain reaction (PCR) of DNA from dodder collected from several sites in this region indicated that plants were mostly C. gronovii, but with some C. campestris and possibly C. compacta co-occurring (Ghantous et al. 2012). Because we did not identify dodder to species level, we will refer to the parasite by genus name only.

3.3.2 Plant propagation and herbivore rearing

We planted tomato seeds using Black Gold seedling mix (Sun Gro Horticulture Distribution Inc., Agawam, MA USA) in 72-plug trays on 10 April 2014, and trays were placed in a mist house at 65°C day and night. Plants were repotted into 10 cm pots using Black Gold potting mix (Sun Gro Horticulture Distribution Inc., Agawam, MA, USA) on 25 April 2014. Pots were placed in 10 cm saucers for bottom watering to prevent dislodging dodder seeds. Plants for the herbivory experiment were propagated in the same way the following year, and were started on 23 September 2015 and repotted on 8 October 2015.

Dodder seeds were scarified in batches of 100 (0.01 g) in a 2 mL microcentrifuge tube for approximately 3 minutes using a small dremel tool (Ghantous and Sandler 2012). Seeds were then rinsed with tap water using a fine mesh strainer, placed in Petri dishes lined with 90 mm moistened filter paper, sealed with Parafilm (Bemis Company Inc.,
Oshkosh, WI, USA), and placed in an incubator at 23°C until seed germination approximately 2 days later.

Tobacco hornworm larvae (*Manduca sexta*) were obtained from a research laboratory (L. Schwartz, Department of Biology, University of Massachusetts, Amherst, MA; the colony was initiated with eggs obtained from APHIS, USDA, Otis, Buzzards Bay, MA, USA). Prior to the experiment, larvae were reared on an artificial diet (Bell and Joachim 1976) and maintained at 27°C at a 16h: 8h light-dark photoperiod.

3.3.3. Experimental design and methods

We conducted two separate greenhouse experiments in consecutive years, 2014 and 2015. The first experiment, hereafter referred to as the *prior dodder experiment*, tested the effects of previous dodder parasitism on subsequent dodder preference across 6 tomato cultivars. The second experiment, hereafter referred to as the *prior herbivory experiment*, asked how herbivory and mechanical damage affected subsequent dodder preference using a single tomato cultivar.

3.3.3.1 Prior dodder experiment

We asked how initial dodder parasitism affected subsequent dodder attachment using three treatments. Control plants had no previous dodder attachment. Dodder-removed plants had an initial dodder seedling attached, removed by hand from the stem of the host, and left in the same pot to simulate what would happen in nature if the parasite died. Some of the early intervention of controlling dodder involves dodder removal by farmers, and dodder could be left behind in bogs in this way. Dodder-continuous plants had an initial dodder seedling that remained attached and not disturbed. Following these treatments, a second dodder seedling was added to assess how initial
interactions affected subsequent attachment as the response. With this design, we can compare (1) initial dodder attachment rate for all six tomato cultivars, using the initial dodder attachment as a measure of dodder preference, and (2) subsequent (second) dodder attachment due to both treatment and host cultivar.

The experiment used a randomized complete block design, with plants placed in blocks based on similarity in height. Each of the 30 experimental blocks consisted of 3 dodder treatments x 6 cultivars, for a total of 540 plants. The initial dodder seedling was placed 1 cm away from host stem in the dodder-continuous treatment and dodder-removed treatments. Although it was not the primary goal of our experiment, we monitored plants daily and recorded the date when this initial dodder attached or died as a measure of dodder preference across tomato cultivars. Two days after the first dodder attached, dodder was removed and left in the pot in the dodder-removed treatment, and left attached on the dodder-continuous plants. On the same day that the first dodder seedling was removed, another dodder seedling was added to measure how dodder responds to previous attachment. Similarly, after two days of the first dodder attachment, a second dodder seedling was added to dodder-continuous treatments. Control plants received dodder seedlings on the same day that the dodder-continuous plants in their block received the second dodder. Plants were monitored daily, and the date when dodder attached or died was recorded as a measure of dodder preference. To analyze subsequent dodder attachment, we ultimately included only three cultivars from the original six because of low attachment by the initial dodder seedling on three cultivars, resulting in low replication (fewer than 10 replicates per treatment for these cultivars). We measured plant height on the day we added the first dodder to all plants as a covariate in analyses.
3.3.3.2 Prior herbivory experiment

The experiment compared dodder attachment rate for mechanically damaged plants, caterpillar-damaged plants and control plants on one tomato cultivar, ‘H5608,’ chosen due to its high success of dodder attachment. Host plants were placed in 50 blocks based on height with the one plant of each treatment (control, mechanical damage and caterpillar damage) per block for a total of 150 host plants. To damage the plants, we bagged all plants using fine mesh bags (17.5 cm x 13 cm). We bagged 35 blocks on one day and the remaining 15 blocks on the next day due to limited bags. One second-instar tobacco hornworm larva was added to plants in the caterpillar damage treatment. Plants in other treatments were bagged without larvae. Larvae fed on the plants for a day before removing bags. Any larvae that died were replaced and left for an additional day. Once the bags were removed, we used a pair of dissecting scissors in the mechanical damage treatment to simulate the same amount and distribution of herbivory as the caterpillar-damaged plant in that block. Bags were removed from control plants at the same time as other plants in that block. One day after removing the bags, we added a single dodder seedling 1 cm away from all host stems in that block. Plants were monitored daily, and the day when dodder attached or died was recorded as a measure of dodder preference. We measured host height and leaf length (mid-vein length of host’s longest leaf) on the same day we added dodder to use as covariates in analysis.

3.3.4 Statistical analysis

We used R version 3.2.1 for Mac (R Core Team 2014) to carry out all statistical analyses. In both experiments, our responses were rate of dodder attachment, analyzed using a Cox proportional hazards mixed-effects model with maximum likelihood
parameter estimation (Therneau 2015). The rate of dodder attachment was analyzed as a survival object that included both whether the plant attached or died, and days until that event.

3.3.4.1 Prior dodder experiment

We tested for differences in initial (pre-treatment) rate of dodder attachment across all six cultivars as a measure of dodder preference. The model included cultivar as a fixed independent variable and block as a random factor. To analyze rate of attachment for the second, post-treatment dodder seedling, we ran a separate Cox mixed-effects model including treatment as an additional fixed independent variable. For this second Cox model, we included only 3 cultivars due to low rates of dodder attachment by the initial dodder seedling in the other cultivars. Plant height was initially included as a covariate, but removed from both models because it did not explain significant variation in attachment rate ($P > 0.62$ for both). We conducted pairwise contrasts using the function glht in the package multcomp in R to test for differences in treatments and cultivars at $\alpha = 0.05$ (Hothorn et al. 2008).

3.3.4.2 Prior herbivory experiment

As in the dodder experiment, we used a Cox mixed-effects model with rate of attachment as the response variable, treatment as fixed independent variable, block as a random factor, and plant height and leaf length as covariates. Again, plant height was not statistically significant and was excluded ($P = 0.62$), but leaf length was statistically significant and retained in the model.
3.4 Results

3.4.1 Prior dodder experiment

3.4.1.1 Cultivar differences in initial dodder attachment

Attachment rates of the initial dodder seedling used for the manipulation differed with cultivar (Cultivar: \( \chi^2 = 65.85, \text{df} = 5, P < 0.001 \)). Dodder attached fastest to susceptible cultivar H5608, slowest on cultivars H9553 and H9992 and intermediate for cultivars H8504, H3402 and H9492 (Figure 3.1).

3.4.1.2 Effects on initial attachment on subsequent dodder attachment

Previous dodder parasitism affected subsequent dodder attachment (Treatment: \( \chi^2 = 10.12, \text{df} = 2, P = 0.0064 \)). Hosts that had the initial dodder seedling attached and then removed experienced significantly slower attachment by the second dodder (Dodder-removed: hazard ratio = 0.48, \( Z = -2.96, P = 0.0031 \)) compared to hosts in the control or continuous dodder attachment treatments (Dodder-continuous: hazard ratio = 0.97, \( Z = -0.13, P = 0.900 \); Figure 3.2). By day 3, only about 63% of dodder was attached to hosts in the dodder-removed treatment, compared to 90% of attached dodder to control hosts and 85% attached in the dodder-continuous treatment (Figure 3.2). Dodder attachment rate also varied across cultivars (Cultivar: \( \chi^2 = 11.23, \text{df} = 2, P < 0.0037 \); Figure S3: 1).

3.4.1.3 Prior herbivory experiment

There was no effect of herbivory on dodder attachment but (Treatment: \( \chi^2 = 0.28, \text{df} = 2, P < 0.87 \); Figure 3.3; Herbivory: hazard ratio = 1.10, \( Z = 0.36, P = 0.72 \); Mechanical: hazard ratio = 1.14, \( Z = 0.51, P = 0.62 \)). However, the covariate leaf length was marginally significant (Leaf length: \( \chi^2 = 2.91, \text{df} = 1 \)).
3.5 Discussion

Dodder attached more slowly to hosts with previously attached dodder that were removed, compared to control hosts or hosts with continuous dodder attachment (Figure 3.2). We hypothesized that previous dodder attachment would induce host plant changes that affect subsequent dodder attachment, so this result was not entirely surprising. However, we also expected that any previous parasite attachment would deter subsequent parasitism, regardless of whether the parasite was then removed or remained attached. It is possible that hosts with dodder removed released a different scent profile, repelling subsequent dodder plants compared to other treatments. Additionally, since we left the removed dodder seedling in the pot, it is possible that the dying or dead dodder released a scent that deterred other dodder seedlings.

Volatile released from dodder itself could mediate attachment of subsequent dodder. Dodder (C. pentagona) can distinguish between preferred tomato host plants (S. lycopersicum) and non-preferred wheat host plants (Triticum aestivum) based on host scent. If slow attachment was caused by repellent effects of the initial dying dodder seedling, then the effects of the dodder-removed treatment should still be observed even if the initial seedling does not attach. This hypothesis could be tested by comparing dodder responses to the effects of removing dodder completely after it attaches, versus removing dodder after it attaches but leaving the dying dodder in the pot. If the presence of dying dodder in the pot is sufficient to repel subsequent dodder attachment, then adding dead or decaying dodder to areas with high parasite infestations could provide environmentally friendly protection to valuable crops.
Dodder may have also have a context-dependent aversion to the scent of dying dodder in the presence of a host. For example, moths can be attracted to carbon dioxide in the presence of flower volatiles but carbon dioxide alone is not attractive to moths (Goyret et al. 2008). The scent of dying dodder against the volatile background of a host could indicate a poor or well-defended host unsuitable for attachment.

Induced host defenses could also mediate interactions between previous dodder and future attachment. Previous work in tomato has shown that host defenses mediated by phytohormones can impact parasite performance (Pennings and Callaway 2002, Irving and Cameron 2009). For example, *C. pentagona* grew larger on both jasmonic-insensitive and salicylic-deficient tomato hosts, suggesting that both salicylic (SA) and jasmonic (JA) responses may be effective against parasitic plants. JA-SA mutants generally lacked a noticeable hypersensitive response (HR), which could be an effective mechanism against dodder parasitism. Both JA and SA are involved in the HR response, which is an effective defense against *C. reflexa* (Ihl et al. 1988, Goldwasser et al. 2001). Future research could investigate the role of host induced defenses such as JA and SA against dodder attachment using JA-SA insensitive mutant host plants. If slow attachment of dodder is due to induced defenses, then we would expect that for JA- or SA-signaling mutant hosts, there would be no effect of initial dodder parasitism on subsequent attachment.

Dodder attached more slowly to hosts with dodder removed compared to hosts with continuous dodder attachment, which was a surprising result. Although this has not been demonstrated, we speculate that perhaps dodder can suppress or manipulate host defenses when attached to the host but not after removal, allowing plants to activate their
defenses following unsuccessful attachment. Suppression of host defenses by herbivores is not uncommon (Schwartzberg et al. 2011, Ali and Agrawal 2012, Stam et al. 2014, Kant et al. 2015). For example, feeding or application of oral secretions of *Manduca sexta* larvae to leaf punctures suppressed production of nicotine in *Nicotiana attenuata* plants, accompanied by other changes in plant defenses, compared to mechanically damaged plants (Kahl et al. 2000). In a more recent study, jasmonic acid accumulated in response to artificial damage but was suppressed by feeding of the pea aphid *Acyrthosiphon pisum* in broad beans, *Vicia faba* (Schwartzberg and Tumlinson 2014). Future studies quantifying host JA and SA defenses in both dodder-continuous and dodder-removal treatments could provide some insights into the mechanisms mediating these interactions. Based on the results of our study, we predict that compared to dodder attachment and removal, continuous dodder attachment would suppress phytohormonal responses in hosts.

The different effects of the dodder-removed and dodder-continuous treatment on subsequent parasitism warrant further investigation to elucidate their ecological effects. The differences between these treatments suggest that the effects of prior interactions depend not just on the identity of the antagonist, but also on the outcome of the interaction. Given that prior parasite attachment has been shown to reduce subsequent herbivory but also deter pollinators (Prider et al. 2011), we suggest experiments testing whether these two dodder induction treatments vary in their effects on subsequent herbivore attraction, pollinator attraction, and fruit yield. Although tomato plants are largely self-fertile, pollinators are required to yield fruit (Morandin et al. 2001), so the effects of parasites on pollinators are particularly relevant in agriculture. In addition,
because dodder attachment has been shown to induce SA-based phytohormonal responses (Runyon et al. 2010) normally associated with resistance to pathogens (Glazebrook 2005, Thaler et al. 2012), the effects of parasitic plants on susceptibility to bacterial and fungal tomato diseases such as fungal blights and bacterial canker could provide insight into the specificity of induction and variation according to the outcomes of previous parasitism.

Cultivars differed in the rate of dodder attachment, with dodder attaching faster to cultivar H5608 than compared to other cultivars (Figure 3.1). This suggests that cultivar H5608 is more susceptible to dodder compared to the other cultivars, which has not been demonstrated to our knowledge. Resistance of at least 3 cultivars, H9492, H9553, and H9992 to dodder parasitism has already been established. Resistance in these cultivars was accompanied by a hypersensitive response (HR), which typically involves swelling, rapid cell death and lignification of host cells blocking successful haustorial penetration (Goldwasser et al. 2001). Screening for cultivars showing HR could be useful in selecting cultivars that display dodder resistance.

Prior mechanical damage and herbivory did not affect subsequent dodder preference (Figure 3.3). Many studies report that both caterpillar and mechanical damage can affect subsequent herbivore preference and performance (Karban and Baldwin 1997). We expected that herbivory would affect subsequent dodder preference because herbivorous insects often release oral secretions while feeding that can elicit stronger responses in plants compared to mechanical damage (Korth and Dixon 1997, Kahl et al. 2000). Our results demonstrate that herbivory and prior dodder attachments have markedly different effects on subsequent dodder attachment.
We speculate that dodder may induce different plant defenses from herbivory in tomato, which could explain why dodder removal affected subsequent dodder attachment but not herbivory. Plants can respond differently to specific herbivores, which influence subsequent interactions with the same host plant (Karban and Baldwin 1997, Van Zandt and Agrawal 2004, Thaler et al. 2010). For example, two specialist herbivores preferred and performed better when fed leaves damaged by conspecifics than heterospecifics, and elicited qualitatively different secondary compound profiles across three Solidago altissima genotypes (Uesugi et al. 2013). In contrast, a third specialist herbivore equally avoided all types of damage and its performance was not affected by damage type (Uesugi et al. 2013). In another study, herbivory by two caterpillar species produced distinct volatile blends, and an egg parasitoid was attracted only to volatile blends produced by its host (Moraes et al. 2005). It is therefore possible that the dodder-removed treatment and tobacco hornworm damage elicited different defense responses in tomato that affect subsequent dodder attachments.

The differences in phytohormonal responses to parasitic plants and herbivores may provide an additional explanation for why herbivory did not affect subsequent parasitism. Tjiurutue et al. (2016) showed that dodder parasitism induced SA production in cranberry hosts and both SA and JA signaling pathways in tomato (Runyon et al. 2010). Generally, chewing insects such as caterpillars activate the JA-signaling pathways (Thaler et al. 2012). Since in our current study we used a chewing herbivore (M. sexta), we expect that herbivory should have induced JA production. Perhaps the activation of the SA signaling pathway is also required as a defense mechanism against dodder parasitism in tomato. Collectively, our results show that prior herbivory and parasitism
are important in shaping interactions between herbivores and parasitic plants with the same shared host.

In conclusion, we show that the effects of prior antagonistic interactions on dodder attachment depend not just on the identity of the previous antagonist, but also on the outcome of the previous interaction. Specifically, removal of dodder slowed subsequent dodder attachment, suggesting that removal of early dodder plants or adding dead dodder might provide agricultural avenues for controlling dodder. Cultivars also differed in their resistance, allowing for selection and use of the resistant cultivars in areas of high dodder infestations. Overall, our results are indicative that tomato responds differently to different antagonists, which could further shape interactions with other species in the community.

3.6 Acknowledgements

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3.7 Notes


Figure 3.1: Differences in dodder attachment for six tomato cultivars

Lines shows dodder seedlings remaining unattached over time. Cultivar ‘H5608’ had significantly higher attachment rate compared to other cultivars.
Figure 3.2: Effects of prior dodder parasitism on subsequent dodder attachment on 3 tomato cultivars

Lines indicate proportion of dodder seedlings unattached at each time point. The ‘continuous attachment’ treatment line stops at day 4 because all dodder that did not attach by this point had died. Plants with the initial dodder removed had significantly lower attachment rate compared to control and plants with continuous dodder attachment.
Figure 3.3: Effects of prior herbivory on subsequent dodder attachment on ‘H5608’ tomato cultivar

Lines indicate proportion of dodder seedlings remaining unattached at each time point.

There were no significant differences between treatments.
Figure S3.1: Effects of prior dodder parasitism on subsequent dodder attachment for 3 tomato cultivars

Lines indicate proportion of dodder seedlings unattached at each time point. Plants that had dodder removed had significantly lower attachment rate compared to control and plants that had dodder plants continuously across cultivars.
CHAPTER 4

MESSAGES FROM THE OTHER SIDE: PARASITES RECEIVE DAMAGE
CUES FROM THEIR HOST PLANTS

4.1 Abstract

As sessile organisms, plants often rely on their environment for cues indicating imminent herbivory. These warning cues can originate from tissues on the same plant or from different individuals. Since parasitic plants form intimate vascular connections with their host, parasites have the additional potential to receive cues from host plants that could allow them to adjust their defenses against future herbivory. Parasitic plants are present in many ecosystems, and play important roles structuring the communities in which they live. However, the role of plant communication between hosts and parasites for herbivore defense remains poorly investigated. Here we examined the effects of lupine host damage (*Lupinus texensis*) on responses of the attached hemiparasitic plant (*Castilleja indivisa*) and a specialist herbivore of the parasite (*Junonia coenia*). Lupines produce alkaloids as defenses against herbivores, and these compounds can be taken up by the parasite. We found that damage to host plants by beet armyworm (*Spodoptera exigua*) significantly increased jasmonic acid (JA) levels in both host and parasite, suggesting uptake of phytohormones or priming of parasite defenses using host cues. In contrast, host damage did not induce changes in alkaloid levels in either the host or attached parasites. Interestingly, the parasite had higher levels of JA and alkaloids compared to host plants.
Interestingly, herbivores consumed more leaf tissue on parasites attached to damaged plants compared to undamaged hosts, although host damage did not affect herbivore relative growth rate. It is possible that increased JA due to damage induced higher production of iridoid glycosides in the parasite, which act as feeding stimulant for the specialist herbivore. Our results demonstrate that damage to hosts may affect both parasite responses and associated herbivores, demonstrating cascading effects of host damage on multiple trophic levels.

**Key Words**- Alkaloids, herbivory, parasitism, plant communication, plant-plant interactions, performance, phytohormones.

### 4.2 Introduction

Plants can prime herbivore defenses in response to compounds released from other tissues from the same individual, or by responding to volatile cues released by neighboring individuals that are under attack (Karban and Baldwin 1997, Karban et al. 2006, Karban et al. 2014). For example, sagebrush (*Artemisia tridentata*) experienced reduced herbivory after exposure to volatiles released from clipped conspecific neighbors (Karban et al. 2006). Moreover, unrelated individuals and species can also “eavesdrop” on cues released by other plants (Karban et al. 2013). For example, wild tobacco (*Nicotiana attenuata*) had higher induced plant defenses and experienced less herbivory when growing next to clipped compared to unclipped sagebrush neighbors (Karban et al. 2000). Thus, cues from neighboring plants may provide important information that allows plants to defend against likely attack.
Parasitic plants can acquire nutrients (Phoenix and Press 2005) and defensive compounds from their host via haustorial connections (Adler and Wink 2001, Lehtonen et al. 2005, Cabezas et al. 2009). Due to the close physical proximity and vascular connection between parasitic plants and hosts, parasitic plants could receive chemical cues associated with herbivory indirectly via released volatiles from damaged host plants or directly from their host via uptake of phytohormones or defensive compounds. Induced host volatiles could be perceived by neighboring parasites, inducing defensive responses. If parasites take up induced phytohormones or chemical defenses from damaged hosts through vascular connections, this could increase the parasite’s own resistance to herbivory and reduce parasite damage. For example, *Castilleja indivisa* hemiparasites grown with *Lupinus albus* hosts containing high alkaloid levels experienced less herbivory, higher pollinator visits and higher seed set compared to parasites grown with low alkaloid hosts (Adler et al. 2001). High pollinator visits were due to reduced damage to flower buds, which resulted in more open flowers that attracted more pollinators (Adler et al. 2001). Thus, uptake of defensive compounds from hosts can influence parasite reproduction.

Although several studies have examined the effects of alkaloid-producing hosts on herbivores of parasites (Stermitz et al. 1989, Marvier 1996, Marvier 1998, Adler 2002), the question of whether host damage mediates interactions between parasites and their herbivores has not been addressed. Herbivore induced host responses could alter parasite species interactions, leading to dynamic changes in food web and community structure (Stam et al. 2014). Moreover, metabolite uptake from the host to the parasite may have implications for biocontrol management of parasitic weeds, since biocontrol
species would need to tolerate both the host and parasite defenses (Smith et al. 2013). Findings from these studies may have both ecological and agricultural implications by helping us understand the mechanisms that mediate interactions between hosts, parasites, and herbivores.

The hemiparasite Castilleja indivisa (Orobanchaceae; hereafter Indian paintbrush) and host Lupinus texensis (Fabaceae; hereafter lupine) were used to study the effects of host damage and secondary metabolite uptake on herbivory in the parasite. Lupine is a native, common annual species in Texas that frequently grows and flowers with Indian paintbrush (Loughmiller et al. 1984). Indian paintbrush is an annual root hemiparasite endemic to Texas (Kuijt 1969, Loughmiller et al. 1984) that does not make its own alkaloids, but takes up the alkaloid lupanine when parasitizing lupine hosts (Adler 2000). The parasite, however, produces iridoid glycosides as herbivore defense compounds (Stermitz and Pomeroy 1992). Junonia coenia (Lepidoptera: Nymphalidae), or buckeye, is a specialist herbivore that feeds on plants that produce iridoid glycosides (Bowers 1984), including Castilleja species (Adler 2000). Iridoid glycosides act as feeding and oviposition stimulants (Bowers 1984) and also make the herbivores unpalatable to predators (Theodoratus and Bowers 1999).

To examine the effects of damage on host defenses, parasite defenses and parasite herbivory, we conducted a greenhouse study to ask:

1. Does herbivory induce changes in phytohormones and alkaloid levels in both lupine hosts and attached parasites?

2. Does herbivory to lupine hosts reduce herbivore performance on attached parasites?
4.3 Methods and materials

4.3.1 Experimental design

Each replicate pot contained 2 lupine hosts and one parasite. Half of these pots \( n = 30 \) (treatment) were used to measure chemical induction and the other half was used for herbivore performance assays. This experiment had 60 replicate pots x two treatments (damage vs non-damaged hosts), for a total of 120 pots.

4.3.2 Plant propagation

Lupine seeds were purchased from Seedville USA (Massillon, Ohio, USA) and Indian paintbrush seeds were purchased from Native American Seed (Junction, Texas, USA). Lupine seeds were scarified by soaking seeds in concentrated sulfuric acid for 3 hours, followed by rinsing with tap water. Lupine seeds were then transferred to petri dishes lined with moistened filter paper before sealing with parafilm to prevent drying until germination. Germinated seedlings were soaked in a rhizobium inoculant (Gourmet Seed International, Tatum, New Mexico, USA) before planting into 24-cell plugs in Black Gold seeding germination mix (Sun Gro Horticulture, Agawam, MA, USA). Once seedlings established roots, seedlings were repotted into 10 cm pots in a 1:1 Fafard professional potting mix: course vermiculite (Conrad Affairs, Inc, Agawam, MA, USA; Whittemore Company Inc, Lawrence MA, USA) on 30 May 2014. Lupine plants were ultimately repotted into 50 cm pots with 2 hosts per pot on 02 July 2014, and kept in the greenhouse at 65°C constant temperature.

To germinate Indian paintbrush seeds, 72-cell trays with Black Gold seedling germination mix moistened with tap water were used. Indian paintbrush seeds were sprinkled on top of the soil and covered with plastic wrap to maintain moisture on 29
June 2014 and placed in a growth chamber with mean temperatures of 18°C and 16:8 D:N. The seeds were sprayed with tap water using a spray bottle as necessary to stay moist. Once seedlings germinated, the tray plugs were transferred to the greenhouse with 65°C constant temperature. Seedlings were transplanted on 30 August 2014 into pots with 2 lupine hosts. Once established, seedlings were thinned to one per pot by clipping extra parasites at soil level to avoid disturbing roots.

4.3.3 Induction experiment

Due to parasite mortality, a total of 54 from the original 60 pots was used to assess host defense induction and compound uptake by the parasite. Each pot contained 2 lupine hosts and one parasite. In half of the pots, host plants were bagged with third instar beet armyworm larvae (*Spodoptera exigua*; Benzon Research Inc, Carlisle, PA). Larvae were reared on artificial diet of soy flour (39.0 g/l) and wheat germ (34.0 g/l) and kept in the laboratory at room temperature before the experiment. Control hosts were bagged at the same time without herbivores. Leaf tissue was collected from both host and parasite for analysis of phytohormones and alkaloids after 24 hrs. Leaves were cut from both parasite and lupine using a razor blade, which is less likely to induce host responses (Thaler et al. 2010). Collected leaf tissue was immediately placed in liquid nitrogen before storage at -80°C until phytohormone analysis. The remaining leaf tissue from both host and parasite were collected for alkaloid analysis and placed in separate brown paper bags and dried in the oven at 45°C for one week. Due to insufficient leaf material, we pooled parasite leaves together for a total of 10 samples (5 x 2 treatments) just for the alkaloid analysis. Host leaves were not pooled (54 samples; 27 x 2 treatments).
4.3.3.1 Phytohormone analysis

We measured leaf JA, SA and ABA hormones from damaged and control lupine hosts and attached parasites using a subsample of 15 plants per treatment for both the host and parasite, for a total of 60 samples. Phytohormone extraction and analysis were based on Thaler et al. (2010). About 200-300 mg of frozen leaf tissue was transferred into a 2 ml screw cap tube containing pre-weighed 0.9 g silica beads (BioSpec, Bartelsville, OK, USA) and leaves were crushed into small particles inside the tubes. We added 100 µl of d4-SA and d5-JA (800 pg ml⁻¹ each) as internal standards (CDN Isotopes, Point-Claire, Canada) with 1 ml extraction buffer (iso-propanol:water:hydrochloric acid 2:1:0.005 by volume) and homogenized the tissue in a FastPrep homogenizer (MP Biomedicals, Solon, Ohio, USA) at 6 m/s for 45 seconds. Samples were centrifuged at 4 °C for 20 min at 20,800 x g (14,000 rpm). The supernatant of each sample was carefully transferred into a fresh 2 ml tube, added 1 ml of dichloromethane and vortexed for 30 min. We then centrifuged the samples again at 4 °C for 20 min at 12,000 x g for 2 min for phase separation. The separated aqueous (top) and middle layer were completely removed and discarded before evaporation of samples overnight under a fume hood. Samples were dissolved in 200 ml methanol and filtered through a 0.45 mm syringe filter (13 mm diameter) into 2 ml HPLC vial with insert. This remaining 15 µl solvent was analyzed on a triple-quadrupole LC-MS/MS system (Quantum Access; Thermo Scientific, Waltham, Massachusetts, USA). A C18 reversed-phase HPLC column (Gemini-NX, 3µ, 150 x 2.00 mm; Phenomenex, Torrance, California, USA) was used to separate compounds using a solution of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) at a flow rate of 300 µl/min. Separation of compounds were performed using
a gradient of increasing solvent B content. The initial gradient of solvent B was maintained at 10% for 2 min and then increased linearly to 100% at 20 min.

Phytohormones were analyzed using negative electrospray ionization (spray voltage: 3.5 kV; sheath gas: 15; auxiliary gas: 15; capillary temperature: 350°C), collision-induced dissociation (argon CID gas pressure 1.3 mTorr [1.3 micron Hg], CID energy 16 V) and selected reaction monitoring (SRM) of compound-specific parent/product ion transitions: SA 137→93; d4-SA 141→97; JA 209→59; d5-JA 214→62 (Thaler et al. 2010).

4.3.3.2 Alkaloid analysis

Alkaloids of Indian paintbrush parasites and lupine hosts were extracted as described in Adler (2000). Briefly, leaves were dried at 45°C for one week in the incubator. Following this, the dried leaves were grounded to fine powder using a Wiley Mill (Thomas Scientific, Swedesboro, N.J.) with a 40-mesh screen. Extraction of alkaloids was achieved by adding 0.5 M HLC to approximately 0.5 g of dry weight for each sample and vortexed until all leaf tissue was covered in solution. Following this, samples were sonicated for 10 min and left to stand for 1 hr before sonicating again for another 10 min. About 3 ml of NaOH, was added to separate out alkaloids as free bases. The samples were then filtered through extrelut columns (Extrelut NT 20 ml, item number 115096; EMD Milipore Corporation, Darmstadt, Germany) filled with hydromatrix (Agilent technologies Inc., California, USA). About 30 ml CH₂Cl₂ was added to each of the extrelut columns and collected into small pre-labeled beakers. The collected filtrates were allowed to dry overnight in the fume hood. About 2 ml of CH₂Cl₂ was added to the beakers to re-dissolve the dried filtrate before transfer to a 2 ml GC vial and left to dry overnight. Plant extracts were re-dissolved in 1 ml of methanol containing
500µg of dodecyl acetate as an internal standard. The samples were diluted further 100x in methanol before analysis using an Agilent 6890 gas chromatograph fitted with a DB-5 fused silica capillary column (30 m length, 0.25 mm diameter, 0.25 µm film thickness, Agilent Technologies LDA, Stockport, Cheshire, UK) and coupled to an Agilent 5973 mass spectrometer. Carrier gas was helium at a constant flow rate of 1 ml/min. The column temperature was held at 60°C for 2 min, and then programmed to 240°C at 6°C/min. Compounds were identified and quantified using the NIST Mass Spectral Database and by comparison to a commercial standard of lupanine (Sigma-Aldrich Company Ltd, Dorset SP8 4XT, UK).

4.3.3.3 Larval performance experiment

To determine whether host damage affects herbivores on parasites, a total of 54 pots, each containing 2 lupine hosts and one parasite, was used to assess insect performance on the parasite in a no-choice assay. Host plants were damaged with generalist beet armyworm as described earlier. After removal of larvae from host plants, leaves were collected from parasites attached to damaged versus non-damaged hosts plants. Leaves were placed in Petri dishes lined with moistened filter paper containing pre-weighed 2nd instar specialist buckeye larvae (Junonia coenia; Shady Oak Butterfly Farm, Inc., Brooker, Florida, USA) that were allowed to feed on leaves from a single parasite for 24 hours. Buckeyes were fed on Plantago lanceolata leaves before the experiment, deprived of food for 12 hours and then weighed prior to the trial. After 24 hours of feeding, larvae were removed and weighed. Relative growth rate (RGR = [final wet weight – initial wet weight]/initial wet weight) and dry and proportional amounts of leaves consumed were used as measures of larval consumption and performance.
4.3.4 Statistical analysis

R for Macintosh version 3.2.1 (R Core Team 2014) was used to carry out all statistical analyses.

4.3.4.1 Induction experiment

We ran three separate MANOVAs, one for parasite phytohormone responses and two separate MANOVAs for host and parasite alkaloid responses. For host phytohormones, JA residuals were not normally distributed, and so we used separate ANOVAs for SA and ABA responses, and used the Kruskal-Wallis rank sum test for host JA responses. Each analysis included damage treatment as the independent fixed factor. Responses for phytohormones were JA, SA and ABA, and responses for alkaloids included lupanine, 13-oxolupanine, 17-hydroxylupanine and one unknown lupanine compound.

Significant MANOVAs were followed by one-way ANOVAs. All data were tested for normality prior to analysis and we log-transformed host and parasite alkaloid responses to improve normality of residuals. We deleted 2 parasite outliers from the damage treatment from all analyses (12SD and 5SD above mean for JA); including outliers did not change the result.

4.3.4.2 Larval performance

We analyzed larval performance using ANOVA with larval RGR as the response and damage treatment as a fixed independent factor. At first we included parasite leaf mass as a covariate, but it was not significant and was removed from the model. Two outliers were deleted, one each from the control (20SD below mean) and damage (13SD above mean) treatment to comply with normality assumptions of ANOVA (Including the
outliers in the analysis did not change the results). We also analyzed larval consumption as the dry weight of tissue consumed and the proportion of leaves consumed. We included the latter measure because some larvae consumed all leaf tissues, and so might have consumed more if it had been available. We used ANOVA with dry weight of leaves consumed and proportion of leaves consumed as responses, and damage treatment as an independent fixed factor. We deleted two outliers from proportion of leaves consumed (one each 4SD below and above the mean in the damage treatment) that violated assumptions of normality. Similarly, one outlier for the control treatment (4SD above mean) was deleted from dry weight consumed.

4.4 Results

4.4.1 Induction experiment

4.4.1.1 Phytohormones

JA levels increased by ~58% in damaged host plants compared to controls (Kruskal-Wallis: df = 1, $\chi^2 = 8.93$, $P = 0.0028$; Figure 4.1A), but there was no effect of damage on host SA or ABA ($F_{1,28} < 0.25$, $P > 0.62$ for both; Figure 4.1C, D). Host damage affected parasite hormone levels (MANOVA, Pillai’s trace = 0.32, $F_{1,26} = 3.72$, $P = 0.025$). JA levels more than tripled when attached to damaged vs. control hosts ($F_{1,26} = 6.20$, $P = 0.020$; Figure 4.1B). Damage did not affect parasite SA or ABA ($F_{1,26} < 1.88$, $P > 0.18$ for both; Figure 4.1C, D). Interestingly, the parasite also had JA levels 5 to 10 times higher than host JA levels (compare Figs.1A and B).

4.4.1.2 Alkaloids

Damage did not affect host (MANOVA, Pillai’s trace = 0.075, $F_{4,49} = 0.99$, $P = 0.42$) or parasite alkaloid levels (MANOVA, Pillai’s trace = 0.57, $F_{4,5} = 1.64$, $P = 0.30$;
Figure 4. 2). It is interesting to observe that, although the parasite does not produce alkaloids, it had substantially higher levels of alkaloids than hosts. Specifically, parasite levels of lupanine and oxolupanine were 6-8 times higher than host levels (Figures. 4. 2A, C). In general, lupanine was the most dominant alkaloid in both host and parasite plant tissues (Figure 2A).

4.4.1.3 Larval performance experiment

Larvae consumed a higher proportion of parasite leaves from damaged compared to undamaged hosts ($F_{1,47} = 4.39; P = 0.042; \text{Figure 4.} 3A$) but there was no significant difference in dry weight consumed ($F_{1,49} = 0.47; P = 0.50$) or larval RGR ($F_{1,49} = 0.59, P = 0.45; \text{Figure 34.} B$).

4.5 Discussion

4.5.1 Does herbivory induce changes in phytohormones and alkaloid levels in both lupine hosts and attached parasites?

Damage to lupine hosts increased JA levels by ~50% in hosts and by ~320% in attached parasites (Figure 4. 1A & B). This suggests that parasites are either taking up host JA or using host cues to prime their own defenses against herbivory. A few studies have examined the uptake of phytohormones by parasitic plants from their hosts. Tomato plants sequentially increased JA and SA levels in response to dodder (*Cuscuta pentagona*) parasitism, but there was no increase in these hormones in dodder collected from the site of infection (Runyon et al. 2010). In a separate study, caterpillar damage increased JA in tomato hosts, but not in attached parasites (Runyon et al. 2008). This suggests that *Cuscuta* do not respond to or passively take up phytohormones of their
hosts, or that host compounds are degraded before reaching the parasite in that system. Induced defenses can influence herbivore preference and performance, and alter herbivore community composition and structure (Agrawal 1999, Thaler et al. 2001, Poelman et al. 2008). Thus, increased JA in attached parasites due to host damage could influence other species interacting with the parasite as well as host.

The assimilation of host alkaloids by parasitic plants has been shown in several parasite taxa including Cuscuta, Castilleja, Pedicularis, Tristerix, Loranthus and Orobanche, and phenolic and cardenolide transfer has been shown in Cuscuta, Santalum and Nerium species (Smith et al. 2013). However, it is not known whether the parasite takes up more defenses in response to host damage in these systems. In our study, we surprisingly found no effect of host damage on alkaloid levels for either host or parasite. This is surprising because quinolizidine alkaloids are typically inducible compounds (Wink 1983, Chludil et al. 2013) and we expected that damage would induce higher concentrations of these compounds. We collected leaf samples 24 hrs after host damage, and it is possible that we did not allow enough time for defense compounds to accumulate and detect changes in induction levels, or that beet armyworm used to damage hosts do not elicit a strong defense response in lupines. It is also possible that damage did induce biosynthesis of alkaloids but they were all assimilated by the parasite. Although damage did not affect alkaloid levels, parasites had consistently higher levels of alkaloids than their hosts (Figure 4. 2A). This could be a “crafty” mechanism employed by the parasite to obtain host defenses and for protection from imminent herbivory. The role of alkaloids in insect defense is well known (Lattanzio et al. 2006, Mithöfer and Boland 2012), and a few studies have shown that secondary metabolite transfer confers
benefits to the parasite (Smith et al. 2013). For example, Indian paintbrush attached to a high alkaloid lupine genotype had less herbivory, more open flowers, increased pollinator visitation and higher fruit set compared to Indian paintbrush attached to a low alkaloid genotype (Adler 2000, Adler et al. 2001). In a more recent study, R. serotinus acquired defensive mycotoxins produced by a symbiotic endophytic fungus that lives within a shared grass host (Lehtonen et al. 2005). Parasites grown with endophyte-infected hosts had increased resistance and supported lower aphid performance compared to parasites that were grown with uninfected endophyte hosts. These studies suggest that there could be fitness benefits to parasites that selectively uptake, or increase concentrations of, host-derived defense compounds.

Interestingly, we found that parasites had higher JA (Figure 4. 1A, B) as well as dominant lupanine (Figure 4. 2A) alkaloid concentrations compared to host concentrations. Indian paintbrush and similar parasites have a high density of stomata that enables them to maintain a negative water potential in relation to host water potential, allowing the parasite to draw water and nutrients from their host vascular system (Press and Graves 1995). Since Indian paintbrush does not synthesize alkaloids (Stermitz and Pomeroy 1992), it is plausible that the parasite lacks the capacity to degrade these compounds, allowing them to accumulate in the parasite. However, the high levels of JA in the parasite relative to host suggest that other compounds may also become concentrated, either because they are not metabolized quickly or because host-derived JA induces JA production in the parasite. Because alkaloids play major roles in defense against herbivores (Mithöfer and Boland 2012), the uptake and concentration of alkaloids by the parasite may exert strong impacts on herbivores and other species that
interact with the parasite. We do not expect this alkaloid uptake to directly influence pollinators because alkaloids taken up by *C. indivisa* attached to lupine hosts were not present in nectar (Adler and Wink 2001). However, alkaloids were found to accumulate in pollen of *L. mutabilis*, resulting in markedly smaller male bees (Arnold et al. 2014). Hence, it is possible that alkaloid taken up by the parasite from its host could accumulate in parasite pollen and potentially affect pollinators.

**4.5.2 Does herbivory to lupine hosts reduce herbivore performance on attached parasites?**

Herbivores fed on parasites attached to damaged hosts consumed proportionally about 30% more leaf material compared to controls (Figure 4.3A). Although not significant, herbivores that were fed on parasites attached to damaged hosts also had twice the RGR of herbivores fed on parasites attached to undamaged hosts (Figure 4.3B). This is surprising because we expected that host damage would induce higher defenses in hosts and attached parasites, reducing herbivore consumption and performance. Furthermore, higher JA in parasites on damaged hosts suggests induction of host defenses or assimilation from host. However, damage did not affect alkaloid concentrations in hosts or parasites, suggesting that alkaloids are not the mechanism increasing herbivore consumption. Paintbrush parasites produce iridoid glycosides that act as feeding stimulants to buckeye caterpillars (Bowers 1984). One possibility is that JA induced higher levels of iridoid glycosides, increasing herbivore consumption. It is also possible that other changes occurred due to host damage, such as the release of nutrients due to stress (Karban and Myers 1989, Nykanen and Koricheva 2004). Future studies comparing performance or consumption by both generalist and specialist herbivores may provide
mechanistic insights. If both generalist and specialist herbivores consume more of the parasites attached to damaged compared to control hosts, this could indicate increased plant quality. If only the specialist herbivore consumes more of the parasite attached to damaged vs control hosts, this suggests induction of iridoid glycosides, which would deter the generalist herbivore. Regardless of the mechanism, host induced responses affected both attached parasites and their associated herbivores.

Changes in host defenses due to damage, and consequences for parasites, could have various outcomes on interacting herbivores and pollinators of both host and parasites. If parasites attached to damaged hosts experience more damage it could lower parasite growth, which may in turn affect other species interacting with these parasites. Additionally, damage could increase production of defenses in the parasite or alter floral traits that could deter pollinators (Strauss et al. 1999, Erb et al. 2011), ultimately reducing parasite reproduction. However, increase in plant defenses could also attract pollinators by reducing floral damage (Adler et al. 2001). In addition, if the host plant and parasite share pollinators due to similarities in floral displays (Moeller 2004) reduced visitation to the parasite may also reduce visitation to the host, impacting community dynamics (Callaway 1995, Palmer et al. 2003). Host plants may benefit from herbivory if parasites are eaten more as we found, potentially reducing the impacts of parasitism and increasing host reproduction and survival. This is especially important in agricultural settings, where farmers could simulate herbivory to the host plants by spraying JA (Thaler et al. 2001). Our study provides a clear demonstration of the importance of host responses to damage on parasites and their herbivores, which could impact populations and community composition. Our results further suggest that parasites could use host signals
to obtain information about the host’s environment, potentially priming their own
defenses in anticipation of future herbivory.

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4.7 Notes


Figure 4.1: Damage effects on phytohormone levels in hosts and attached parasites

(A) Host JA, (B) parasite JA, (C) host and parasite SA and (D) host and parasite ABA levels. Host damage significantly increased JA in both hosts and attached parasites. Note the different scale for (A) and (B). Different letters above the bars indicate significant differences between treatments within the host or parasite ($P < 0.05$). Error bars represent standard error.
Figure 4.3: Effects of damage on alkaloid levels of hosts and attached parasites

(A) Lupanine, (B) 13-hydroxylupanine, (C) 17-oxolupanine and (D) unknown lupanine.

Different letters above the bars indicate significant differences between treatments within the host or parasite ($P < 0.05$). Error bars represent standard error.
Figure 4.3: Differences in herbivore consumption and performance feeding on parasites attached to control and damage hosts

(A) Proportion of leaves consumed by larvae and (B) relative growth rate (RGR) of larvae. Different letters above the bars indicate significant differences \((P < 0.05)\). Error bars represent standard error.
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