Evidence that treehoppers induce susceptibility of oak trees to dietary generalist caterpillars

By

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Abstract

Theory on the evolutionary ecology of plant-herbivore interactions is predominantly based on studies of these interactions in isolation from the rest of the ecological community. Yet in real ecosystems, plants are often attacked by multiple species and guilds of herbivores. This reality influences the predictions of plant defense theory, as plants’ inducible resistance traits consist of defense mechanisms that target specific feeding guilds that are attacking them. The induction of anti-herbivore resistance traits is limited by antagonistic crosstalk between two phylogenetically conserved biochemical pathways mediated by the phytohormones salicyclic acid (SA) and jasmonic acid (JA), respectively. The induction of SA-mediated plant resistance by phloem-feeding herbivores has been shown to inhibit JA-mediated inducible resistance against leaf-chewing herbivores, a phenomenon called ‘induced susceptibility’. The existence and broader effects of induced susceptibility in ecological communities are virtually
unstudied. To address this gap, we study the possibility that phloem-feeding herbivores induce susceptibility to leaf-chewing herbivores in Connecticut forests. We hypothesize that induced susceptibility will benefit dietary generalist leaf-chewers more than dietary specialist leaf-chewers because the latter are expected to possess specific counteradaptations to plant resistance traits. We test this induced susceptibility hypothesis for white oak (Quercus alba) and its community of insect herbivores because white oak is frequently co-colonized by the phloem-feeding guild, which largely consists of dietary specialist treehoppers (Membracidae), and the leaf-chewing guild, which consists largely of caterpillars (Lepidoptera) that range widely in dietary specialization. We compared community structure and the growth performance of dietary generalist and specialist caterpillars that fed on white oak leaves that were experimentally manipulated by 1) treehopper removal, 2) JA addition, and 3) inactivation of tannin chemical defenses. Dietary generalist caterpillars were more abundant on branches that were also hosting sap-sucking treehoppers, whereas dietary specialist caterpillars were less abundant. Assays of caterpillar growth showed that treehopper colonization of oak branches increased the growth performance of dietary generalist caterpillars, and this growth benefit was removed by JA addition to experimental branches. In contrast, JA addition did not reduce the growth performance of dietary specialist caterpillars. Experimental inactivation of tannin defenses benefited dietary generalist caterpillar growth, but not that of dietary specialists. Evidence from this study suggests that treehoppers induce localized inhibition in leaf tannin production, thereby inducing susceptibility of white oak to dietary generalist caterpillars.
Introduction

One of the greatest natural challenges to plant fitness is the threat of herbivore assault. The taxa that bear the greatest responsibility for these insistent attacks are the vast array of insect herbivores (Farha-Rehman et al., 2010). Terrestrial plants and the insect herbivores that consume them have shared a long evolutionary history of ecological interaction. The fossil record indicates that arthropod herbivores have been feeding on plant tissues since the late Silurian, long before the evolution of Tetrapods and their colonization of land (Labandeira, 2007). Over the past 417 million years, plants have evolved resistance mechanisms that deter consumption by reducing palatability, decreasing nutritional value, and increasing toxicity (Schoonhoven, Van Loon, & Dicke, 2005). In turn, insect herbivores have adapted to plant resistance mechanisms through co-evolutionary shifts in behavior, morphology, and physiology (War et al., 2012).

Plant-insect coevolution

Coevolution is a course of microevolution in which two or more species become interlocked in reciprocal bouts of natural selection that result in the adaptation of interacting traits (Janzen, 1980). The coevolution that has occurred between plant and insect herbivores has helped to drive an arms race in plant defense traits and herbivore "offense" traits (Ehrlich and Raven, 1964; Karban and Agrawal 2002). Natural selection acts through the relentless onslaught of herbivore attackers that cumulatively reduce plant fitness (Heil and Baldwin, 2012).
This selection from herbivores spurs the adaptive evolution of plant tolerance and resistance traits that decrease the plants’ vulnerability to their natural enemies.

Resistance traits of plant defense have been a focus of this coevolutionary process between herbivores and plants. These traits work to prevent or limit damage from herbivore attack (as reviewed by Baldwin, 1996; Stout, 2013). One of many resistance mechanisms is the production of secondary metabolites (Bennett and Wallsgrove, 1994). These chemical resistance traits primarily function as deterrents or toxins to herbivores (Bryant et al., 1991; Pickett, Simley & Woodcock, 1999; Bruce, 2014). Despite their effectiveness, the evolution of plant secondary chemistry has not provided the most well defended plants a complete reprieve from herbivory. Herbivores have largely overcome plant secondary metabolites with specific counteradaptations, which are typically accompanied by the evolution of dietary specialization (Termonia et al. 2001; Wahlberg, 2001; Zarate, Kempema & Walling, 2007).

**Diet breadth of insect herbivores**

Herbivore dietary specialization is a common evolutionary response to plant resistance traits (Ehrlich and Raven, 1964; Bernays and Chapman, 1994; Cornell and Hawkins, 2003). Although rarely demonstrated in the literature, herbivores that evolve the ability to counter a specific resistance mechanism of their host plant are expected to become less adapted to alternative resistance traits of other host plants (Whittaker and Feeny, 1971; Dyer, 1995; Cornell and Hawkins, 2003; Britt, Hicks & Bennet, 2006; Berumen and Pratchett, 2006; Tremmel and Müller, 2014; Schapers et al., 2016). If true, a consequence of this trade off is that dietary specialists will become ecologically constrained, feeding preferentially on plant species for
which they have specific behavioral, physiological, and morphological adaptations (Futuyma and Moreno, 1988; Paiva, 2000; Sidorovich, Polozov, & Zalewski, 2009, Forister et al., 2012).

Because multiple species of herbivores compete for the same set of plant resources, it is useful to define these consumers within the context of their diet breadth (Morse, and Farrell, 2005; Finlay-Doney, and Walter, 2012). The diet breadth continuum ranges from herbivores that feed exclusively on one plant species to those that feed on dozens of plant lineages (e.g. taxonomic families) (Shipley, Forbey & Moore, 2009; Ruffino et al. 2011; Forister et al., 2015).

Dietary generalist herbivores are not expected to be well adapted in thwarting acute resistance traits, but presumably have the advantage of feeding readily on a whole suite of host plants that are available in the community (Forister et al., 2012; Tremmel and Müller, 2014). This is in stark contrast to dietary specialist herbivores, which typically feed on plant species that are either closely related biochemically or phylogenetically (Futuyma and Moreno, 1988; Ali and Agrawal, 2012). The specialist-generalist paradigm is a conceptual model that compares and predicts an organism’s peak performance based on the number of resources it utilizes. Dietary specialists that exploit the fewest resources are predicted to have a narrower range of optimal performance, yet their maximum performance capability will eclipse that of any dietary generalist. Selection for either dietary specialization or generalization among insect herbivores is pinned to the trade-off between resource accessibility and peak performance. Furthermore the dietary specialization and specific counteradaptations of herbivores generate ongoing selection on plants for more effective resistance (Cornell and Hawkins, 2003; Wise and Rausher, 2013). This coevolutionary process predicts an escalation of antagonistic traits of plants and their specialist herbivores (Ehrlich and Raven, 1964; Petschenka and Agrawal, 2016). It also
predicts ongoing challenges for dietary generalist herbivores in coping with plant resistance traits, such as secondary metabolites (Cornell and Hawkins, 2003).

**Feeding guilds**

Beyond the dietary specialization that results from bi-trophic coevolution, plants must also contend with a variety of herbivore feeding guilds that utilize different feeding niches. Herbivore feeding guilds describe where and how an organism must eat and process food (Root, 1967; Simberloff and Dayan, 1991). Each feeding guild produces a unique form of tissue damage to the plant (DeClerck and Shorthouse, 1985; Carvalho et al., 2014; Walling, 2000). When multiple feeding guilds coincide on the same host plant, the combined damage sustained can have a larger effect on the plant’s fitness (Hougen-Eitzman, and Rausher, 1994). Beyond just increased tissue damage, plants must also cater their defensive response to match each type of attack. When a specific resistance pathway is initiated by a plant, it can have far-reaching effects on other herbivores both temporally and spatially (Denno et al., 2000; Kessler & Baldwin, 2004). These defenses require the plant to divest from growth and reproduction and reallocate these resources to mount multiple resistance pathways (Baldwin and Preston, 1999). It has also been long understood that the secondary compounds that are mounted against one herbivore assault can impart deleterious effects on subsequent attackers (Conrath et al. 2001; Kessler and Baldwin, 2004; Kessler and Halitschke, 2007) However, not until recently has there been an increased interest in how a resistance mechanism against one herbivore guild can have interacting effects on another (Poelman et al., 2010; Schweiger et al., 2014; Sotelo et al. 2014;
de Rijk et al., 2016). Two of the most abundant and vigorously studied insect herbivore feeding guilds are the leaf-chewers and phloem-feeders.

**Phloem-feeding herbivory**

Phloem-feeders are Hemiptera that have piercing-sucking mouthparts that penetrate the plant's surface and vascular system to gain access to the carbohydrate-rich phloem sap (Tjallingii and Esch, 1993; Walling, 2008). The tissue damage caused by the insertion of the herbivore's mouthparts in phloem feeding is acute and localized at the cellular level (Walling, 2000). Plants respond defensively to this damage with a long-lived, systemic pathogenesis related (PR) form of induced resistance that is variable between both plant species and genotype (Zarate, Kempema & Walling, 2007). Typically the secondary chemistry associated with PR plant resistance is either enzymatic (i.e. pathogen degrading enzymes) or oxidative (i.e. reactive oxygen species) (Arimura et al. 2011). Insects that make their living as phloem-feeders experience a high level of host intimacy. For prolonged periods of time large portions of the phloem-feeder's body surface area (e.g. mouthparts and digestive tract) remain directly exposed to plant tissue (Benard-Dagan, 2013). Over extended periods of exposure to defended phloem the reactive oxygen species can result in soft tissue damage to the phloem-feeding herbivore (Walling, 2000). Oxidative stressors and the high degree of host intimacy among phloem-feeders has made PR resistance effective against phloem-feeding herbivores, demonstrated in multiple agricultural systems–(Walling, 2008).

**Leaf-chewing herbivory**
Unlike their phloem-feeding counterparts, externally feeding leaf-chewing insect herbivores do not necessarily share the same degree of host intimacy (Benard-Dagan, 2013). Members of this feeding guild use their sharp sclerotized mandibulate mouthparts to cut and masticate leaf tissue for consumption (Schoonhoven, Van Loon, & Dicke, 2005). This method of consumption limits the leaf-chewer's body surface area that is exposed to only the short period of time when their mandibles are cutting and dislodging foliar tissue (Benard-Dagan, 2013).

The leaf-chewing feeding guild is responsible for creating prodigious amounts of plant damage (Labandeira, 2012). On average, 20% of the total leaf mass in temperate deciduous forests is lost to insect leaf-chewers (Crawley, 1989; Cunningham, Pullen & Collof, 2009). In response to leaf-chewing damage, plants have evolved many resistance mechanisms. These defenses can be constitutively present in the plant's leaf tissue as byproducts and waste material of photosynthesis (Barbehenn and Constabel, 2011). Constitutive secondary compounds most often resist herbivory through aversive taste that inhibits insect feeding behavior and digestibility-reducing toxins that decrease herbivore growth or survival. Some of these compounds are toxins that are mobilized through resistance induction provoked by damage incurred by the different feeding guilds (Kaloshian and Walling, 2005).

**Feeding guilds and the corresponding phytohormones**

The amount and type of tissue damage resulting from herbivory from each feeding guild is perceived by plants through mechanisms that monitor cell death, herbivore specific elicitors, and hydraulic stresses that result from phloem-feeding insects (Walling, 2008). When damage is detected, plants initiate signaling pathways that cause the transcription of a suite of genes
responsible for wound healing and herbivore resistance (Thompson and Goggin, 2006). A large cohort of resistance genes provides the plant with the ability to mount tightly regulated inducible defenses against both herbivore and pathogen alike (Karban and Baldwin, 1997).

The energetic cost that is associated with implementing these resistance traits is high and therefore is a limiting factor in the metabolic budget of the plant (Heil and Baldwin, 2002; Vos, Pieterse and Van Wees, 2013). To minimize the metabolic costs of a resistance mechanism, plants have evolved inducible chemical signaling networks that activate the most energy-demanding defenses only when necessary (Stamp, 2003). Several plant hormones have been identified as the signals that initiate these induced responses. These hormones include jasmonic acid (JA) and salicylic acid (SA), which work in concert to mobilize and suppress complex networks of induced resistance that are responsible for targeting herbivores and pathogens (Rojo, Solano and Sánchez-Serrano, 2003).

**Jasmonic acid**

Jasmonic acid (JA) is responsive to necrotic wounds and the deposition of chemical elicitors sustained from leaf-chewing herbivores (Rojo, Solano and Sánchez-Serrano, 2003; Armura, Ozawa, & Maffei, 2011). When mediating the defensive signaling pathways, JA induces the gene expression of wound response proteins and the downstream production of secondary metabolites (Wasternack and Hause, 2013). JA is responsible for mediating induction of secondary metabolites, including terpenoids, nitrogen-containing compounds, and phenolics (Karban and Baldwin, 1997). Some of these metabolites have been shown to play an active role
in the suppression of growth and development of leaf-chewing insect herbivores, such as larval Lepidoptera (Felton et al., 1989; Ryan, 2000; Chen et al., 2005; Alves et al. 2014).

*Salicylic acid*

The phytohormone SA is similar to JA in that it mediates plant resistance mechanisms; however, SA’s downstream targets are not the same. SA induces a cascade of biochemical reactions that culminate in a plant-wide (root to shoot) systemic acquired resistance (SAR) (Stewart et al., 2016). The SAR is considered an acquired defense because after the initial resistance induction the host plant becomes primed with a hypersensitivity to SA and future induction (Aranega-Bou et al., 2014). Once primed, SA and the SAR pathways regulate the expression of a broad assortment of pathogen defense-related (PR) genes (Uknes et al. 1992; Zarate, Kempema & Walling, 2007). The downstream products of these PR genes are proteins and enzymes that provide a broad-spectrum defense against both invading pathogens and sap-feeding herbivores, which often vector plant pathogens (Durrant and Dong, 2004; Arimura et al. 2011; War, Paulraj, & War, 2011a).

When phloem-feeding insects attack plants with their piercing mouthparts, the tissue damage they produce is similar to that of an assault by fungal hypae (Tjallingii, and Esch, 1993; Zhao et al., 2009 ). It is this microbial-like damage that initiates the SA induction of the pathogenesis response (Durrant and Dong, 2004; Walling, 2008). The increased concentration of the SAR mediating hormone can induce the plant’s resistance within two hours of the initial penetration of the aphid’s mouthparts. Furthermore the resistance has been shown to remain
active for 48 hours after phloem-feeding has been halted, making it a long-lived induction (Walling, 2000).

The orchestration of multiple induced biochemical pathways in the mediation of plant defenses is only partially understood (Chen et al., 2016). The complexity of plant biochemical synthesis makes tracing individual resistance pathways difficult (Depuydt and Hardtke, 2011). The inducible JA and SA hormone signaling pathways that respond to the damage of specific feeding guilds have been well studied in isolation (e.g. Berenbaum and Zangerl, 1994; Mackenzie, 1996; Thaler et al., 1996; Nykanen and Koricheva, 2004; Varenhorst, McCarville, and O’Neal, 2015). Yet, few empirical studies have explored the synergistic and antagonistic interactions that may occur due to simultaneous attacks by multiple feeding guilds (e.g. Stout et al., 1998; Bostock, 1999; Koornneef and Pieterse, 2008; Pieterse et al., 2012; Ohri et al. 2016). Under these conditions plants must adjust and accurately balance their defensive responses to maximize their resistance to the greatest herbivore threat (Walling, 2011). The orchestration of multiple induced biochemical pathways in the mediation of plant defenses is only partially understood (Chen et al., 2016). The complexity of plant biochemical synthesis makes tracing individual resistance pathways difficult (Depuydt and Hardtke, 2011). Plant hormone signaling is a highly conserved trait of plant evolution (War et al., 2012), which has also forced plants to rely on a small set of hormones for numerous functions beyond the induction of resistance traits (Wasternack and Hause, 2013). Downstream responses to phytohormones are often the result of pleiotropy. Multiple hormone networks and resistance pathways have become entangled as a result of gene sharing and overlap in the biosynthesis (Barbehenn and Constabel, 2011). Hormone responses are often dose-dependent and both
synergistic and antagonistic relationships can occur between the same set of hormones (Thaler, Fidantsef, & Bostock, 2002). Crosstalk between hormones refers to a chemical antagonistic interaction in which the induction of one plant resistance response is limited by the induction of other resistance pathways (Harms, Ramirez, & Peña-Cortés, 1998). An ecologically and agriculturally noteworthy example of this crosstalk interaction is the disruption of JA-mediated gene expression by induction of the SA-mediated pathway in response to phloem-feeders (Koornneef and Pieterse, 2008; Chen et al., 2016). While this crosstalk may be adaptive in that it reduces the expression of unnecessary energy-intensive defenses, it may also leave the plant vulnerable to induced susceptibility.

Induced susceptibility is the consequence of hormonal cross-regulation and is the manifestation of induced resistance that no longer matches the current herbivore threat (Zarate, Kempema, & Walling, 2007). When herbivory induces a resistance pathway, a legacy of that resistance remains and acts to thwart later attacks (Denno et al., 2000; Sauge et al., 2006). This legacy of resistance may actually become beneficial to some herbivores under certain situations. As herbivore feeding guilds shift or attack a plant concurrently, the affected host plant may experience a window of vulnerability towards the subsequent attackers (Mewis et al., 2006; Arimura et al. 2011). Because antagonistic chemical cross-talk between SA and JA limits a plant’s induced resistance from responding to both phloem-feeding and leaf-chewing herbivory, simultaneous assault renders the plant unprotected from one of the attacking feeding guilds (Zarate, Kempema, & Walling, 2007; Orhi et al., 2016). Examples of this phenomenon have been documented in many pest-ridden agricultural crops such as soy, tomato and peaches, yet few studies have explored this hormone-mediated defensive loophole.
outside of agricultural systems (Doares et al., 1995; Thaler et al., 1996; Sauge et al., 2006; Varenhorst, McCarville, & O’Neal, 2015).

Previous studies that have investigated induced susceptibility have focused on model organisms and simplified ecological communities, e.g. white radish (Agrawal and Sherriffs, 2001), Arabidopsis (Groen et al., 2013), and wild tobacco (Kallenbach et al., 2012). While plant hormones are highly conserved traits in plant evolution, the networks and interactions they mediate can vary widely among different plant species in different ecological contexts (Karban and Baldwin, 1997; Ju et al., 2015). Due to the plasticity of induction pathways (i.e. changes in plant hormone sensitivity across temporal and spatial periods of development) and great variety of ecological communities in which plants are embedded, it remains unknown how the evidence from model organisms tested under simplified conditions translates to much wider ecological systems. Most model plants are herbaceous and have life history traits that conveniently lend themselves to laboratory experimentation. Large woody plants present challenges to laboratory breeding and experimentation because they require expansive amounts of time and space to reach maturity. Furthermore, their immense vascular networks have made localized inductions difficult to regulate and monitor (Takahashi, 1986). Therefore, the study of induced susceptibility in woody plants requires a different experimental approach than the conventional approaches used for model organisms.

Uncertainty in the generality of the induced susceptibility hypothesis has stemmed from an overall scarcity in the number of projects that address this dynamic feeding guild interaction in trees and forest ecosystems. Because deciduous trees typically support larger insect
communities than their herbaceous counterparts, their induced responses to insect herbivores will potentially have impacts on a larger set of insect species with farther-reaching effects on the whole community (Schoonhoven, Van Loon, & Dicke, 2005). A temporary loss or variation of resistance due to induced susceptibility is predicted to lead to changes in insect behavior and performance (Walling, 2008). It is ecologically important to begin to understand induced responses of trees and the effects of induced susceptibility on larger ecological communities (Nykanen, and Koricheva, 2004).

Here we test the hypothesis that phloem-feeding treehoppers induce susceptibility to caterpillars in white oak (Quercus alba) trees in Connecticut forests. White oak’s primary resistance against leaf-chewing herbivores, such as caterpillars, centers on its capacity to produce and store phenolic compounds as both constitutive and induced defenses (Salminen et al., 2004). The two primary phenolic groups produced by white oak are condensed tannins (CT) and hydrolyzable tannins (HT) (War et al., 2012). In woody plants, both CTs and HTs are under continuous production as consequence of normally occurring Calvin cycle carbohydrate degradation (Barbehenn and Constabel, 2011). The rate of HT production however, can be dramatically increased by JA-mediated pathways in response to leaf-chewing tissue damage (Salminen and Karonen, 2011).

Condensed tannins deter plant consumption by imparting unpalatability and by reducing digestion under certain chemical conditions (Martin and Martin, 1983; War et al., 2012). As oligomers of flavonoids, CTs readily become esters of proteins, making the proteins insoluble and indigestible (Kao, Harding, & Tsai, 2002). Traditional understanding of tannins'
functional role in insect herbivore resistance has drawn many of its inferences from studies that examined the effects on larger browsing mammals (Barbehenn and Constabel, 2011). This body of literature has lent the belief that tannins defend plants against leaf chewing caterpillars by impairing their digestion of plant proteins (Feeny, and Bostock, 1968; Rhodes and Cates, 1976; Hemming and Lindroth, 2000; Lill and Marquis, 2001). While there is strong experimental evidence that CTs are digestibility-reducing mechanisms that decrease ruminant performance, there remains a deficit of empirical support that substantiates this claim for leaf-chewing insects such as caterpillars (Osier, Hwang, & Lindroth, 2000; Osier and Lindroth, 2004). In fact, many chemical ecologists have called into question CT’s ability to bind protein in the alkaline environment of a caterpillar’s mid-gut (Appel, 1993; Salminen, and Karonen, 2011; Barbehenn and Constabel, 2011).

A common misconception that is repeated throughout the literature is that tannins as a whole act in a uniform manner. Not only are tannin biological effects dependent on their molecular structure but they also represent a disproportionate amount of the diversity in the hierarchy of plant secondary chemistry (Hartmann, 2007; War, Paulraj, & War, 2011b; Barbehenn and Constabel, 2011). Their high level of complexity has hindered the development of practical analytic techniques that facilitate the accurate resolution of tannin structure (i.e. hydrolyzable tannins vs. condensed tannins) (Salminen and Karonen, 2011). This has forced most plant-animal interaction studies to take a quantitative approach that only recognizes total phenolics. Within an ecological context, tannin structure is far more relevant than general tannin assays (Barbehenn and Constabel, 2011). This general lack of methodology has allowed the protein-precipitation hypothesis to persist in the scientific culture, despite more than 30
years of conflicting evidence (Martin, Martin, & Bernays, 1987; Appel, 1993; Barbehenn and Costabel, 2011). An excellent review by Salminen and Karonen (2011) has implored both ecologists and chemists alike to reinvent the way we approach the study of tannins and their role in resisting insect herbivory. Instead of addressing tannins as a whole, Salminen and Karonen (2011) propose that specific analytic tools need to become more widely available for the detection of hydrolyzable tannins.

Hydrolyzable tannins are highly unstable oxidative couplings of galloyl groups to a polyol core (War et al., 2012) (Salminen and Karonen, 2011) (Box 1). The chemical fate of HTs is highly dependent on their physical environment. Both pH and redox-potentials play a significant role in the reactive outcome of HT digestion (Vickers et al., 2009). Hydrolyzable tannins become strong pro-oxidizers under alkaline conditions or in the presence of redox-active iron, both of which match the physiochemical environment known to exist in insect herbivore guts (Barbehenn et al., 2005; Barbehenn and Costabel, 2011). Although there remains a deficit in experimental studies that have directly tested this hypothesis, it is this oxidative potential that is now believed to have the strongest deterrent and toxic effects on insect herbivores (Salminen and Karonen, 2011). Theoretical studies predict HTs oxidize in the lumen of the caterpillar’s digestive tract and cause tissue lesions (War et al., 2012). Previous studies that have explored HTs' role in plant resistance have used artificial diets that contain concentrated extracts, which fail to translate to the greater ecological context. Thus, ecologically relevant studies that utilize not only qualitative approaches and natural diets would be helpful to resolve some questions about how tannins defend plants against insect herbivores.
In this study we begin to address this dearth of knowledge by using a novel experimental approach in testing HTs' physiological impact on insect-plant interactions. We attempt to manipulate tannin defenses of oak leaves by treating leaves with polyethylene glycol (PEG), a commonly used industrial and agricultural tannin-binding agent. For over a half-century PEG has been added to livestock diets to remove tannins and aid in protein digestion (Min et al., 2003). However, to the best of our knowledge, PEG had never been used to supplement insect diets. Here we use PEG to artificially inactivate tannins to experimentally knock out the tannin defense of white oak leaves. This new methodology is significant beyond this study because it offers further opportunities to test direct causation between tannins and insect herbivore performance.

**Box 1. Structural chemical diagrams of hydrolyzable and condensed tannin**

![Diagram of hydrolyzable and condensed tannin](image)

(Salminen and Karonen, 2011)

In this study we aim to test if early season phloem-feeding insects induce susceptibility to leaf-chewing herbivores of white oak. If induced susceptibility occurs, we predict that: 1)
herbivory by phloem-feeders would increase the abundance of dietary generalist caterpillars, which are expected to be less well-adapted to the induced defenses of their host plants than dietary specialists; 2) phloem-feeding would improve the food quality of host plants for dietary generalist caterpillars, but not necessarily for dietary specialists; 3) the food quality benefits due to sap-feeding can be removed when JA is artificially added; 4) the induced resistance mediated by JA will have stronger effects on dietary generalists than dietary specialists; and 5) the negative effects of JA on dietary generalists is due to the induction of tannin production in leaves. We test these predictions with field and laboratory experiments, the latter consisting of feeding assays that include caterpillars of varying degrees of dietary specialization.

**Methods**

*Plant Community*

While the deciduous forests that make up the three field sites are taxonomically well mixed, the focal plant species of the present study is *Quercus alba* (white oak). As a member of the Fagaceae family, white oak is a long lived, slow growing intermittently shade tolerate tree (Little, 1980; Burns, and Honkala, 1990; Petrides, and Wehr, 1998). With its tall broad crown it remains a co-dominant member of the upland forests of Connecticut (Little, 1980). Despite its recent decline in numbers, the white oak's massive seed banks fortify against disturbance and forest secession (Abrams, 2003). White oaks waiting for light gap opportunities can persist as saplings for a century in the understory before they experience release (Jeffries, Marquis, and Forkner, 2006). White oak's ability to endure is partially attributed to its prodigious root
system. A deep tap root provides white oaks the tolerance to a wide range of conditions; however, they perform optimally in dry well drained soils (Walker, 1991).

White oak is a favorable species for the study of induced susceptibility as it is host to a diverse herbivore community (Jeffries, Marquis, and Forkner, 2006). Furthermore, oaks are known for their tannins as anti-herbivore defenses (Ostrofsky, 1993). White oak especially has one of the highest tannin concentrations of all native New England tree species. Foliar tannins account for 16.2% of white oak's dry leaf weight, double the average of other local tree species (Suberkropp, Godshelk & Klug, 1976). Of these tannins, the inducible hydrolyzable ellagintannins are the most abundant during late spring and early summer (Salminen et al., 2004; Yarnes et al., 2006). This peak in herbivore resistance coincides with a window of overlap between two insect herbivore feeding guilds: leaf-chewing larval Lepidoptera and phloem-feeding treehoppers (Family: Membracidae).

*Caterpillar community*

The current study’s focus is on larval Lepidoptera that feed externally on the foliage of white oak. To date, observations by Singer and Wagner have identified 72 local caterpillar species representing 9 families of Lepidoptera that have been confirmed as natural consumers of white oak (M.S. Singer, D.L. Wagner, unpublished data). Caterpillars, like other members of the leaf-chewing feeding guild, range widely in their degree of dietary specialization. For the purpose of this study, we classified dietary specialists as caterpillar species limited to feeding and developing on the leaves of host trees in the oak family (Fagaceae). In contrast, dietary
generalist caterpillar species feed on leaves from one or more tree families in addition to Fagaceae.

**Treehopper community**

Treehoppers (Family Membracidae) are a diverse group of phloem-feeding insects that inhabit all of the continents besides Antarctica. In the northeastern United states as many as 23 species have been recorded to feed on white oak (Wallace, 2008), seven of which have been recorded at our study sites (R.E. Clark, unpublished data). Similar to aphids, treehoppers have piercing-sucking mouthparts that allow them to tap into the phloem sap of their host plants.

Treehoppers are flightless until adulthood. Once mature, female treehoppers oviposit their eggs into slits that they form in the cambium tissue of their host tree (Wallace, 2008). Eggs overwinter and hatch in the spring directly following bud break and leaf expansion (Wood, 1993; Lopes, 1995). In late May and early June, treehopper nymphs are regularly observed feeding on the expanding shoots (leaves and stems) of white oak (M. Comerford, personal observation). This behavioral aspect of their ecology constrains the earliest portions of their life history to localized areas of their host branch, which lends itself well to experimental manipulation.

Past studies have demonstrated that treehopper feeding and oviposition activates the induction of the SA resistance pathway in other species of tree (Wallings, 2008). While no study has specifically tested this in white oak, we hypothesize the same is true for white oak. Besides the activation of induced resistance traits, treehoppers are known to attract predators, which elevate the predatory risk for other insect herbivores (Styrsky and Eubanks, 2007). As an
adaptive response to the high amounts of water and sucrose in tree phloem, treehoppers frequently excrete a concentrated sugar solution called honeydew. Insectivorous predators, such as ants, wasps, and birds, take advantage of the undefended sugar source in a food-for-protection mutualism (Douglas, 2006; Morales and Beal, 2006). Ants, in particular, tend and protect treehoppers in exchange for honeydew. Tending is a form of foraging in which the ant removes other potential competitors from the area in order to obtain sole access to the honeydew (Grinath et al. 2012). Tending is beneficial to both ant and phloem-feeder. Ants gain an essential resource while the phloem-feeders are allowed to feed unmolested by their natural enemies (Way, 1963). However, caterpillars, can be negatively affected by the increased density of ants that accompany this interaction (Zhang, Zhang, & Ma, 2011). When tending, ants actively remove the entire arthropod community from the local branch including caterpillars (Styrsky, and Eubanks, 2007; Rudgers, and Clay, 2008). The larger caterpillars are simply dislodged from their feeding sites, while smaller caterpillars may be killed and brought back to the nest for consumption (Sendoya, Oliveira, & Hambäck, 2015). Therefore when considering the net effect of treehopper-mediated induced susceptibilities on caterpillars it is important to account for ant tending behavior. For this reason, it is necessary that branches be experimentally isolated and protected from predators to isolate interactions between treehoppers and caterpillars, the subject of this study.

Methodology

Field observations of associations between caterpillars and treehoppers
To test for positive associations between phloem-feeding herbivores and the abundance of dietary generalist caterpillars in the field (Prediction 1), we analyzed data on caterpillar abundance and treehopper presence/absence from a predator-exclusion field experiment conducted in 2011 and 2012. Ants and birds were excluded from tree branches to measure the effect of ant and bird predation on dietary generalist and specialist caterpillars. Treehoppers and other phloem-feeders were recorded as being present or absent, but they were not experimentally manipulated. Although the design included multiple tree species (Lichter-Mark et al., 2015, Clark et al, in review), here we use the data from white oak branches (N=133) that were distributed among three field sites in Middlesex County, Connecticut: Cockaponset State Forest (Haddam), Hurd State Park (East Hampton), and Millers Pond State Park (Durham). Each site was separated by at least 10 km (Singer et al. 2012). A sticky resin barrier (Tanglefoot, Contech Inc.) was used to prevent ants from gaining access to the branch on half of the branches in both 2011 (N = 30) and 2012 (N = 36). Additionally, bird barriers in the form of fine nylon netting were placed over 30 of the branches in 2011. There were four experimental treatments in 2011: bird-exclusion/ant-exclusion, bird-exclusion/ant access, bird access/ant-exclusion, bird access/ant access. The factorial design in 2011 meant that ant and bird effects could be analyzed for additive (independent) and interactive effects. There were two experimental treatments in 2012: ant-exclusion, ant access (controls). Each branch was sampled twice during each year. The number of caterpillars of each species and the number of sap-feeding herbivores were recorded in each sample.

We implemented three Poisson Generalized Linear Models (GLMs) to analyze total caterpillar abundance, dietary generalist caterpillar abundance, and dietary specialist caterpillar
abundance, respectively, with the following predictor variables: 1) sap-feeder presence, 2) year, 3) branch leaf area, 4) field site, 5) sample period, 6) ant exclusion, and 7) bird exclusion.

Because predation was a strong predictor of caterpillar abundance, we ran the same models using only branches that were ant-excluded minus predictor variable 6. All statistical tests were performed using JMP (version 11 pro; SAS Institute, Carry, NC.). The deviances were scaled to correct for overdispersion using a Poisson distribution function, and $\chi^2$ was used for analysis of significance.

_Caterpillar rearing for experiments 1-3_

The caterpillars that were used for feeding experiments were reared from egg clutches that were laid by wild-caught gravid female moths (collected April-June 2015). A list of pre-selected taxonomically closely related Lepidoptera target species that represented a range of dietary specialization was created using field guides and diet breadth studies conducted by Singer, and Wagner (Wagner, 2005; Wagner et al. 2011). Female moths were captured at night using mercury vapor light and bait traps at locations throughout southern New England and North Carolina. Field guides and local lepidopterists (David Wagner, Michael S. Singer, and Eric Quinter) were used to positively identify moths at the species level (Wagner, 2005; Beadle and Leckie, 2012). Females that represented targeted species were transferred to 0.5 l glass rearing jars (Ball inc. Bloomfield, CO.) with ventilated lids. To facilitate oviposition, leaf debris and wax paper strips were included in the rearing jars as a laying substrate (Walker and Allen, 2010). For the duration of oviposition, moths were provided nectar food sources and kept at ambient laboratory temperature to prevent thermal disruption of the oviposition period (Stamp and
Bowers, 1994; Raitanen et al. 2014). After oviposition was complete, moths were removed and local moths were released, while the North Carolinian moths were culled by freezing. When eggs hatched, neonate larvae were transferred into plastic specimen jars (BioQuip products, Inc.) by groups of four. Caterpillars were maintained on a plentiful diet of fresh field-collected *Q. alba* leaves. To reduce any food competition between siblings, leaves were replaced daily. Prior to each 24-hour feeding period the partially consumed leaves and the day’s accumulation of frass were removed to prevent disease. Caterpillars were housed individually in jars when they reached a body mass greater than 30 milligrams.

The fresh *Q. alba* leaves were harvested daily from sapling trees that populate a newly established stand of trees adjacent to Wesleyan’s Long Lane Farm (Middletown, CT). This site was selected to minimize the confounding effects that may be associated with pesticide loads that are pervasive in many urban forests due to the high degree of fragmentation (Dormann, et al., 2007). In addition, this site’s white oak trees hosted a minimal herbivore load (i.e. treehoppers and caterpillars) providing us with a source of relatively undamaged and presumably non-induced leaves for collection. Leaf collection was accomplished using a pair of clipping shears to excise young visually undamaged leaves along the petiole to avoid inducing a chemical defense in the leaf (Sutter, and Muller, 2011; Tang, Zhao, and Gao, 2013).

*Experiment 1: Effects of treehopper removal and JA addition on the growth of a dietary generalist caterpillar*

To test if sap-feeding by treehoppers improves plant quality specifically for dietary generalist caterpillars (prediction 2) we manipulated naturally occurring treehopper
communities on replicated white oak branches located at two field sites (Cockaponset State Forest, Hurd State Park, Middlesex County, Connecticut). We factorially manipulated these branches with treehopper removal and jasmonic acid (JA) addition to test if the plant quality benefits associated with treehoppers would be removed when JA was added (prediction 3). The first factor of this 2 x 2 factorial design was the treehopper removal and replacement treatment. The presence of feeding treehoppers is predicted to provide a window of induced susceptibility for other herbivore feeding guilds. If true, physically removing these treehoppers (Treehopper removed) should diminish the effect of induced susceptibility. To identify experimental white oak branches, the arthropod community was beat-sampled white oak branches within reach (1-2 m above ground). A stick was used to gently beat the branch for two ten-second intervals. White 1 m² collection sheets (BioQuip inc.) were placed directly under the branch during the beating procedure to capture any dislodged arthropods. Upon visual observation of the collection sheets, branches identified as hosting treehopper nymphs were selected to be included in the experiment. Each treehopper-removal branch was paired with a branch with similar proximity and ecology. Control branches (Treehopper replaced) had three of their dislodged treehoppers replaced by hand, allowing for any possible induced susceptibility to continue. The second factor of this manipulation was the addition of jasmonic acid (JA). A 0.5 mM solution of (±)-Jasmonic acid BioReagent (Sigma-Aldrich co. St. Louis, MO) suspended in a 99% aqueous solution of EtOH was sprayed onto the treatment branches (JA+). The addition of JA is intended to force induction of plant defenses that are normally induced by leaf-chewing herbivores and override any induced susceptibility provided by the treehopper manipulation. Each JA+ branch was paired with a control branch (JA-) that was selected for
ecological similarity and spatial proximity to the treatment branch. JA- branches were sprayed with only 99% EtOH. Each branch received seven sprays applied to the underside of the branches to prevent the treatment from being washed away in subsequent rains.

Each experimental branch was stocked with one lab-reared 4th instar caterpillar that ranged from 80-230 mg to induce the JA pathway and mobilize defensive plant compounds such as hydrolyzable tannins. A protective 55 x 70 cm$^2$ nylon mesh bag (BioQuip Products, Inc.) that ties at both ends and zips along its long axis was used to cover the branch for the remainder of the five-day manipulation period. The bag served the dual purposes of preventing re-colonization of the branch by herbivores and predators while confining our experimental arthropod community to the branch. After five days, we returned to the branches and removed any undamaged leaves for use in laboratory assays (see below). Only branches that still maintained their stocked herbivore community were included in the experiment. Leaf collection was performed by clipping the petiole of the leaf with garden sheers to prevent any further induction due to leaf damage. Leaves were packaged in plastic 3.785 L bags (Ziploc; S.C. Johnson and Sons inc.) between damp sheets of paper towel. The bags were stored in a cooler with ice for transport from the field to the laboratory.

Growth assay protocol

The experimental leaves were then fed to lab-reared larval Himella fidelis, an abundant dietary generalist caterpillar in Connecticut that feeds on tree species in at least ten families, including Fagaceae (Wagner, 2005). The 3$^{rd}$ to 4$^{th}$ instar caterpillars were starved for 48 h prior to the growth assay. This starvation period ensured that each caterpillar cleared its gut
contents prior to the experiment and increased its readiness to feed at the start of the growth assay. At the close of the starvation period caterpillars were weighed using an electronic scale (Sartorius; Data Weighing Systems inc. Elk Grove, IL.) to the nearest thousandth of a gram to determine the initial mass. The caterpillars were then placed individually into 10 cm diameter x 7.4 cm depth plastic feeding arenas (Fabri-KAL Corp. Kalamazoo, MI). A test-tube was filled with 8 ml of DI water and secured to the side of the feeding arena to maintain humidity. An experimental Q. alba leaf was placed into the feeding arena and the container was covered and stored at ambient laboratory temperature. Each experimental leaf was replaced after 24 h. Each growth assay trial ended at 48 h, whereupon the experimental leaf was removed and the caterpillar starved for another 48 h before being weighed again to determine the final mass. We calculated growth rate as the final mass minus the initial mass.

Experiment 2: Test of JA addition on growth of dietary generalist and specialist caterpillars.

This experiment tests prediction 4 if our experimentally manipulated JA addition was, in fact, inducing plant resistance against caterpillars, and that this induction affected dietary generalist caterpillar more than specialists. To test this, we conducted both laboratory and field-based feeding assays on leaves from branches that were manipulated with JA additions. The branches utilized for this feeding assay were the branches that were found not to host treehoppers in our original branch beating sample. Once identified, each branch received the same JA or control spray manipulation as in experiment 1. As before, branches were protected from predation and competition with mesh nylon bags. Experimental branches were then stocked with a single caterpillar to induce resistance. We used the stocked induction
caterpillars for the field-based growth assay. Five species of inducer caterpillars were employed across this assay (Table 1).

The same species of caterpillars were included in laboratory growth assays that utilized leaves collected from the field growth assay. Caterpillar species that were incorporated in this experiment represented several points along the diet breadth spectrum (3 dietary generalists, 2 specialists). As in experiment 1, each experimental caterpillar was starved for 48 h before and after the growth assay, and weighed to obtain its initial and final mass. The growth assay was conducted in the same manner is in experiment 1. We calculated each caterpillar's growth rate during the assay by subtracting the initial mass from the final mass.

**Experiment 3: Tannin binding experiment**

To test if the negative effect of JA addition on the dietary generalist is due to induction of tannins in leaves (prediction 5) we conducted caterpillar growth assays using JA-induced leaves treated with a PEG (a tannin binding agent). While it is possible to use signal inhibitors that block tannin production, this method could elicit unintended downstream consequences in plant resistance networks (Lortzing and Steppuhn, 2016). Instead we have chosen to inactivate tannins in vivo during the feeding and growth assays. PEG is known to form strong insoluble complexes with tannins and other poly acids resulting from the direct esterification of hydroxyl and carboxyl functional groups (Makkar, 2003; Shirazi, Imani & Sharifi, 2011; Etuk et al., 2012). Besides its ability to bind tannins, PEG is highly biocompatible. Industry commonly utilizes PEG in medicine and agriculture because it is inert and has a low level of toxicity (Shirazi, Imani & Sharifi, 2011).
In the current study, we conducted a growth assay on three species of laboratory-reared caterpillars. Each species occupied its own unique rung of the diet breadth spectrum. *Acronicta impleta* (Noctuidae) is a dietary generalist; *Nadata gibbosa* (Notodontidae) is a Fagaceae specialist; *Heterocampa umbrata* (Notodontidae) is a strict oak specialist that is known to feed on different *Quercus* species, but does not feed on other members of the Fagaceae family, such as American beech (*Fagus grandifolia*). Each caterpillar was included in the same feeding assay as the previously described experiment 2: test of JA addition on growth of dietary generalist and specialist caterpillars. However, in this experiment an additional treatment was added in a factorial manner. Besides feeding on control and JA-induced leaves, a subset of each species of caterpillar also fed on these treatment leaves bathed in solution of 1% Poly(ethylene glycol) Bio Ultra 4000 (Sigma Aldrich co. St. Louis, MO.). The purpose of the bath is to coat the leaves in PEG so that upon consumption PEG binds the tannins while in the digestive tract of the caterpillar.

*Determination of concentration of a novel treatment (PEG)*

Prior to the experiment we conducted a preliminary feeding assay on a group of caterpillars collected from the wild. The intent of this feeding assay was to determine a concentration of PEG large enough to inactivate tannins and improve caterpillar growth, but small enough to not inhibit feeding or caterpillar survival. This preliminary study was necessary because no previous study has supplemented PEG in any insect diet. Over a one-week feeding assay, 60 caterpillars fed on leaves collected from the same branch. Each caterpillar was randomly assigned to one of six PEG treatment groups. For each treatment group, the
caterpillars fed on leaves that underwent a 30-second bath in one of several concentrations of PEG in DI water. The first group fed on leaves bathed in 20% PEG solution. Each subsequent treatment group fed on leaves that bathed in an incrementally decreasing concentration of PEG solution (10%, 5%, 1%, 0.5%). The final group of caterpillars was fed a control diet of the collected leaves bathed in only DI water. Caterpillar survival and the mean mass gain were analyzed using ANOVA. From this analysis the 1% solution was determined as the optimal concentration to increase caterpillar growth and assure survival.

*Analysis of caterpillar growth in experiments 1-3*

All statistical tests were performed using JMP (version 11 pro; SAS Institute, Cary, NC). In the analysis of growth assays, each statistical model included the mass gain of caterpillars as the dependent variable. In addition to experimental treatments, each model included the mass of caterpillar feces (frass) and initial mass of caterpillars as independent variables because the former indicates the amount of leaf material consumed and the latter determines the potential consumption rate. Each model also included field site as an independent variable because local field conditions, such as soil or microclimate may affect the food quality of leaves fed to caterpillars during the assay. Temperature, moisture and sunlight have all been shown to influence leaf chemistry (Shimizu et al., 2005; Meier and Leushner, 2008). Finally, each model included trial period as an independent variable because of differences in the timing of assays through the growing season that might affect leaf quality. As we did not collect leaves from branches at the same time intervals, some branches experienced longer induction periods than
others. Similarly, the timing of assays and leaf collection could have been influenced by seasonal changes in leaf quality (Wold and Marquis 1997).

To test effects of treehopper removal and JA addition on mass gain by the dietary generalist *Himella fidelis*, analysis of variance (ANOVA) was used (mass gain was evenly distributed and an F-test was used in analysis of significance). The ANOVA modeled mass gain as a function of treehopper removal, JA addition, frass mass, the initial caterpillar mass, field site, and all two-way interactions test if there is any synergistic effect from the interaction of independent variables. The least-square means were compared using standard error for effect size.

To test the effect of JA addition on the growth of dietary specialist and generalist caterpillars in laboratory assays, ANOVA was used to analyze the mass gain of caterpillars as a function of JA addition, initial mass, field site, trial period, and the frass mass. To test the effect of JA addition on caterpillar growth in the field assays, we used the same model except that frass mass was not included as an independent variable because we did not collect caterpillar frass in field assays. To test the effects of tannin binding (PEG) and JA addition on caterpillar growth, we used ANOVA analyzing the mass gain of caterpillars as a function of JA addition and PEG application, the JA x PEG interaction, trial period, initial caterpillar mass, field site, frass mass and trial period.

In all statistical models analyzing caterpillar growth, a simplification of factor levels was performed by using an F-test to evaluate whether combining factor levels into a single level resulted in a significant increase in deviance of the model (Grandison, Piper, & Partridge, 2009).
In addition, a Student’s t-test comparing the least square means of caterpillar mass gain was conducted on all applicable analyses. This post hoc analysis was chosen in response to limited sample size. A more conservative Tukey test was utilized on models that had sample sizes greater than 10 caterpillars per treatment group.

Results

Field observations of associations between caterpillars and treehoppers

In 2011 and 2012, there was a 21% increase in caterpillar abundance on white oak branches in the presence of treehoppers compared to caterpillar abundance in the absence of treehoppers (Fig. 1; Poisson GLM, $P = 0.0316$; Table 1). Dietary generalist caterpillars ($N = 269$) far outnumbered dietary specialist caterpillars ($N = 35$) in both their overall abundance and population density. Indeed, the increased abundance of caterpillars in the presence of treehoppers resulted from elevated abundance of dietary generalist caterpillars (Fig. 2.; Poisson GLM, $P = 0.0357$; Table 2), consistent with prediction 1 of the induced susceptibility hypothesis. In contrast, dietary specialist caterpillars showed reduced abundance in the presence of treehoppers (Fig. 3.; Poisson GLM, $P = 0.0234$; Table 3), a pattern that disappears when the analysis includes branches that have had the ant community excluded (Poisson GLM, $P = 0.365$). The latter result suggests that the negative association between treehoppers and dietary specialist caterpillars is an indirect effect that is mediated by ants.

Test of treehopper removal and JA addition on growth of dietary generalists
In the laboratory assay testing predictions 2 and 3 of the induced susceptibility hypothesis, the interaction between treehopper removal and the addition of JA varied the growth rate of dietary generalist caterpillars (*H. fidelis*) that consumed experimentally manipulated leaves (ANOVA Treehopper removal x JA addition, $F_{1, 42} = 4.412, P = 0.0452$; Table 4). The Tukey test showed that caterpillars only experienced the predicted increased mass gain on treehopper replacement branches in the absence of JA (Fig. 4), indicating that the JA addition nullified the induced susceptibility effect.

**Test of JA addition on growth of dietary specialists and generalists**

In the field, JA addition had no significant effect on dietary generalist caterpillar growth (ANOVA, $F_{1, 30} = 0.1305, P = 0.7214$; Table 5). This was also true for dietary specialist caterpillars (ANOVA, $F_{1, 33} = 0.1563, P = 0.6959$; Table 6). There was an observed growth effect in response to JA addition in the laboratory feeding assays, and this effect was diet-breadth specific (Fig. 5). The oak specialist, *Herterocampa umbrata*, experienced a positive growth effect from the addition of JA (ANOVA, $F_{1, 22} = 6.6574, P = 0.0218$; Table 6). No significant effect was observed on the growth of the Fagaceae specialist, *Nadata gibbosa* (ANOVA, $F_{1, 56} = 1.687, P = 0.2007$; Table 6) Conversely, the addition of JA had a significant negative effect on the dietary generalist, *Acronicta impleta* (ANOVA, $F_{1, 23} = 10.9663, P = 0.0079$; Table 6).

**Tannin binding experiment**

The addition of PEG in the diets of caterpillars showed evidence that tannins in white oak leaves provide resistance against dietary generalist caterpillars but not against dietary specialists. As predicted, the mean mass gain of the most dietary generalized caterpillar of the
feeding assay experiment (*A. impleta*) was increased by the PEG treatment (Fig. 6; ANOVA, $F_{1,23} = 6.9045, P = 0.0253$; Table 7). As found previously, JA addition reduced the growth rate of the dietary generalist (Table 8). There was however, no interaction between the PEG and JA treatments on the growth rate of *A. impleta* (ANOVA, $F_{1,19} = 0.5152, P = 0.8262$; Table 7), indicating that effects of tannins and JA acted independently (Fig. 6). In contrast, the PEG and JA treatments had no effect on the growth of the Fagaceae specialist caterpillar, *N. gibbosa* (ANOVA, $F_{1,59} = 0.7714, P = 0.3345$ (Fig. 7, Table 8). There was also no significant JA x PEG interaction effect on growth (Table 7). The Student’s t-test showed no difference in mean mass gain between treatments (Fig. 7). Likewise, the PEG treatment had no effect on the growth of the oak specialist caterpillar, *H. umbrata*, either independently or interactively (Fig. 8, Table 7). However, JA addition increased oak specialist growth independently (Fig. 5). The Student’s t-test that followed showed that only control branches treated with H$_2$O differed significantly from the JA branches treated with PEG (Fig. 8).
Figures and Tables

Table 1. Poisson GLM of total caterpillar abundance per branch

<table>
<thead>
<tr>
<th>Term</th>
<th>$\chi^2$</th>
<th>df</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site</td>
<td>0.424</td>
<td>1,2</td>
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<td>Sample period</td>
<td>0.0179</td>
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<td>Year</td>
<td>60.223</td>
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<td>Treehoppers</td>
<td>4.621</td>
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<td>0.0316*</td>
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<td>Ant exclusion</td>
<td>6.717</td>
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<td>0.0095*</td>
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<td>Bird exclusion</td>
<td>70.363</td>
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<td>&lt;0.0001*</td>
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<tr>
<td>Leaf area</td>
<td>18.062</td>
<td>1,130</td>
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$N = 261$ samples of insect community from 130 branches. * for $P<0.05$
Table 2. Poisson GLM of dietary generalist caterpillar abundance per branch

<table>
<thead>
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<th>Term</th>
<th>$\chi^2$</th>
<th>Df</th>
<th>P</th>
</tr>
</thead>
<tbody>
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<td>Site</td>
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<tr>
<td>Treehoppers</td>
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<td>Bird exclusion</td>
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<td>Leaf area</td>
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N=261 samples of insect community from 130 branches

Fig. 2. Representation of treehoppers’ effects on dietary generalist caterpillar abundance. Bar height is the least-square mean of caterpillar abundance per branch and error bars show standard error of the mean.
Table 3. Poisson GLM of dietary specialist caterpillar abundance per branch

<table>
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<th>Term</th>
<th>All branches</th>
<th>Ant excluded branches only</th>
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<tbody>
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<td></td>
<td>$\chi^2$</td>
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<tr>
<td>Site</td>
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<td>Sample period</td>
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<tr>
<td>Year</td>
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<td>Ant exclusion</td>
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All branches $N = 261$, Ant excluded branches only $N = 130$

Fig. 3. Representation of treehoppers effects on dietary specialist caterpillar abundance. Bar height is the least-square mean of caterpillar abundance per branch and error bars show standard error of the mean.
Table 4. ANOVA on growth rate of *H. fidelis* (dietary generalist)

<table>
<thead>
<tr>
<th>Term</th>
<th>F</th>
<th>df</th>
<th>P</th>
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<tbody>
<tr>
<td>JA addition</td>
<td>8.7365</td>
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<td>0.0064*</td>
</tr>
<tr>
<td>Treehoppers</td>
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<td>0.0239*</td>
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<tr>
<td>Site</td>
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<td>0.6745</td>
</tr>
<tr>
<td>Initial mass</td>
<td>13.687</td>
<td>1,41</td>
<td>0.0010*</td>
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<tr>
<td>Frass</td>
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<td>JA x Treehopper</td>
<td>4.4119</td>
<td>1,41</td>
<td>0.0452*</td>
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*N = 42 caterpillars included in growth assay.*
Table 5. ANOVA of growth of dietary generalist caterpillars in field experiment

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<tr>
<td>Trial duration</td>
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<td>Site</td>
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<tr>
<td>JA addition</td>
<td>0.0069</td>
<td>1,23</td>
<td>0.9349</td>
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</table>

N = 23 caterpillars included in growth assay. Species included in the assay: *Acronicta impleta*, *Acronicta americana*, *Schizura ipomeae*.

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Table 6 ANOVA of growth of dietary specialist caterpillars in field experiment

<table>
<thead>
<tr>
<th>Term</th>
<th>$F$</th>
<th>df</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial mass</td>
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<td>1,32</td>
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<tr>
<td>Trial duration</td>
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<tr>
<td>Site</td>
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<td>1,32</td>
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</tr>
<tr>
<td>JA addition</td>
<td>0.5002</td>
<td>1,32</td>
<td>0.4852</td>
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</table>

N = 33 caterpillars included in growth assay. Species included in assay: *H. umbrata*, *N. gibbosa*.
Fig. 5. JA addition’s effect on the growth of a dietary generalist (*A. impleta*), b Fagaceae specialist (*N. gibbose*), and c oak specialist (*H. umbrata*). Bar height is the least square means of caterpillar mass gain and the error bars show standard error of the mean. The numbers at the base of the column describe the number of caterpillars included in the feeding assay. *P<0.05  **P<0.01"
### Table 7. ANOVA on caterpillar growth in experiment 3

<table>
<thead>
<tr>
<th>Term</th>
<th>Dietary generalist</th>
<th>Fagaceae specialist</th>
<th>Oak specialist</th>
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<tbody>
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<td></td>
<td>F</td>
<td>df</td>
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<td>JA addition</td>
<td>10.966</td>
<td>1,18</td>
<td>0.0079*</td>
</tr>
<tr>
<td>PEG</td>
<td>6.905</td>
<td>1,18</td>
<td>0.0253*</td>
</tr>
<tr>
<td>Initial mass</td>
<td>3.882</td>
<td>1,18</td>
<td>0.0771</td>
</tr>
<tr>
<td>Trial length</td>
<td>5.5369</td>
<td>1,18</td>
<td>0.0404*</td>
</tr>
<tr>
<td>Frass</td>
<td>19.969</td>
<td>1.18</td>
<td>0.0012*</td>
</tr>
<tr>
<td>Site</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>JA x PEG</td>
<td>0.5152</td>
<td>1,18</td>
<td>0.4893</td>
</tr>
</tbody>
</table>

Dietary generalist is *A. impleta* (*N* = 19); Fagaceae specialist is *N. gibbosa* (*N* = 60); Oak specialist is *H. umbrata* (*N* = 22).
Fig. 6. Treatment effects of a JA addition and b tannin binding manipulation, on the growth of the dietary generalist caterpillar *A. impleta*. Bar height is the least square mean of caterpillar mass gain and the error bars show the standard error of the mean. The numbers at the base of the column describe the number of caterpillars included in the feeding assay.
Fig. 7. Treatment effects on the growth of Fagaceae specialist *N. gibbosa*. Bar height is the least—square mean of caterpillar mass gain and error bars show standard error of the mean. The number at the base of the column describes the number of caterpillars included in the feeding assay. Letters above the error bars correspond with Tukey test. Columns not sharing letters are significantly different.
Fig. 8. Treatment effects on the growth of oak specialist *H. umbrata*. Bar height is the least-squares mean of caterpillar mass gain and error bars show standard error of the mean. The number at the base of the column describes the number of caterpillars included in the feeding assay. Letters above the error bars correspond with Tukey test. Columns not sharing letters are significantly different.
**Discussion**

Our results support the hypothesis that phloem-feeding herbivores induce susceptibility to herbivory by dietary generalist caterpillars in white oak trees. First, the prediction that herbivory by phloem-feeders would increase the abundance of dietary generalist caterpillars was substantiated by observational evidence from 2011 and 2012. Second, a laboratory growth assay of a dietary generalist caterpillar supports the prediction that phloem-feeding would improve the food quality of host plants for dietary generalist caterpillars, but not necessarily for dietary specialists. Third, this growth assay confirmed the prediction that the food quality benefits due to sap-feeding can be removed when JA is artificially added. Fourth, evidence from controlled laboratory feeding assays supported the prediction that the induced resistance mediated by JA will have stronger effects on dietary generalists than dietary specialists. Lastly, laboratory growth assays found partial support for the prediction that the negative effects of JA on dietary generalists were due to the induction of tannin production in leaves.

*Caterpillar Abundance*

We found observational evidence that caterpillar abundance on white oak increased in the presence of treehoppers (Fig. 1). This increase in abundance resulted from the predicted increase in dietary generalist caterpillars and not dietary specialists (Tables 2, 3). The increase in the generalists' abundance is thought to be the consequence of increased performance due to elevated plant quality (Slansky, 1993). It is likely that mobile dietary generalist caterpillars accumulate in areas of high-quality food as they feed more and disperse less often than in areas of low-quality food. This mechanism is not only supported by our own feeding assays but is
consistent with previous studies (Ayres et al. 1997; Wold and Marquis 1997). In contrast to dietary generalists, treehoppers may have indirectly reduced the abundance of dietary specialist caterpillars. According to our results, predatory ants mediate this negative association between treehoppers and dietary specialist caterpillars. The abundance of dietary specialist caterpillars was not affected by the presence of treehoppers when caterpillars were not subjected to ant predation (Table 3). However, studies have repeatedly demonstrated that ants are recruited to branches where honeydew is provided as a reward (Way, 1963; Wimp and Whitham, 2001; Morales and Beal, 2006; Yoo and Holway, 2011; Grinath, 2012). The branches in the 2011-2012 study had a greater number of ants on branches that also hosted treehoppers (Clark et al., in review). Our analysis demonstrated that these ant-rich treehopper branches also had fewer dietary specialist caterpillars (Table 3), suggesting that deterrence or predation by ants was responsible for the reduced abundance of specialists in the presence of treehoppers.

Treehopper-mediated induced susceptibility

Our experimental evidence supports the hypothesis that treehoppers induce susceptibility to dietary generalist caterpillars on white oak. When dietary generalist caterpillars consumed leaves collected from branches that hosted treehoppers (treehopper replaced), they experienced increased growth rates compared to caterpillars that fed on branches with treehoppers removed (Fig. 4), presumably because treehoppers' induction of the SA pathway inhibited caterpillars' induction of the JA pathway. The increased caterpillar performance indicates higher quality leaves (i.e. more nutritious and/or less chemically defended) on branches with treehoppers. Results of JA addition suggest that the increase in host-plant quality
was due, at least in part, from a reduction in induced chemical resistance. The JA addition removed the elevated performance of the dietary generalist caterpillars that fed on leaves from treehopper replacement branches (Fig. 4, Table 4), presumably by inducing chemical resistance despite putative crosstalk from SA-mediated induction by treehoppers. These findings are consistent with the inhibition of JA resistance due to the induction of SA in other studies (Dores et al., 1995; Stout et al., 1998; Schweiger et al., 2014). While our study did show caterpillar growth patterns that matched the predicted outcome, the effect of treehopper-mediated induced susceptibility was short lived, as there was no effect of JA addition on treehopper removal branches (Table 4). This implies that the feeding by the stocked caterpillar was enough to induce the JA pathway within five days of treehopper removal. Because all of the branches included in this experiment had hosted treehoppers directly prior to experimental treatments, the levels of SA must have dissipated enough over the five-day field trial so that SA-JA crosstalk disappeared.

*JA additions' effect on dietary generalist and specialist caterpillars*

Our experimental evidence from caterpillar growth assays run in the field fail to support the prediction that JA addition reduced the growth performance of dietary generalist caterpillars (Tables 5, 6). However the JA addition treatment did reduce the growth of dietary generalist caterpillars in the laboratory assay (discussed later). Perhaps the contradiction in these findings may be the result of compensatory feeding behaviors exhibited in the field but not in the laboratory. Past studies have shown that insect herbivores increase consumption rates in response to low quality foods (Yang and Joern, 1995; Denno et al. 2000; Berner, 2005).
Because we were unable to collect frass measurements from the field assays, we cannot know if caterpillars adjusted their consumption levels to account for decreased food quality. If compensatory feeding did occur, then similarities in mass gain do not reflect true food quality of each branch or treatment. Furthermore, field caterpillars were not restricted to one leaf, but could instead move about the branch and sample from multiple leaves. There is behavioral evidence that insect herbivores respond to food plant quality by selectively feeding on high quality food and moving away from poor quality food (Bernays and Chapman, 1994). Although JA induction is recognized as producing localized effects, it remains unknown in white oak if differences in food quality exist between leaves on the same branch (Herde et al., 1996; Tian et al. 2003).

**Effects of JA addition on dietary generalist and specialist caterpillars**

As predicted, JA addition caused white oak leaves to become resistant to dietary generalist caterpillars, but not to dietary specialists. In laboratory growth assays, JA addition decreased the growth performance of dietary generalist caterpillars (*H. fidelis, A. impleta*) compared to control diets (Fig. 5, Table 7). However, we saw no change in the growth performance of the Fagaceae specialist, *N. gibbosa*, from the addition of JA (Fig. 7, Table 7). Interestingly, JA addition increased the growth performance of the oak specialist, *H. umbrata* (Fig. 8, Table 7). Previous studies have demonstrated secondary compounds such as tannins can act as feeding stimulants for caterpillar species that are well adapted to plants with tannin-rich leaves (Shultz, 1989; Chew and Renwick, 1995; Whittstock and Gerhenzon, 2002; and reviewed by Barbehenn and Constabel, 2011). Because oaks are known for their high tannin profiles, oak
specialists must be highly adapted to the tannins that are normally present in the leaves of their hosts. For this reason we speculate that our JA addition caused increased levels of induced tannins, and that these tannins caused resistance toward the dietary generalists, but acted as a feeding stimulant resulting in elevated mass gain for the oak specialist.

Role of tannins in induced resistance mediated by JA

In support of our prediction that tannins cause the JA-mediated induced resistance, PEG increased the growth performance of the dietary generalist, A. impleta, that fed on JA-treated leaves (Fig. 6). However, the positive effect of PEG on caterpillars given leaves without JA addition suggests that constitutive (non-inducible) tannins also contribute to host-plant resistance against A. impleta or that the JA-free leaves might have been induced by natural causes in the field. While the dietary generalist's growth performance matched our predictions, it was the oak specialist's growth performance that was more telling. For H. umbrata, the positive effect of JA on its growth was increased by the application of PEG. Presumably, dietary specialist caterpillars increased consumption as a result of tannins acting as a feeding stimulant, and that PEG increased the plant quality by inactivating those tannins after consumption. Because condensed tannins are constitutively expressed and hydrolyzable tannins are induced by JA we can deduce that PEG-associated increases in performance are the result of inactivated hydrolyzable tannins (Wold and Marquis, 1997; Elder, 2013). Therefore, we conclude that treehoppers induce white oak's susceptibility to dietary generalist caterpillars, and that the inhibition of JA-mediated hydrolyzable tannin production is at least partly responsible for the observed positive growth effect.
Specialist-generalist paradigm

Empirical evidence in support the specialist-generalist paradigm has remained elusive. Despite the fact that there are studies that show an increased performance of dietary specialists on host resources (Wink and Theile, 2002; Sorensen, Turnbull and Dearing, 2004) there remains many previous studies of insect diet breadth that have failed to demonstrate any specific performance advantage associated with dietary specialization (Agosta and Klemens, 2009; Barkae et al., 2012; Garcia-Robledo and Horvitz, 2012). This has led many to challenge the usefulness of the specialist-generalist paradigm as an ecological model (Ali and Agrawal 2012). These mixed results might mean that some previous tests have failed to consider complicating factors, such as effects of induced plant defenses, or other components of fitness, such as risk of attack from natural enemies. Regardless of the inconsistencies in the literature it remains generally accepted that selection can drive species toward both extremes of the diet breadth spectrum (Forister et al., 2012). Perhaps the greatest obstacle in the evaluation of the S-P paradigm is the previous lack of sophistication in exploring multidimensional factors that influence variation in diet breadth performance. Outside factors such as predation risk and overlap between feeding guilds may better explain previous failures to identify performance advantages resulting from dietary specialization. Our results show a gradient of performance across the diet breadth spectrum that supports the S-P paradigm. The downstream resistance product resulting from jasmonic acid additions decreased generalist performance significantly in laboratory feeding trial, yet the Fagaceae specialist was unaffected. In contrast, the oak specialist increased its
performance on leaves that received the JA addition. This finding suggests that either the oak specialist has co-opted tannins in such a way that enhances the caterpillar's metabolic performance, or that the caterpillar has adapted to use tannins as a feeding cue. For the former to be true the supplemental addition of PEG would need to remove this performance benefit, because the tannins would no longer be biologically active. Instead we saw no change in performance, indicating that tannins may instead be stimulating feeding in the more specialized caterpillars. Previous studies have suggested that while specialists may have increased tolerance against chemical plant resistance they are not impervious to their negative effects (Ali and Agrawal 2012). If white oak's secondary chemistry is, in fact, acting as a feeding cue then there would have to be a performance advantage that supersedes any deleterious effect of the tannins themselves.

Our results from the 2011-2012 abundance data provide a possible mechanism that would select for oak-specialist caterpillars that prefer JA-induced those that were uninduced or SA-induced. This hypothetical mechanism is that JA or its downstream products could serve as a chemical cue for enemy-free space for dietary specialist caterpillars. Because we showed that the abundance of dietary specialist caterpillars was decreased in the presence of phloem-feeding treehoppers only when ants had access to the branch, we infer that phloem-feeding by treehoppers increases predation risk for specialist caterpillars. As our other experiments support the hypothesis that treehoppers induce susceptibility by inhibiting JA, we reason that the presence of JA or its downstream products could be a highly detectable chemical cue for an area with reduced predation risk for specialist caterpillars. Consequently, using JA or its
downstream products as a feeding cue can provide oak specialist with a means of choosing branches that pose the least amount of predation risk. The advantage of escaping predation may overcome the negative effects that accompany feeding on a highly chemically defended food source. These findings not only lend support the S-P paradigm but suggest that predicted increases in performance are multidimensional and manifest outside of growth performance alone.

*Future Outlook*

The evidence for induced susceptibility in this study suggests its broader importance in ecological communities. While induced susceptibility has begun to catch the attention of ecologists, the underlying mechanisms (i.e. the actual secondary chemistry structures) have remained poorly tested (Lortzing and Sterphunn, 2016). There has been increased desire among the chemical ecologists to expand our understanding of the specific tannins that are in play in order to fully understand and predict how overlapping feeding guilds will influence herbivore community (Salminen and Karonen, 2011). Obtaining a tannin’s identity and mode of action would not only elucidate the underpinning of white oak's vulnerability to dietary generalist caterpillars, but would also increase the depth of knowledge regarding tannins interaction with insect herbivory. At present, we can only indirectly infer that hydrolyzable tannins are the mechanism that resists leaf-chewing caterpillar attack. The future challenge is to find quantitative ways of confirming their biological, ecological, and evolutionary relevance. Chemical analysis of each treatment’s leaves would greatly enhance the resolution of future induced susceptibility studies. Furthermore, the growth rates and performance responses to
plant resistance can be species specific (Lill and Marquis, 2001). Admittedly our study struggled to produce enough species diversity to characterize a wide range of dietary specialization, but we attempted to maximize our supporting evidence by choosing species that spanned a range of diet breadth in white oak herbivores. Replication of this experiment with a larger number of species representing the diet breadth spectrum would greatly contribute to our understanding of the generality of the S-G paradigm and the mechanism of induced plant susceptibility and resistance in this system.

Conclusion

The results of these experiments provide evidence for the mechanism of induced susceptibility: treehopper feeding inhibiting JA-mediated induced resistance, which consists at least in part of inducible tannins in white oak leaves. Furthermore, according to our analysis of the insect herbivore community on white oak, the effects of phloem-feeding herbivores, such as treehoppers, can affect abundance distributions of dietary generalist and specialist caterpillars in distinctive ways. Dietary specialists do not appear to benefit from treehopper-mediated changes in white oak leaf quality. If anything, the presence of phloem-feeding herbivores negatively affects the dietary specialist leaf-chewers in the context of the broader insect community, which includes predatory ants. Thus, this study provides insight into the ecological mechanisms by which phloem-feeders affect plant-caterpillar interactions on white oak. More studies of other tree species that experience temporal and spatial overlap of multiple herbivore feeding guilds are required to test the generality of these findings.
Works cited


