

# Constitutive and Induced Chemical Defenses as a Function of Leaf Age in *Quercus rubra* (Red Oak)

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**Abstract** Limited resource availability results in a trade-off between growth and defense in plants. Natural selection has favored strategies in plants that efficiently allocate resources to achieve a high net benefit to cost ratio. As integral parts of their defense strategy, plants have evolved not only to have constitutive chemical defenses, but also the ability to induce defenses. Constitutive levels and inducibility may vary among leaves within a tree due to varying net benefit to cost ratio of defending different leaves. In this study, we examined the effect of leaf age on constitutive levels and inducibility of phenolics following a mechanical attack in *Quercus rubra* (Red Oak). Young leaves possessed higher constitutive levels of phenolics compared to older leaves on the same trees, which were sampled eight days later. Inducibility did not differ between leaf age categories. Constitutive phenolics, but not inducibility, was negatively correlated with leaf nitrogen and positively correlated with leaf C:N ratio. Our results suggests that natural selection may have favored the evolution of spring-feeding herbivores in part because of the inability of early season leaves to induce higher levels of chemical defenses.

## INTRODUCTION

Limited resource availability prevents plants from optimally investing in all activities that promote their survival and reproduction. Because allocating resources to one function precludes plants from allocating them to other functions, plants routinely encounter resource-based trade-offs. Since natural selection favors those with highest fitness, it is reasonable to assume that plants have evolved to efficiently allocate resources to achieve the highest net benefit to cost ratio.

As a result of millions of years coevolving with herbivores, plants have evolved an array of mechanical and chemical defenses to protect against herbivory. Plant chemical defenses exist primarily in two states. Constitutive defenses are always present and offer continuous protection against herbivory (Wittstock 2002). When these defenses fail, plants may produce higher levels chemical defenses, known as induction. Inducible defenses include phenolics, which are a taxonomically widespread class of carbon-based chemical defenses produced within leaf tissues (Bonga 1982). Because they are produced only when necessary, induced defenses are viewed as an efficient resource allocation strategy that increases net benefit to cost ratios (Moreira et al. 2012).

Because resources are limited, allocating resources toward defense often prevents those resources from being used for growth; thus trade-offs often occur between defense and growth. The Optimal Defense Theory suggests that plants allocate resources for defense to a leaf based on the net benefits the leaf can potentially bring to that plant (Moreira et al. 2012). The calculation of the net benefit to cost ratio may be a function of many factors, such as leaf age and cost of defense.

The net benefits to costs ratio resulting from resource investments to growth or defense may change with age because emphasis on growth may vary for plants over their lifespan. For example (this is not an example of growth vs. defense), phenolic compounds in young leaf tissues may be more likely to result in self-damage. Because the cells in young leaves are more frequently undergoing mitosis, their DNA spends a larger amount of time exposed and vulnerable to toxic mutation. The production of toxic chemicals meant to deter herbivores could harm the plants that produce them if those toxins target cell types that also occur within the plant, a threat known as autotoxicity (Singh et al. 1999). Phenolics vary in their effect on target cells, with some inhibiting growth and others interfering with energy synthesis (Choi and Gu 2009).

Other phenols, such as tannins, bind to proteins to reduce digestibility (Rossiter et al. 1988). Because toxins can be harmful to the plants producing them, investing in chemical defenses may be a more damaging investment than initially believed. As the tissues of younger leaves might be more susceptible to autotoxicity, phenolic production may pose a greater risk to plants aside from just postponing growth. Because young leaves focus resources on growth rather than defense, we expect that young leaves will have lower constitutive levels of phenolics and also induce less. what do other studies show?

Although both growth-defense and autotoxicity-defense trade-offs may be more severe in young leaves, as leaves age the ratio of net benefits to net costs of defense could change to favor increased inducibility. A previous study by Quintero and Bowers (2011) that focused on short-lived perennial plant species indicated that herbivore-induced plant responses can change substantially as a function of plant developmental stage and previous history of damage. We question whether the change in inducibility due to aging is present in all perennial plant species, and if inducibility correlates with age. better to save questions for end of Intro

Because the cells of older leaves divide less frequently, we would presume that mature leaves are less vulnerable to autotoxicity. We believe that mature leaves may contribute more to plant fitness as well, due to their larger size, increased sunlight exposure, and overall photosynthetic capabilities (Kriedemann 1970). Past research on pin oak, red maple, and sugar maple found a strong correlation between leaf area and mass, and net photosynthesis (Reich and Walters 1990). With the assumption that this is true in other plant species, it would indicate that mature leaves are more valuable to plants, supporting our hypothesis that plants should invest more in older leaves. The Optimal Defense Theory predicts that tissues most valuable to a plant's fitness should have highest levels of chemical defenses (Moreira et al. 2012). Because we presume there are lower net costs of induced defenses in older leaves and higher net benefits in protecting them, our hypothesis would align with the Optimal Defense Theory.

Although chemical analysis has shown that phenolics contain carbon, the extent to which nitrogen and carbon availability limit phenolic production remains unclear. Like growth, the production of phenolics requires carbon and nitrogen since the genes and enzymes required for induced defenses must be transcribed and translated, both of which are nitrogen-intensive processes (Lou and Baldwin 2004). It would seem that inducibility and constitutive levels of phenolics are nitrogen-limited. A study on birch trees examined the relationship between

nitrogen availability and phenolic production in the soil (Riipi et al. 2002). Findings indicated that low levels of nitrogen resulted in lower phenolic production. However, the study did not look directly at nitrogen concentrations within the birch trees, but instead analyzed nitrogen availability within the soil. We are uncertain that nitrogen availability within the soil accurately reflects the amount of within-leaf nitrogen available to the plants for induction. Consequently, we are interested in nitrogen concentrations specifically within leaf tissues, and aim to examine the relationships between nitrogen in the leaves, constitutive phenolic levels, and inducibility.

Additionally, little research has been done examining whether constitutive levels and inducibility are primarily determined by nitrogen availability, carbon availability, or the ratio of carbon to nitrogen. We seek to determine if any of these variables is a key determinant of phenolic production. Furthermore, we question how well these variables predict phenolic concentrations when examined concurrently with leaf age. If nitrogen does in fact limit inducibility, for example, we ask whether the influence of nitrogen availability remains constant throughout a leaf's early developmental stages.

Therefore, we ask about the factors and mechanisms that influence phenolic production in *Quercus rubra*, red oak. Known to produce phenolics, red oak was abundantly available during the time of our study (Rossiter et al. 1988). Red oak was also expanding its leaves over the course of our study, allowing us to examine changes in chemical defenses during periods of growth, particularly in young leaves. In this study, we ask how plants manage the trade-off between investment in growth and in defense. Oak species produce phenolic compounds following attack by herbivores (Makkar 1990) important to mention whether this was done in old or young leaves. The investment in defense and assumed consequential loss of resources devoted to growth during induction indicates that red oak may experience a resource-based trade-off (Heil et al. 2002). To our knowledge, there has not been research addressing induction of phenolics in young leaves of red oak. In examining the interactions of nitrogen availability, carbon availability, and leaf age on constitutive and induced levels of chemical defenses in red oak, we would like to see how plants manage trade-offs made unavoidable by limited resources. Abundant in Northern Michigan, red oak has presumably been successful due to natural selection's favoring of efficient resource allocation strategies. Consequently, we would like to examine what these allocation strategies entail.

Ultimately, we seek to answer three questions:

1. Do constitutive levels of phenolics differ between young and older leaves?
2. Is inducibility influenced by leaf age?
3. Are constitutive levels and inducibility a function of nitrogen content?

## **MATERIALS AND METHODS**

To determine whether leaf age has an effect on the concentration of phenolics in red oak, we selected 20 trees from the UVB field and surrounding trails at the University of Michigan Biological Station in Pellston, Michigan. On each tree, one young leaf was sampled 8 days before the other older leaf. The two branches were similar in height, aspect, and sunlight exposure. The two leaves selected from each tree held the same position on each branch and were similar in proximity to the trunk. Leaf length and width were measured from apex to petiole and across the widest lobe as an indicator of leaf age. Older leaves were in fact eight days older than the young; additionally, casual observation suggested that the leaf continued to expand throughout the duration of our experimental collection. Prior to induction, the diameter at breast height (DBH) and sampled branch aspect for each tree were recorded. Additionally, we measured temperature of both the adaxial and abaxial leaf surfaces.

### *Sampling for phenolics in constitutive and induced leaves*

We sampled young leaves on May 25, 2013 using scissors to remove one half of the leaf to serve as our constitutive sample while leaving the midrib intact. Each sample was placed in a glassine envelope and an iced cooler. To mimic herbivore damage, a garlic press was used to mechanically puncture the remaining half of the leaf without removing any leaf material. Within 45 minutes of constitutive sample collection, the glassine envelopes with enclosed leaf tissues were placed in a freezer at  $-80^{\circ}\text{C}$  for a minimum of two hours. The envelopes were then freeze dried for a minimum of 24 hours, ground under liquid nitrogen in a mortar and pestle, placed in a drying oven at  $40^{\circ}\text{C}$  to remove water gained during grinding, and stored in a microcentrifuge tube for later chemical analyses.

Between 52 and 54 hours after mechanical damage, we collected the damaged remaining half of each young leaf repeating the process described above for constitutive samples.

On June 2, eight days after collecting young constitutive samples, constitutive samples from older leaves were collected and the remaining leaf half was damaged using the procedures described above for young leaves. We also measured leaf dimensions and temperatures as before.

#### *Phenolic Assay*

Approximately 10 mg of ground leaf tissue from each young and old constitutive and induced sample (n = 80 total) was weighed, placed in a separate microcentrifuge tube containing 0.5 mL of 70:30 acetone:deionized water as the extraction solvent, vortexed for 5 seconds, placed in a sonicator for 10 minutes, and then centrifuged for 5 minutes at 6,000 rpm. The supernatant of each sample was transferred to a new microcentrifuge tube. The pellet was resuspended in another 0.5 mL of 70:30 acetone:deionized water and vortexed, sonicated and centrifuged as before. The two supernatants from each leaf sample were combined and brought to a total volume of 1.5 mL with 70:30 acetone:deionized water.

Phenolic levels were measured by a modified Folin-Denis method developed by Mike Grant and Dave Karowe. To a borosilicate glass tube, we first added the 2.59 mL of deionized water (2.6 mL for the blank). We then added 100  $\mu$ L of Folin-Denis reagent diluted 1:2 with deionized water and 10  $\mu$ L of phenolic extract. Each tube was vortexed before and after adding 300  $\mu$ L of 4N  $\text{Na}_2\text{CO}_3$  and placed in the drying oven at 40° C for 30 minutes to allow color development. All samples were then transferred to cuvettes and absorbance at 700 nm was read using a Spectronic Genesys 2 spectrometer.

Phenolic levels were expressed as tannic acid equivalents (TAE) by interpolating from a 6-point standard curve of tannic acid concentration ranging from 7.8 to 501 ppm.

#### *Elemental analysis*

To determine the content of nitrogen and carbon, 1.5 - 2.0 mg of the remaining ground leaf tissue was weighed into tin capsules and given to J. Croskey for nitrogen and carbon elemental analysis using a Costech Elemental Combustion System chromatographer.

#### *Statistical Analysis*

To determine if there was a difference in the concentration of phenolic compounds between young and older leaves while attempting to standardize variables that could be noisy or

confounding, we used a paired t-test. We also ran multiple regression analyses to examine the relationships between various variables.

## RESULTS

### *Constitutive phenolic levels as a function of leaf age*

The constitutive phenolic levels were higher in young leaves than in older leaves. Young leaves had constitutive levels of 8.11 tannic acid equivalence (TAE)/mg of phenolics. Older leaves had constitutive levels averaging 5.29 TAE/mg. The constitutive levels in young leaves were on average 34.8% higher than those in older leaves. We found this difference to be statistically significant ( $t=13.24$ ;  $df=19$ ;  $p<0.001$ ; Figure 1).

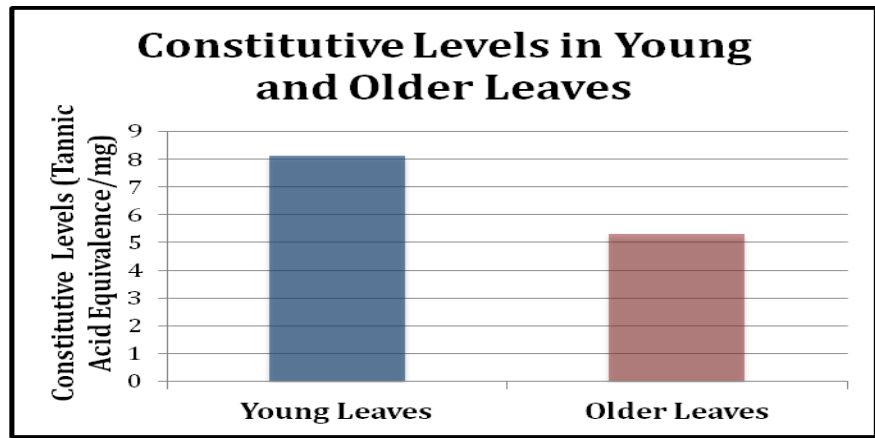


Figure 1.

### *Inducibility and leaf age*

Our results indicated that induction did not occur in young leaves during our experiment. The difference in means was not statistically significant (paired t-test;  $t=-0.187$ ;  $df=19$ ;  $p=0.9$ ;

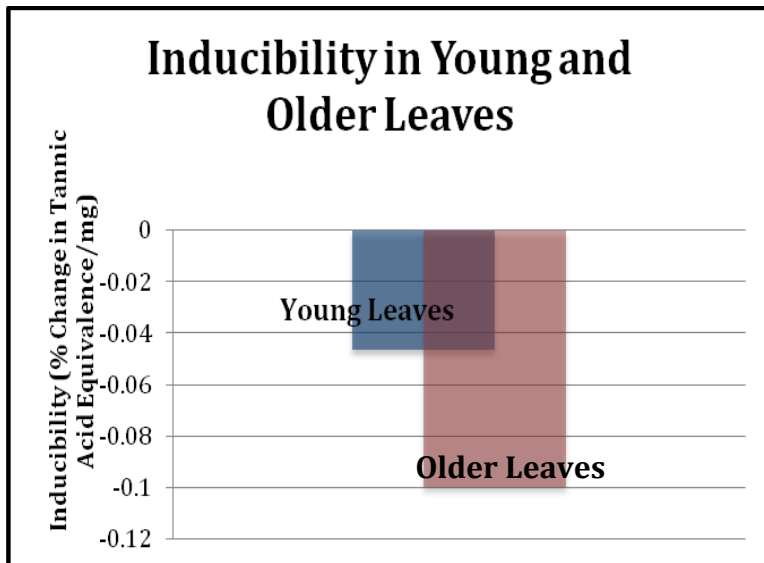


Figure 2). Older leaves did not appear to induce either (paired t-test;  $t=-0.670$ ;  $df=19$ ;  $p=0.5$ ; Figure 2). A comparison between inducibility in young leaves and older leaves indicated that inducibility was not a function of age (paired t-test;  $t=0.199$ ;  $df=19$ ;  $p=0.8$ ; Figure 2).

Figure 2.

*Leaf nitrogen content as a function of leaf age*

We found that nitrogen concentrations were higher in young leaves than in older leaves (paired t-test;  $t=5.75$ ;  $df=19$ ;  $p<0.001$ ; Figure 3). Nitrogen content in young leaves was 21.9% higher than in older leaves.

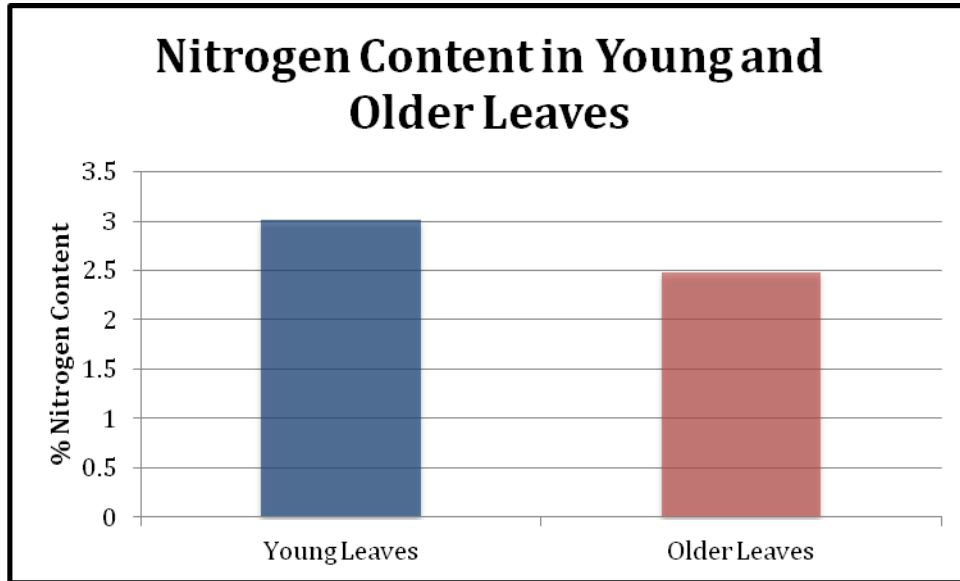


Figure 3.

*Constitutive phenolic levels as a function of leaf nitrogen content*

Our results indicated that there was a significant, negative relationship between nitrogen content and constitutive phenolic levels in young leaves (regression analysis;  $R^2=0.298$ ;  $df=19$ ;  $p=0.013$ ; Figure 4). Higher concentrations of nitrogen in leaves were associated with lower constitutive phenolic levels in older leaves as well (regression analysis;  $R^2=0.227$ ;  $df=19$ ;  $p=0.034$ ; Figure 4). Our results indicated a negative correlation between nitrogen content and constitutive phenolic levels for both age groups.

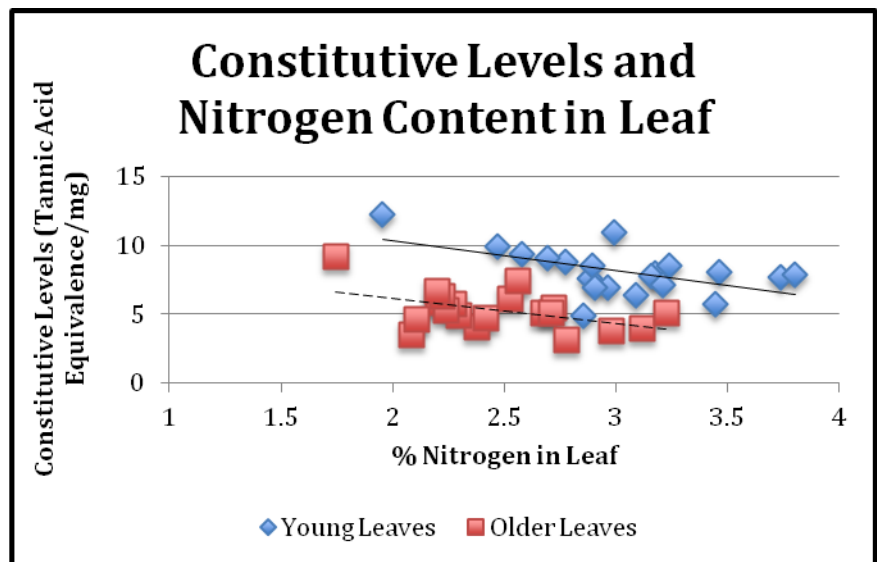


Figure 4.



### *Inducibility and leaf nitrogen content*

We found no significant relationship between nitrogen content in young leaves and inducibility (regression analysis;  $R^2=0.005$ ;  $df=19$ ;  $p=0.8$ ; Figure 5). Our results also indicated there was no significant relationship between nitrogen content and inducibility in the older leaves (regression analysis;  $R^2=0.126$ ;  $df=19$ ;  $p=0.124$ ; Figure 5).

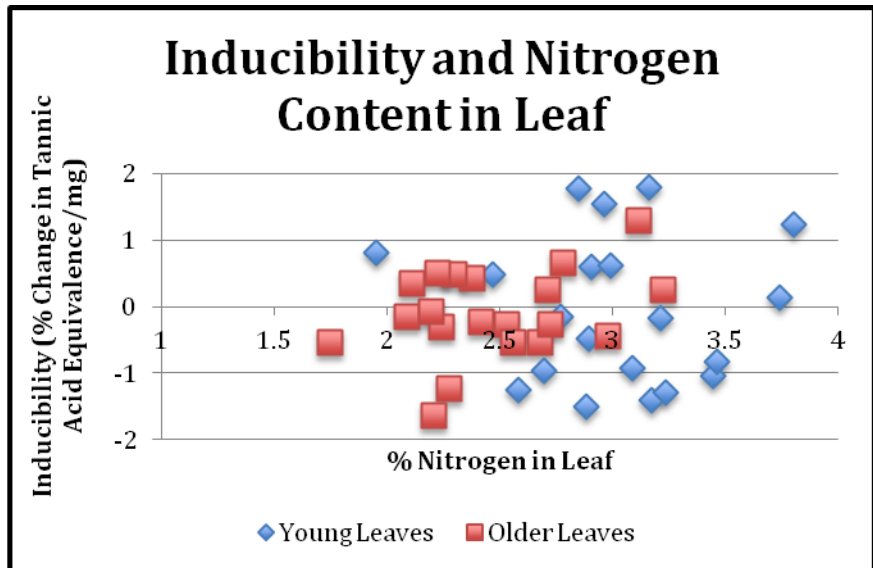


Figure 5.

### *Constitutive phenolic levels as a function of leaf C:N ratio*

We found that there was a significant, positive relationship between the C:N ratio in young leaves and constitutive phenolic levels (regression analysis;  $R^2=0.469$ ;  $df=19$ ;  $p=0.001$ ; Figure 6). A significant, positive relationship between the C:N ratio and constitutive phenolic levels was also found in older leaves (regression analysis;  $R^2=0.268$ ;  $df=19$ ;  $p=0.019$ ; Figure 6). For both young and older leaves, higher constitutive levels of phenolics were positively correlated with increased C:N ratios.

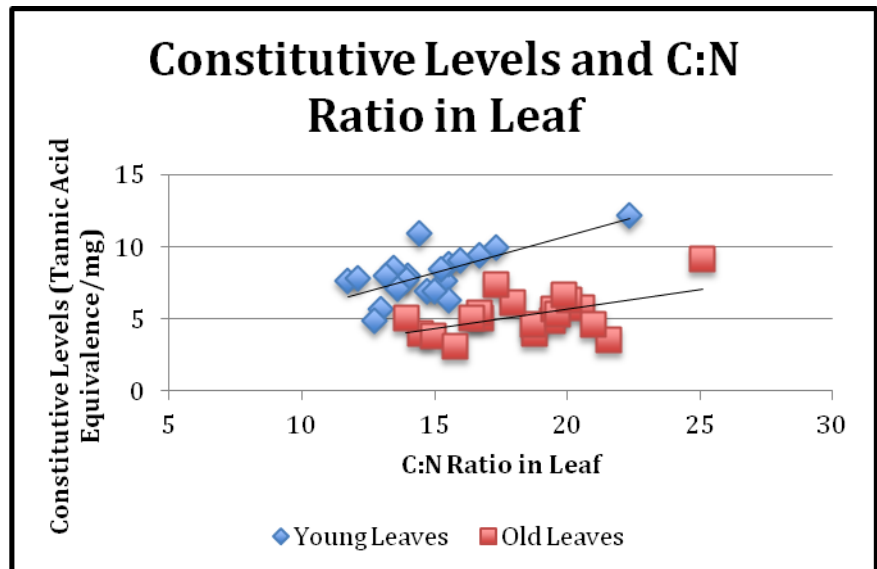


Figure 6.

### *Inducibility and leaf C:N ratio*

We found no significant relationship between inducibility and the C:N ratio in young leaves (regression analysis;  $R^2=0.000$ ;  $df=19$ ;  $p=0.9$ ; Figure 7). We also did not observe a significant relationship between inducibility and the C:N ratio in older leaves (regression analysis;  $R^2=0.122$ ;  $df=19$ ;  $p=0.132$ ; Figure 7).

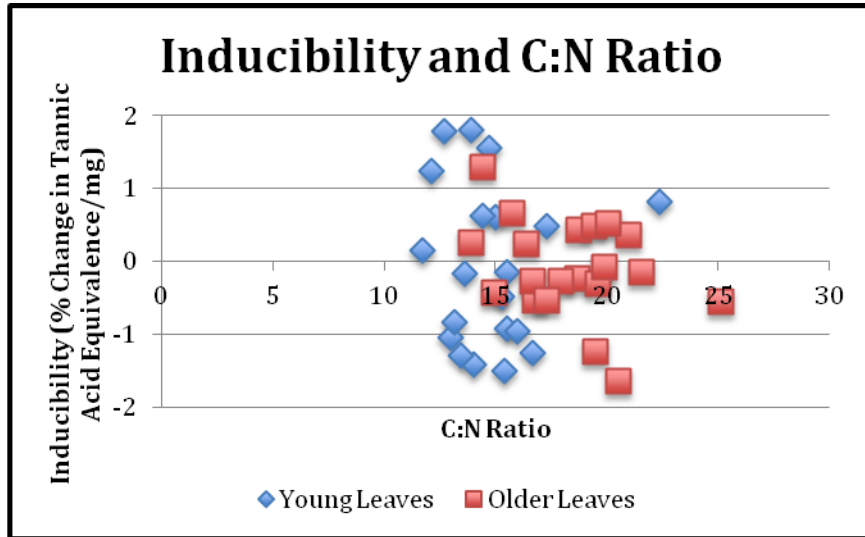


Figure 7.

### *Constitutive phenolic levels as a function of leaf size*

Our results indicated that there was a significant, negative relationship between leaf surface area and constitutive levels of phenolics (regression analysis;  $R^2=0.231$ ;  $df=39$ ;  $p=0.002$ ; Figure 8). These results aligned with those from Figure 1 in which we compared constitutive phenolic levels and leaf age.

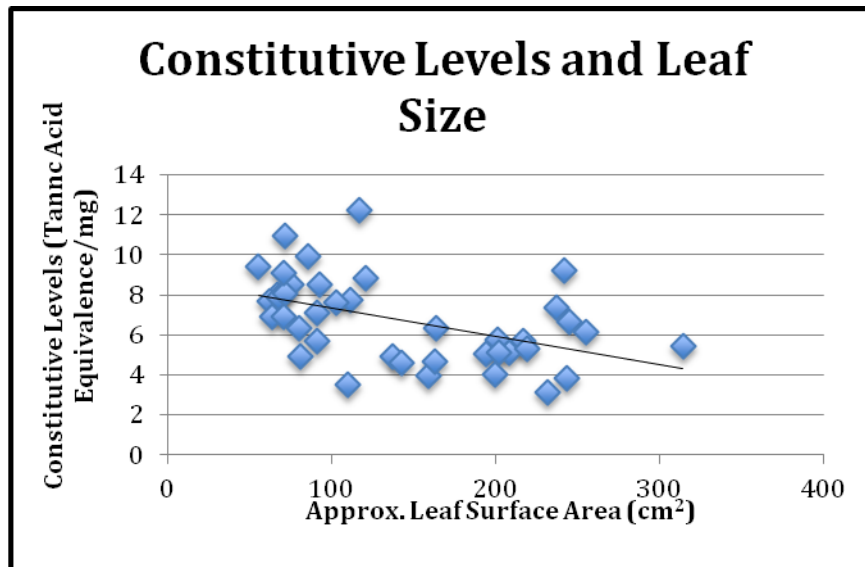


Figure 8.

### *Inducibility and leaf size*

We found no significant relationship between the inducibility and the surface area of a leaf (regression analysis;  $R^2=0.008$ ;  $df=39$ ;  $p=0.6$ ; Figure 9). This corresponded with our results from Figure 2.

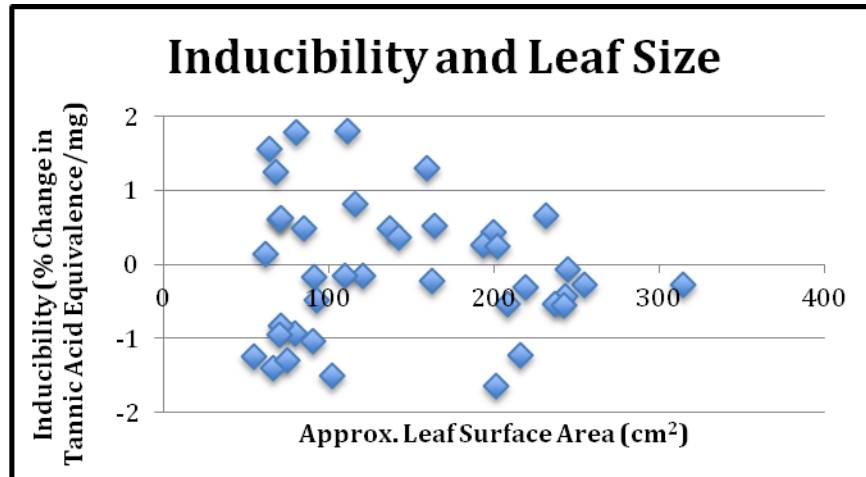


Figure 9.

## **DISCUSSION**

Contrary to our predictions that constitutive levels would be higher in older leaves due to their greater surface area and presumed increased photosynthetic capabilities, our results showed that young leaves actually had higher levels of constitutive phenolics. From this, we suspect that young leaves may contribute more to plant fitness, thus having a higher net benefit to cost ratio. Supporting this notion, a study on tropical trees showed that rates of photosynthesis decreased as leaves expanded (Kitajima et al. 1997). We do not know whether this is true of all trees, especially those in temperate deciduous forests, but it may provide an explanation for why our results indicated higher constitutive levels in young leaves. Because young leaves have relatively high constitutive levels of phenolics, autotoxicity may be less of a threat to young leaf tissue than we had predicted. This would mean that the autotoxicity and defense trade-off is not as severe as we had initially thought.

As separate testing indicated, the nitrogen concentration in young leaves was higher than in older leaves. Due to herbivores' preference for more nitrogen-rich tissue, young leaves with higher nitrogen content are more at risk to herbivore attacks (White 1984). The plant-age hypothesis also supports our findings, indicating that the investment in potentially toxic defenses

may be more warranted for young leaves due to increased herbivory (Barton and Koricheva 2010).

Previous studies done on the *Quercus* genus show a direct negative correlation between phenolics and temperature in fall (Chun-ming 2011). While this study may show us that phenolic levels decrease with temperature, there is little research showing the same relationship between temperature and phenolic production earlier in the spring. Additionally, another study found that temperature has an effect on insect-phenolic interactions (Forkner et al. 2004). Because we collected our older constitutive and induced samples on a cooler day than the young, we suspect that insect feeding patterns were reduced due to their susceptibility to low temperatures. This may have resulted in trees producing a decreased amount of phenolics for defense in response to lower herbivory.

Contrary to our initial thinking, perhaps the trade-off between growth and defense is not simply a linear function of age but is a combination of many other factors. Young leaves appear to play an important role in the long-term survival of trees, helping to explain why it is important for the tree to invest more resources in protecting young leaves. Along the lines of these results, we also suspect that young leaves must not be as susceptible to autotoxicity as we had predicted.

Our results showed that induction occurred in neither young nor older leaves. Our predictions about inducibility as a function of leaf age failed to portray our results. Prior to the study, we thought that older leaves provided a higher net benefit to cost ratio because of their presumed higher photosynthetic capability. Thus, it would be efficient to invest in the protection of older leaves through induction. From our results, we may infer that red oaks value growth significantly more than defense during early stages of leaf development. We suspect that in the spring plants prioritize growth because it has at that time a higher net benefit to cost ratio. Seasonal changes may alter the allocation of resources to different functions.

With that being said, our study only spanned the course of eight days. This resulted in our older leaves not being much older than our young leaves. In order to determine age differences between the two samples, leaf size was used as an indicator. The young samples were considerably smaller than the older. However, leaf size is not maximized until at least early July and casual observation noted that our leaves were still expanding (USDA 2002). This could explain why we did not see differences in the inducibility of phenolics when comparing young and older samples. Our subject groups would have been more appropriately labeled “very

young” and “young” instead of “young” and “older” leaves. While we may have allowed for enough time for the leaves to induce, there may not have been enough variance in the age of leaves between the young and older samples for us to draw accurate conclusions as to which age red oaks are able to optimally induce phenolics. Due to time constraints, we were unable to design our study so that the older leaf samples would be differentiate significantly in age from the young leaf samples.

Perhaps our prediction did not match our results simply due to the fact that our collection timing did not synchronize with the period of induction. To our knowledge, optimal time and age of induction in red oaks has not been extensively researched. Because of the lack of literature on red oaks in particular, we used a similar study done on phenolic induction in *Trifolium repens* as a reference for plant induction time when designing our methods. Because phenolics are resource-intensive, induced defenses are sparingly activated. Following an attack, plants typically reach maximum phenolic levels within 48 hours (Gomez et al. 2008). With this in mind, we returned 52 hours after we had damaged the tissue to collect our induced samples for both the young and older induced samples. We are uncertain if allowing 52 hours for induction was an appropriate allowance of time to get a representative sampling of phenolics in the red oak. Again, due to time constraints, we were unable to allow any more time before collecting our induced samples for both young and older leaves. It also seems plausible that both young and older leaves, still early in their development during this experiment, experienced the same vulnerability to autotoxicity. To compensate for this vulnerability while still inducing defenses, leaves may have increased levels of phenolics for a very short period of time following attack and lowered them quickly to minimize the risk of autotoxicity. This would have resulted in us missing the time window in which the leaves had actually induced.

It is also possible that our use of a garlic press did not fully mimic true herbivory. Successful experimentation in the past on *Brassica rapa* using a garlic press led us to believe that mechanical attack would induce phenolic responses just as well as true herbivory (Karowe and Grubb 2011). In this experiment, we assumed that red oaks were able to respond just as well to our mechanical damage as *Brassica rapa* did. If a chemical stimulant released during herbivory had been necessary, then our reliance on only mechanical damage might explain the lack of induction expressed by our results.

As was the case with the constitutive level results, the older leaves may have induced more, but were just constricted by the colder temperatures on the day the study was conducted. The weather a few days after attack may have played a role as well. In our experiment, the days following attack of the older leaves were also considerably colder.

As a result, it could also be possible that there is strong selection favoring spring-feeding herbivores. Because our findings indicated that neither young nor older leaves induced following attack, it is reasonable to assume that spring-feeding herbivores will exploit this opportunity.

Our results indicated that as nitrogen content increased within a leaf, constitutive phenolic levels decreased. This initially seemed counterintuitive given nitrogen's role as a limiting resource in growth and defense. The carbon-nutrient balance hypothesis might offer an explanation for our findings. If plants may only use carbon and nitrogen at a fixed ratio for growth and if carbon is first allocated to growth, then nitrogen limits the use of carbon for growth. As the amount of nitrogen decreases, the ability of the plant to use carbon for growth decreases as well. If the production of phenolics is not constrained by a similar ratio as the one that limits growth, then carbon that would have otherwise been allocated to growth may instead be used for defense (Hamilton et al. 2008). Our results support this hypothesis, showing that as the C:N ratio increased, the constitutive levels of phenolics increased as well. Our findings that examined the relationship between nitrogen content and constitutive phenolic levels further supported this.

Overall, our results suggest that early in the growing season plant defenses do not increase following leaf tissue damage. Our samples were relatively young and did not respond to mechanical attacks. This would seem to leave plants quite susceptible to herbivory near the beginning of their seasonal development. As a result, herbivores able to take advantage of these vulnerable, nitrogen-rich leaves may fare better. Species who feed early in the spring, such as the gypsy moth, may have a competitive advantage over those who exit diapause later in the season (Rossiter et al. 1988). Consequently, natural selection should favor spring-feeding herbivores.

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