OXIDATION OF INGESTED PHENOLIC COMPOUNDS CREATES OXIDATIVE STRESS IN THE MIDGUT TISSUES OF *LYMANTRIA DISPAR* CATERPILLARS

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ABSTRACT

Phenolic compounds are an important component of the oxidative defenses of plants against herbivores. In some cases, phenolic compounds become oxidized when consumed by leaf-feeding insects. These compounds are called prooxidants, and their reaction products are responsible for causing oxidative stress in the digestive tract by producing reactive oxygen species (ROS), such as semiquinone radicals. This has been demonstrated in previous work on *Lymantria dispar*, the gypsy moth caterpillar, which had more peroxide and hydroxyl radicals present in the gut lumen when consuming sugar maple (*Acer saccharum*) leaves than when consuming red oak (*Quercus rubra*) leaves. This distinction was believed to be due to the fact that maple foliage contains higher levels of phenolic compounds than red oak foliage. I hypothesize that (1) maple feeders have higher oxidative stress than oak feeders, (2) oxidative stress is lower in the spring than the summer, and (3) maple feeders have lower performance than oak feeders in both the spring and summer. In my research, I investigate this possibility by using glutathione disulfide (GSSG): total glutathione (GSH) ratios as a marker for oxidative stress. I also examine whether these levels change in caterpillars that feed on foliage in the spring and the summer since it is unknown whether temporal changes in leaf chemistry affect oxidative stress in herbivores. Results supported my first and third hypotheses, but not the second, suggesting an absence of temporal effect on the leaf-feeding larvae. It was concluded that ingested phenolics cause oxidative stress in the midgut tissues. The GSSG: GSH ratios in the midgut contents, however, were not useful as a marker for oxidative stress. It was further concluded that while oxidative stress might reduce the growth rate of leaf-feeding insects in the spring, other factors in mature leaves, such as lower nutritional quality, appear to be more limiting in the summer.

BACKGROUND AND INTRODUCTION

Tannins are phenolic compounds found in the leaves of numerous tree species that are known to defend plants against attack from herbivores (Barbehenn et al., 2006). There are two main types: hydrolyzable tannins and condensed tannins (Hagerman and Carlson, 1998). The foliage of most trees contains mixtures of hydrolyzable and condensed tannins (Baldwin et al., 1987). Although their exact impact on herbivores is not well understood, research has shown these phenolic compounds work by producing ROS (Kalyanaraman et al, 1987). Specifically, tannins get oxidized in the guts of leaf-feeding insects. The products of this oxidation have the potential to damage vital nutrients as well as cause oxidative stress in these organisms (Summers and Felton, 1994).

Lymantria dispar feeds on tree leaves containing both hydrolyzable and condensed tannins (Keating et al, 1989). Research shows that the consumption of these tannins causes adverse reactions in the midguts of *L. dispar* (Barbehenn et al., 2009). These reactions can be detrimental to the performance of the insect because the midgut is the principal site of nutrient digestion and absorption. Furthermore, the midgut acts as the first line of defense against the absorption of hydrogen peroxide and oxidized phenolic compounds. More specifically, ingested allochemicals first encounter oxidizing conditions in the gut lumen and not the tissue. This is because the high alkalinity in the caterpillars' gut (pH 10) creates an environment conducive to phenolic oxidation (Barbehenn et al., 2001, 2005). Thus the effects of oxidized allochemicals can be observed and studied well in caterpillars.

It is important to note that the products of tannin oxidation create adverse effects in the caterpillar, and not so much the phenolic compounds themselves (Appel, 1993). Quinones and semiquinone free radicals are two such products of tannin oxidation. Other harmful ROS are produced

during the oxidation process. These include superoxide anion, hydroxyl radical, and hydrogen peroxide. However, there is a way to combat these toxic compounds, as we will see with *L. dispar* caterpillars.

Just as plants have prooxidants to defend themselves against attack from herbivores, herbivores have antioxidants in their midguts to protect themselves from phenolic compounds (Lindroth, 1991). Antioxidants are important to all organisms because they serve to prevent the oxidation of compounds that have the potential to cause oxidative damage (Halliwell et al., 2000). Oxidative stress occurs when the level of ROS overcomes the antioxidant supply of the host organism (Bulger and Helton, 1998). The depletion of antioxidants can thereby serve as an effective biological marker of oxidative stress (Halliwell and Gutteridge, 1990).

In caterpillars, ingested tannins can get oxidized in the gut lumen. However, the oxidation of ingested phenolics is minimized in caterpillars by co-ingested ascorbate (Felton and Duffey, 1992; Barbehenn et al., 2005). Ascorbate is one type of antioxidant that is essential for normal growth, development, and reproduction. It is relevant to my work because it works best paired with glutathione (GSH), an antioxidant I study in the midgut tissues of *L. dispar*. The preservation of adequate levels of ascorbate in the midgut lumen in insects is explained by the secretion of glutathione into the midgut lumen, thus forming a full ascorbate-recycling system (Figure 1) (Barbehenn et al., 2001). Glutathione is needed because it is used to reduce dehydroascorbate and replenish ascorbate so that the host organism can eliminate ROS.

*CHEMICAL REACTION:

$H_2O_2 \rightarrow H_2O + O_2$ (1)
$AA + H_2O_2 \rightarrow DHA + 2\ H_2O$ (2)
$DHA + 2GSH \rightarrow AA + GSSG$ (2)

*AA = Ascorbic Acid; DHA = Dehydroascorbic Acid; GSSG = Glutathione Disulfide

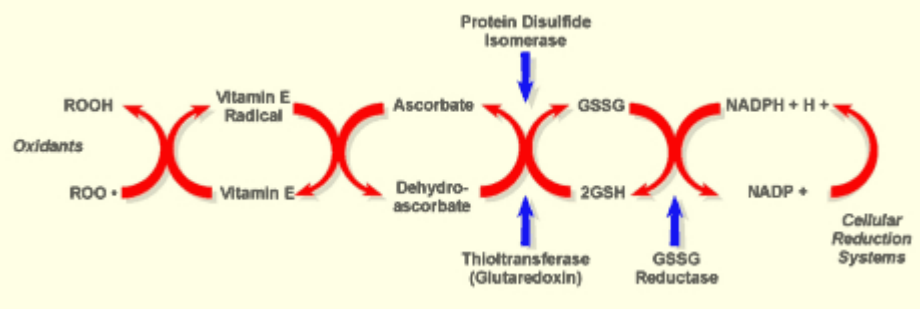

Figure 1 – Ascorbate-recycling system

I am investigating glutathione (Figure 2) in my project because it is one of the most important antioxidants in cells. It is used to detoxify reactive oxygen species and reduce peroxides in the gut. One reason GSH can do this is because it lacks the toxicity identified with cysteine (Woods et al., 1999). This makes GSH suitable as a cellular thiol "redox buffer" to maintain a given thiol/disulfide redox potential. The γ-linkage is important because it is believed to protect the compound from degradation by aminopeptidases (Sies, 1999). Chemicals altering its concentration affect transcription of detoxification enzymes, cell growth, and apoptosis (Jones, 2002). Increasing the concentration of GSH is correlated with growth stimulation and development (Thiboldeaux, 1998). So we see that if the GSH concentration is depleted in tissues by ROS, the host organism may be prevented from developing normally. I study this question using *L. dispar* in my work.

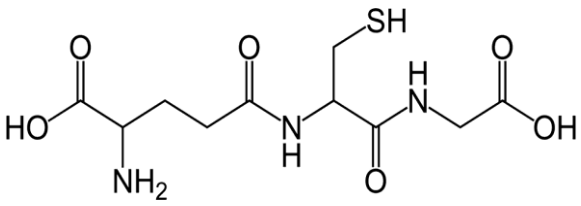

Figure 2 - Glutathione (GSH)

GSH is involved in both enzymatic and non enzymatic biological processes. Figure 1 shows how glutathione reacts nonenzymatically to maintain ascorbate in its reduced and functional forms. However, GSH also reacts rapidly and nonenzymatically with hydroxyl radical, the cytotoxic Fenton reaction product, as well as with N_2O_3 and peroxynitrite, cytotoxic compounds produced from the reaction of nitric oxide with O_2 and superoxide (Griffith, 1999). Enzymatically, GSH acts as an electron donor to detoxify hydrogen peroxides and lipid peroxides in reactions catalyzed by multiple isoforms of GSH peroxidases. These reactions can lead to the production of GSH's oxidized form, glutathione disulfide (GSSG) (Figure 3) (Griffith, 1999).

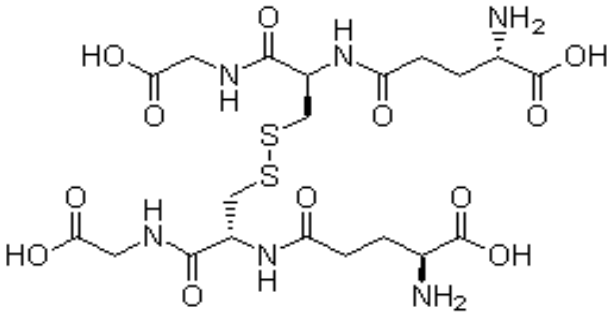

Figure 3 - Glutathione Disulfide (GSSG)

GSH is also involved in another major reaction type involving the toxicity of quinones. Most quinones are able to react with nucleophiles, especially thiols (Finley, 1974). The covalent interaction of quinones with GSH results in the production of thioether, superoxide, and hydrogen peroxide. As a result, GSH becomes depleted and GSSG accumulates, marking oxidative stress. The oxidation of sulfhydryl groups is also critically dependent on pH (Irons and Sawahata, 1985). At pH 10 in the caterpillar midgut, this oxidation can readily occur. This information suggests that the arylation of nucleophiles like GSH may be an effective mechanism for causing cytotoxicity in the host organism (Gant et al., 1988).

Besides becoming arylated by quinones, glutathione also becomes oxidized to glutathione disulfide, a reaction we will see is important in the midgut tissues of *L. dispar*. But how does glutathione disulfide become reduced back to glutathione? Glutathione reductase, an NADPH-dependent enzyme, is responsible for this conversion, and its activity increases as the concentration of GSSG rises in the gut (Jones, 2002). At normal levels of oxidative stress, GSSG reductase activity and NADPH availability are adequate to maintain the proper GSSG: GSH ratio in cellular tissue (Griffith, 1999). When tissues are exposed to increased oxidative stress, the GSSG: GSH ratio will increase as a consequence of GSSG accumulation. Thus the GSSG: GSH ratio is a good indicator of the redox state of glutathione because it reflects the levels of oxidative stress in the tissue.

PREVIOUS WORK

My work follows up on previous studies that investigated the effects of oxidation in the midguts of *L. dispar*. Experiments were conducted in which *L. dispar* fed on either sugar maple leaves or red oak leaves (Barbehenn et al., 2005). Ingested maple leaves produce higher levels of semiquinone radicals in caterpillar midgut fluids than do oak leaves (Barbehenn et al., 2005). This observation can be explained by the foliar composition of sugar maple versus red oak. Specifically, sugar maple contains high levels of hydrolyzable tannins, chemicals known to be active prooxidants in caterpillar midguts. Red oak, on the other hand, contains high levels of the much less active condensed tannins. This is one factor which helps to explain why maple feeders grow less than oak feeders. It also led researchers to hypothesize that increased tannin oxidation would increase oxidative stress in the guts of caterpillars. Oxidative stress would then have a direct negative impact on insect performance. A study done in 2005 showed that maple leaves produce higher levels of oxidative stress in the midgut tissues of *L. dispar* compared with oak leaves. This confirmed that hydrolyzable tannins are responsible for producing oxidative stress in the midgut tissues of caterpillars.

Previous work on *Malacosoma disstria* Hubner (Lasiocampidae) that consumed maple leaves showed that their midguts contained significantly elevated levels of semiquinone radicals, peroxides, and oxidized proteins compared with the midguts of larvae on red oak leaves (Barbehenn et al., 2005). A study was done in 2007 to test the hypothesis that increased levels of phenolic oxidation was associated with increased oxidative stress in *M. disstria* when feeding on red oak and sugar maple (Barbehenn et al., 2008). One marker used was the GSSG: GSH ratio. Both reduced and total glutathione levels were higher in the midgut tissues of larvae on red oak and than on sugar maple. GSSG: GSH ratios were significantly higher in the midgut tissues of *M. disstria* that fed on maple versus oak leaves, consistent with more oxidative stress in larvae on maple (Barbehenn et al., 2005). These results led me to hypothesize that maple feeders would have higher GSSG: GSH ratios than oak feeders in all cases independent of temporal changes.

For my research, I used *L. dispar* to study how feeding on sugar maple versus red oak leaves causes GSSG: GSH ratios to change and what this effect might have on the relative growth rates of these leaf-feeding insects. I also compare these growth rates in samples between May, July, and August because it is well known that the phenolic compositions of tree leaves change seasonally (Salminen et al., 2004), and it was expected that spring leaves would have lower tannin levels (Feeny, 1970). The Barbehenn (2009) study on *L. dispar* showed that they perform better in the summer. In my project, I follow up the 2009 study by examining what happens in the spring and comparing this data to what happens in the summer.

HYPOTHESES

(1) Maple feeders have higher levels of GSSG: GSH ratios than oak feeders.

(2) GSSG: GSH ratios are lower in the spring than the summer.

(3) Maple feeders have lower performance than oak feeders in both the spring and summer as a result of oxidative stress.

MATERIALS AND METHODS

Trees

Red oak and sugar maple trees from the University of Michigan campus were used in the study. Twigs containing terminal leaf clusters from the sunlit portion of trees were randomly chosen and cut with a pole pruner. The twigs were immediately placed in water and returned to the lab for feeding experiments. Oak and maple leaves were washed with water.

Insects

Lymantria dispar eggs were obtained from the United States Department of Agriculture (Otis Air Force Base, Mass.). Larvae were reared on an artificial diet in Petri dishes in a 23°C incubator. When larvae reached the third instar, they were switched to a 16°C incubator to control growth so that larvae would be of comparable size at the fourth instar for the experiment. The caterpillars fed on an artificial diet made according to a protocol in Barbehenn et al. (2001).

Larval Performance

Leaves were patted dry and 2.5 cm in diameter leaf disks were punched out from them with a cork borer. The disks were mixed to randomize variation in leaves before feeding to the caterpillars

(Barbehenn et al., 2009). Thirty fourth instar larvae were weighed and placed at random on maple and oak leaf disks in 35-ml plastic cups with snap cap lids. Larvae were kept in the 23°C incubator. Fifteen larvae per each tree species was set up in this manner. Larval fresh weights were converted to dry weights based on fresh to dry weight ratios of other fourth instar caterpillars that were dried at 70°C. New leaf disks were provided daily. Larval development was determined as the number of days until molting to the fifth instar (±0.5 days). Larval growth was determined as the difference between the dry weights of newly molted fifth instar larvae and the initial dry weights of the fourth instar larvae. This relative growth rate (RGR) was calculated as an average from the larvae on both oak and maple in mg/mg initial dry weight per day.

Larval initial dry weights were calculated based on the fresh to dry weight ratios of newly molted fifth instars that were dried at 70°C. Leaf disks were weighed and fed to each larva on a daily basis. Uneaten leaf remains were collected daily and dried. Daily consumption was measured as the difference between the dry weight of the leaf disks and the dry weight of the uneaten remains. Initial leaf dry weight was estimated from the fresh to dry weight ratios of five representative leaf disks that were collected on each day of feeding. The frass, or caterpillar feces, was allowed to completely dry and the relative consumption rate (RCR) was calculated as mg/mg initial dry weight per day.

Oxidative Stress

Midgut tissue and gut contents were collected from 15 replicate larvae on both oak and maple on 19 May, 12 July, and 19 August 2010. 10-15 replicate larvae per both oak and maple were retrieved on each date. Midgut tissues were weighed and homogenized in 300 µL of 5% meta-phosphoric acid (MPA) containing 1 mM ethylenediaminetetraacetic acid (EDTA). Gut contents were also weighed but not homogenized. Instead, they received 300 µL of 5% MPA containing 1mM EDTA plus 15 mg

polyvinylpyrrolidone. This mixture was placed in a shaker for one hour to mix the contents thoroughly. Both tissue and gut content samples were stored frozen (-80°C) until analyzed with an enzymatic assay. Before assayed, frozen samples and glutathione standards were thawed and mixed. Homogenates were centrifuged (10,000g; 5 min) and kept on ice. GSSG levels were measured in 50 µL aliquots of samples diluted 1:1 with 5% MPA. GSH levels were measured in 50 µL aliquots of tissue samples diluted 10% (vol:vol) in 5% MPA but diluted only 5% (vol:vol) in gut content samples in 5% MPA. 2-vinylpyridine, diluted 75% (vol:vol) in methanol, was added to the wells of samples used to measure GSSG concentrations (2 µL/well) and mixed. 25 µL of triethanolamine (TEA), diluted 22% (vol:vol) in double-distilled water, was next added to each well to neutralize the pH. The microtiter plate was mixed and incubated in the dark for one hour at room temperature. After incubation, 143 µL of the reaction mixture was added to each well using a multichannel pipetter. This mixture was composed of sodium phosphate buffer (pH 7.5, 125 mM), 5, 5'-dithiobis (2-nitrobenzoic acid; 1 mM), and nicotinamide adenine dinucleotide phosphate (NADPH; 3.6 mM). The plate incubated for another five minutes at room temperature. Using the multichannel pipetter, 50 µL of glutathione reductase solution was added to each well to initiate the reaction. This solution consisted of 1.5 U/mL of pH 7.5 sodium phosphate buffer. Reaction rates (mAbs/min) were measured at 415 nm using a kinetic protocol (Microplate Manager 4.0; Bio-Rad, Hercules, CA). Total GSH concentrations were calculated as GSH + 2 x GSSG concentrations. Concentrations of glutathione in both midgut tissue and contents were calculated based on tissue water volumes (80% of tissue fresh weight; 90% of gut contents fresh weight) (Barbehenn et al., 2005).

GLUTATHIONE ASSAY DESIGN:

(a) 2 GSH + DTNB → GSSG + 2 TNB

(b) GSSG + NADPH + H^+ → 2 GSH + $NADP^+$

(c) DTNB + NADPH + H^+ → 2 TNB + $NADP^+$.

GSH reacts non-enzymatically with Ellman's reagent (DTNB, 5, 5'-dithiobis (2-nitrobenzoic acid)) to yield GSSG and 2-nitro-5-thiobenzoic acid (TNB). Then GSSG uses NADPH and glutathione reductase to recycle GSH which can react again in the same manner. The sample is also run with 2-vinylpyridine to conjugate GSH so that only GSSG is detected during the glutathione assay (Griffith, 1983).

Statistics

Comparisons between species of trees and months were made using 2-way analysis of variance (ANOVA).

RESULTS

Glutathione

GSSG: GSH ratios were higher in the midgut tissues of *L. dispar* that consumed sugar maple than red oak leaves. This is consistent with my first hypothesis and shows to be true across May, June, and July (Figure 4a). The same cannot be said for the gut contents of the same larvae. There were no significant differences between GSSG: GSH ratios of oak and maple feeders. Similar patterns were also observed across all months examined (Figure 4b).

The results do not support my second hypothesis. GSSG: GSH ratios are not lower in the spring than summer, as I expected. There were no significant differences when comparing May ratios to either July or August ratios within species, suggesting an absence of temporal effect on oxidative stress in the leaf-feeding larvae.

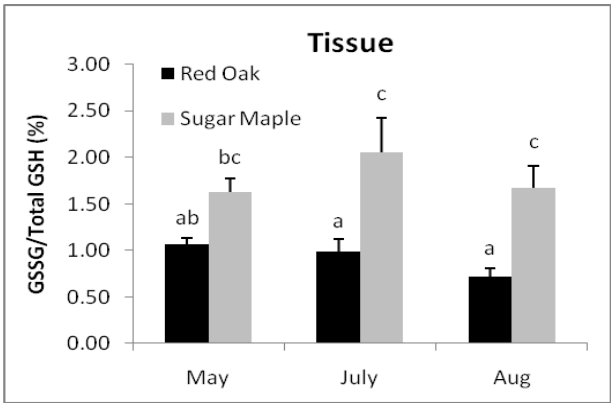

Figure 4a - GSSG:GSH ratios in midgut tissue of *L. dispar* larvae feeding on red oak or sugar maple in May, July, and August.

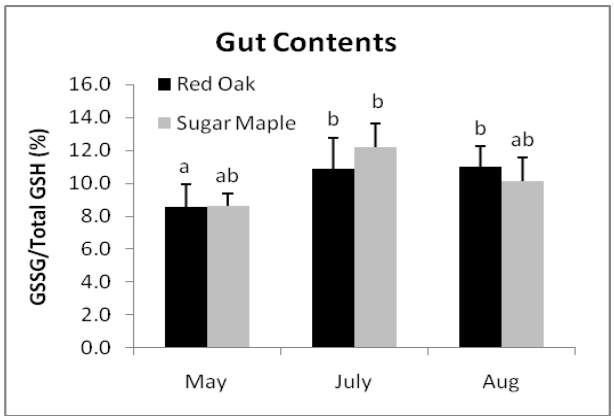

Figure 4b - GSSG:GSH ratios in midgut contents of *L. dispar* larvae feeding on red oak or sugar maple in May, July, and August.

Glutathione levels are higher in oak-feeding larvae in July (Figure 5a). GSH levels are higher in maple-feeding larvae in August. Glutathione disulfide levels are higher in maple feeders than oak feeders in all months examined (Figure 5b).

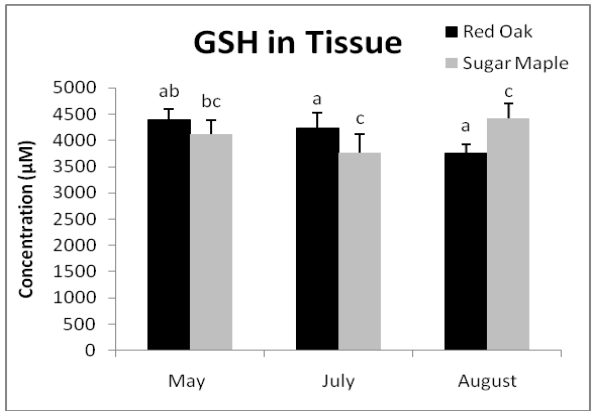

Figure 5a – GSH concentrations in midgut tissue of *L. dispar* larvae feeding on red oak or sugar maple in May, July, and August.

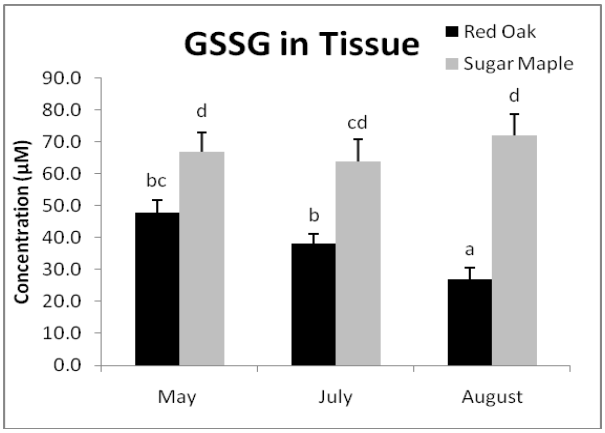

Figure 5b - GSSG concentrations in midgut tissue of *L. dispar* larvae feeding on red oak or sugar maple in May, July, and August.

Performance

As expected, maple feeders grew less in both the spring and summer in comparison to oak feeders (See Table 1). However, the difference was much more pronounced in the spring. The relative growth rate (RGR) of maple feeders in May was almost half that of oak feeders. The RGR was still less in maple feeders in August.

Also important to note are the differences in RGR across *L. dispar* feeding on the same tree species over the spring and summer months. Oak feeders grew more slowly in July and August than

what they grew in May. Maple feeders also grew at a slower rate in July and August than what they grew in May. Overall, the data shows a significant decrease in growth rate of *L. dispar* larvae when they consume summer versus spring foliage.

Relative Growth Rate (RGR)			
Species	May	July	August
Red Oak	0.441±0.022 [d]	0.146±0.002 [ab]	0.156±0.011 [b]
Sugar Maple	0.265±0.006 [c]	0.134±0.004 [ab]	0.123±0.009 [a]

Table 1 – Relative growth rate of oak and maple leaf-feeding larvae in May, July, and August. Statistical comparisons were made using two-way ANOVA. Different letters represent significant differences between species within months.

The above data suggests that oxidative stress may be the cause of reduced performance in maple feeders in the spring. However, we must also examine the amount of food the leaf-feeding larvae consumed in order to eliminate bias that the caterpillars were not growing well because they were not eating well. Here, we denote the amount of food consumed as the relative consumption rate, or RCR. In Table 2, we see that oak feeders consumed less food in July than maple feeders in those months. However, both oak and maple feeders fed on foliage at similar rates in May and August. The data suggests that the RCR is not having an effect on the RGR. Even though oak feeders consumed less than maple feeders in July, oak feeders grew at similar rates to maple feeders in July. Overall then, we can say RCR does not impact RGR in either the spring or summer.

Relative Consumption Rate (RCR)			
Species	May	July	August
Oak	4.48±0.43 [d]	2.20±0.03 [a]	2.72±0.12 [b]
Maple	4.68±0.26 [d]	3.07±0.05 [c]	3.10±0.12 [bc]

Table 2 –Relative consumption rate of oak and maple leaf-feeding larvae in May, July, and August. Statistical comparisons were made using two-way ANCOVA. Different letters represent significant differences between species within months.

Since the GSSG: GSH ratio is a marker for oxidative stress, one would expect that a higher GSSG: GSH ratio would cause the RGR to go down. In Figure six, we see no correlation between RGR and GSSG:

GSH ratios of these leaf-feeding larvae in the summer. However, we do see that that higher GSSG: GSH ratios cause the RGR to fall in the spring. Overall, this graph shows that oxidative stress does not necessarily reduce overall performance, but it could in the spring.

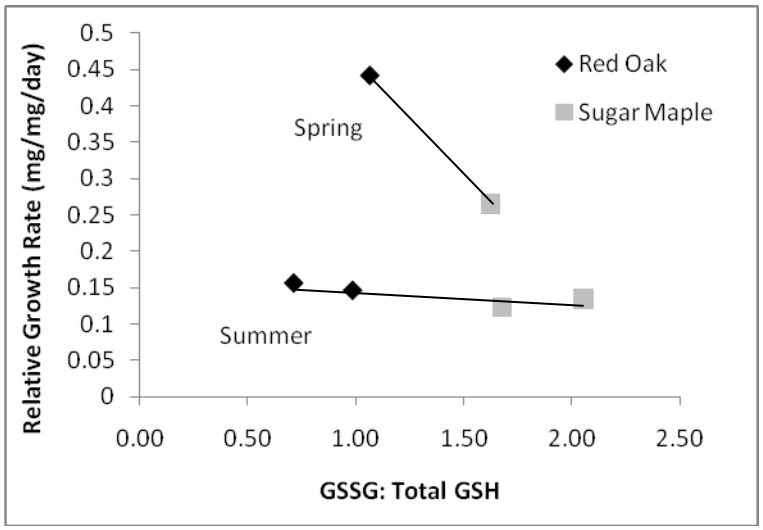

Figure 6 – Correlation Plot showing the relationship between oxidative stress and relative growth rate in *L. dispar* feeding on red oak or sugar maple. Upper line is plotted through spring data, and lower line is plotted through summer data.

DISCUSSION

This study investigated the effects of ingested maple and oak leaves in both the midgut tissues and midgut contents in the leaf-feeding caterpillar, *L. dispar*. Since it is known that maple foliage contains a higher concentration of phenolic compounds than oak foliage, I expected ingested maple foliage to produce more oxidative stress in the caterpillar midgut than ingested oak foliage. Since GSSG: GSH is a marker for oxidative stress, I hypothesized that maple feeders would have higher levels of GSSG: GSH ratios than oak feeders. The data supported this hypothesis, but only for midgut tissues. The GSSG: GSH ratios for the midgut contents did not differ significantly between oak and maple feeders. This observation suggests that the midgut contents are not as useful as midgut tissue in studying oxidative stress specifically using GSSG: GSH ratios as a marker.

This study also determined whether or not glutathione levels, or oxidative stress, changed in the spring versus the summer months. Previous studies investigated how ingested tannins produced oxidative stress in caterpillars feeding in the summer. No previous work was done to investigate the possibility of a seasonal effect on oxidative stress in the caterpillar's consumption of spring versus summer leaves. In the Barbehenn et al. study (2008), both reduced and total glutathione concentrations were higher in the midgut tissues of larvae on red oak versus sugar maple in the summer. In an effort to test the reliability of these results for tent caterpillars, we studied oxidative stress on caterpillars in 2010. I hypothesized that caterpillars feeding in the spring would experience less oxidative stress. I studied the effects of leaf consumption on glutathione ratios both in the midgut tissue and midgut contents in both tree species in May, July, and August. My finding was that caterpillars do not experience more oxidative stress in the spring.

Because of the high level of phenolic compounds in maple foliage, I also hypothesized that maple feeders would not grow as well as oak feeders in both the spring and the summer. A feeding experiment was done in which the relative growth rate was measured in both species across May, July, and August. The results supported my hypothesis, showing a significant decline in performance from the spring to the summer months, in addition to smaller RGR in maple feeders versus oak feeders. The RCR did not affect RGR, so it was determined that the caterpillars' growth was not dependent on the rate of food consumed.

However, while oxidative stress is produced in the midgut tissues of both species, it is not completely responsible for reduced performance in maple feeders in comparison to oak feeders. There may be other components in maple foliage that have negative effects on insect growth. When I analyzed the midgut tissues of larvae feeding on maple leaves using high performance liquid chromatography, I

discovered the presence of a phenolic compound not present in oak feeders (unpublished data). While the identity of this compound is unknown, it may be another source of oxidative stress in maple feeders. It could potentially give rise to the products that react with GSH. Perhaps it is a toxin that decreases RGR but does not cause a rise in GSSG concentration. Future work could be done to identify the compound using mass spectrometry. This could be followed by a dosage experiment on *L. dispar* to determine whether or not the compound affects performance.

GSSG: GSH ratios stayed relatively similar across both the spring and summer months. This result leads one to believe that these leaf-feeding larvae have other ways to escape damage from oxidative stress and maintain normal growth and development. Perhaps the caterpillars can feed more in May because the leaves are lusher. However, I saw that RCR did not impact RGR and I showed that the caterpillars cannot escape plant defenses by feeding in the spring.

I suggest that while oxidative stress is an important factor in insect performance, it is not the only factor involved. More studies could be done to examine what other factors might play a role in insect performance. These could include research on the changes in leaf biochemistry as well as research on the synergistic effect of both oxidative stress and nutritional stress, a condition in which herbivores cannot get the nutrition they need from the foliage they consume**.** Future research should also make comparisons across other tree and herbivore species to be able to generalize about the importance of these results.

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