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Effects of UVB Radiation & Herbivory on Induced Resistance Traits in *Datura Wrightii*

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by

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ABSTRACT OF THE THESIS

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Plants can produce phenolics as a form of protection against ultraviolet B radiation, which act as sunscreens to reduce UVB penetration through the epidermis. Phenolic compounds have also been shown to have deleterious effects against herbivores. This raises questions as to how UVB exposure and herbivory may overlap in the expression of induced plant traits. In this field study I attempt to discern how UVB exposure and herbivory interact in the expression of plant phenolics (rutin and chlorogenic acid), polyphenol oxidase activity, and protease inhibitors in *Datura wrightii*. In the field, cages wrapped in plastic films that differed in UVB transmittance were used to control for UVB exposure. After two or four weeks in the field, treatment plants were exposed to two feeding adult *Lema daturaphila* individuals. Herbivores were removed after 24 hours, and both locally damaged and undamaged systemic tissue adjacent to damaged tissue were subsequently harvested and analyzed. An herbivore performance

bioassay was then used to test for the effects of phenolics on *Lema daturaphila* growth rate. UVB exposure had a significant effect in priming chlorogenic acid, inducing higher concentrations of chlorogenic acid in local and systemic tissue following herbivory. Conversely, herbivory alone induced rutin in locally damaged tissue, but UVB had no effect on rutin induction. Herbivory induced polyphenol oxidase activity and protease inhibitors in locally damaged leaves as well, but UVB did not affect induction of these traits. High concentrations of rutin also had a deleterious effect on the biomass gain of herbviores, while chlorogenic acid had no significant effect. Although UVB had no effect on protease inhibitors on polyphenol oxidase activity, the discrepancies in induction responses found between chlorogenic acid and rutin indicate a clear role for UVB mediated induction in this system.

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Introduction

Plants must cope with a variety of environmental pressures, both biotic and abiotic. Wounding from herbivores, pathogen infection, and stress from drought, salt, and extreme temperatures all activate genetic defensive pathways within plants. Multiple types of stress can interact with one another and in turn influence induced plant traits (Stratmann 2000, Stratmann 2003, Atkinson & Urwin 2012). One such abiotic pressure, UVB exposure, has been shown to play an important role in both causing environmental stress and influencing plant-herbivore interactions (Ballare et al. 1996, Rousseaux et al. 1998, Mazza et al. 1999, Izaguirre et al. 2003, 2007, Foggo et al. 2007, Kuhlman & Mueller 2009, Dinh et al. 2013). Overlaps in induction responses found between UVB exposure and wounding may be explained by UVB causing the accumulation of signaling molecules involved in wound/defense responses such as jasmonic acid (JA), ethylene (ET), and salicylic acid (SA) (Izaguirre et al. 2003, Jenkins 2009). Evidence over the last decade suggests an overlap in JA induced traits in response to both UVB and herbivory, as well as responses that are specific to each type of damage mediated largely through abscisic acid (Conconni et al. 1996, Izaguirre et al. 2003, Izaguirre et al. 2007, Demkura et al. 2010, Atkinson & Urwin 2012). Although induced plant responses have shown both generalized and specific patterns in response to UVB and herbivory, it remains difficult to distinguish between plant responses from a UVB specific pathway as opposed to responses triggered from the accumulation of physical damage (i.e. more generalized signaling pathways) (Jenkins 2009, Dinh et al. 2013).

A consistent induced response to UVB across several plant systems is the accumulation of leaf phenolics. These compounds act as sunscreens to reduce UVB penetration through the epidermis, protecting cells from damage by absorbing UV radiation (Beggs et al. 1985, Li et al. 1993, Middleton & Teramura 1993, Landry et al. 1995, Reuber et al. 1996, Izaguirre et al. 2007, Foggo et al. 2007). UVB exposure can induce phenolic compounds independently from herbivore damage, which may indicate that phenolic induction from UVB exposure is not a JA related response (Izaguirre et al. 2007, Demkura et al. 2010, Dinh et al. 2013). Most studies have analyzed the pool of phenolics being extracted with a solvent rather than individual compounds and/or enzyme activity, which may limit implications regarding specific differences between UVB and herbivore induced responses (Izaguirre et al. 2007).

Phenolic compounds are not only used to combat UVB stress but also have been shown to have defensive functions against herbivores (Haukioja & Niemela 1979, Lindroth & Peterson 1988, Hemming & Lindroth 2000, Ruuhola et al. 2001, Ruuhola et al. 2007, Izaguirre et al. 2007). Individual phenolic compounds can also vary in their effects on insect herbivores (Lindroth & Peters 1988, Roth et al. 1994). For example, phenolic glycosides such as salicin, salicortin and tremulacin have contrasting effects in their influence on gypsy moth larvae performance (Hemming & Lindroth 2000). Phenolic compounds can also change their function due to polymerization by enzymes such as polyphenol oxidases (PPO) (Yang et al. 2007, Ruuhola et al. 2008). Oxidation of phenolic compounds from PPO activity in the insect midgut can produce toxic quinones, which can reduce protein digestibility (Appel 1993, Li & Steffens 2002).

UVB has been shown to induce protease inhibitors (PINS) as well, which typically serve as markers for JA mediated responses (Demkura et al. 2010, Kruidof et al. 2011, Dinh et al. 2013). In *Nicotiana attenuata,* an insect-responsive protease inhibitor (PI) gene was positively regulated by simulated ambient UV-B. Conversely, the PI gene was down regulated by solar UV-B in field grown *Nicotiana longiflora* (Izaguirre et al. 2003). In an analysis of PI protein levels, UV enhances the effect by which herbivores induced PIN synthesis in *N. attenuata* yet PINS were not synthesized in response to wounding or UVB in *N. longiflora (*Izaguirre et al. 2007). UVB alone may not result in significant PIN induction, where as prior UVB irradiation followed by wounding can amplify PIN production, producing different induction responses to multiple stresses in combination compared to induction against the stresses in isolation (Stratmann et al. 2000, Atkinson & Urwin 2012).

Overlaps in plant responses to abiotic and biotic stress may provide ecological benefits of induction that reduce physiological costs associated with mounting a specific response to different pressures. Izaguirre et al. 2007 noted a great deal of convergence in the types of phenolics being produced in two *Nicotiana* species after exposure to UVB and simulated herbivory. Genetically modified plants with an overexpression of protease inhibitors are more tolerant to both salt and drought stress (Srinivasan et al. 2009, Huang 2007). “Choice” and “no choice” bioassays with herbivorous insects demonstrated that ambient UVB exposure can influence insect feeding rates (Ballare et al. 1996, Rousseaux et al. 1998) and larval performance (Izaguirre et al. 2003, Kuhlmann & Muller 2009). Induced plant responses to UVB and herbivory may thus be mediated through the

activation of the same defense signaling cascades (Izaguirre et al. 2003, 2007, Stratmann 2003, Caldwell et al. 2007). In turn, generalized responses to stress may act as mechanisms to "prime" plants against subsequent herbivore attack with amplified induction following herbivory. This could lead to potential additive effects in induction patterns found in plants exposed to multiple pressures at once (Karban & Baldwin 1997, Kessler et al. 2006, Frost et al. 2008, Cipollini & Heil 2010, Rasmann et al. 2012).

The glasshouse is opaque to UVB, emphasizing the potential difficulty of maintaining field plants in a seemingly "uninduced" state, as well as demonstrating the potential dichotomy that may exist between findings in the field and glasshouse. Field studies are necessary to ensure responses to different types of plant stress are in fact realized under natural conditions. Most experiments thus far examining the impact of plant-UVB responses have performed in glasshouse settings with artificial UV sources. As such, UVB emitting lamps used in these experiments may have created spectral ratios between UVB and PAR that do not mirror what we would expect to see under natural conditions (Izaguirre et al. 2003, 2007; Demkura et al. 2010, Dinh et al. 2013). Field experiments that account for more realistic ratios will allow patterns on both induced plant responses and subsequent interactions with the insect community to be interpreted correctly, thereby allowing us to discern what sort of cross talk exists between plant induction in response to different types of stress. Conclusions drawn about the types of responses plants can induce in response to different types of stress cannot be synthesized into an evolutionary context without replicating conditions that we would expect to see in nature.

Assessing how UVB and herbivory induce specific resistance traits in natural settings is necessary to fully understand how these various stressors in turn influence plant-herbivore interactions, as well as the degree by which abiotic and biotic stressors do in fact overlap in induced plant responses. It may be advantageous for plants to produce quantitative and/or qualitative differences in induced compounds (such as phenolics) to effectively combat different types of stresses, both biotic and abiotic. Alternatively, UVB exposure may be advantageous if plants are able to prime for future exposure to herbivory.

Induced plant responses can also occur both in locally damaged tissue and in undamaged systemic leaves, i.e. leaves distant from the area of leaf damage (Karban & Baldwin 1997, Orians 2005). Damage can result in long distance transport of signaling molecules that produce induced changes in distant leaves (Zhang & Baldwin 1997, Stratmann 2003). However, Orians (2005) suggests that species specific vascular restrictions may result in proximately located leaves that are in opposite orthostichies, implying that vascular signals may fail in reaching all parts of a plant. In addition to vascular architecture, differences in phloem transport and volatile production may also influence induced responses in systemic tissue (Orians 2005).

Locally, jimsonweed (*Datura wrightii,* Solanacae) is fed primarily upon by the specialist herbivore *Lema daturaphila*. Previous work on this system indicated that ambient levels of UVB over the course of the growing season were capable of inducing low levels of protease inhibitors in the absence of herbivory (Kruidof et al. 2011). As PINS are typically used as a marker for JA mediated responses, I predicted that UVB

exposure would both a) induce phenolic compounds independently of herbivory in accordance to studies using closely related systems and b) induce JA mediated responses (PINS, PPO) in the absence of herbivory. I thus predicted that UVB exposure would trigger both JA-dependent and JA-independent pathways in the expression of induced defensive traits. Preliminary dye uptake assays have shown poor vascular connectivity in leaves one node proximate to damaged leaves (Hare & Sun unpublished data). I therefore predicted the measured induced traits would be specific to locally damaged tissue and not undamaged systemic tissue in the absence of UVB. UVB independently induced responses, however, should show a similar response between "local" and "systemic" tissue as both leaves are undamaged and receive the same amount of UV exposure in this treatment. Specifically, the phenolic compounds rutin and chlorogenic acid were analyzed, as well as both cysteine and serine protease inhibitors and polyphenol oxidase activity. Finally, the effect of the phenolic compounds rutin and chlorogenic acid on *L. daturaphila* relative growth rate was measured. Given that I predicted rutin and chlorogenic acid would be induced due to UVB, I also predicted they would play a role in UVB rather than anitherbivore defense and thus have little effect on *L. daturaphila* performance.

Materials & Methods

Study System

Fifth generation backcrossed *Datura wrightii* plants of the BCV7 line were grown in the glasshouse. *D. wrightii* is a local perennial plant and is adapted to xeric conditions. *D. wrightii* exhibits two leaf trichome phenotypes within southern California (van Dam & Hare 1998a). The "velvety" phenotype is densely covered with short, non-glandular trichomes while the "sticky" phenotype is less densely covered with glandular trichomes that secrete esters of aliphatic acids and glucose (van Dam et al. 1999). Fifth generation backcross progeny of heterozygous sticky progeny were backcrossed to their original velvety pollen parent. The ratio of adult heterozygous sticky plants to homozygous recessive velvety sibs is 1:1, and the two phenotypes do not differ in their levels of induction (Hare & Walling 2006). After five generations of backcrossing, sibs are expected to be at least 98.4% similar, thereby minimizing variation in responses (Hare 2011). 80 plants were transplanted into the field at Agricultural Operations at the University of California, Riverside. 20"x20"x20" cages were constructed using PVC pipe and placed over the plants. Cages were wrapped with two types of plastic for the exclusion of herbivores/UVB—polyethylene, which does not filter UVB radiation, and polyester which does (Kruidof et al. 2011, Figure 1). A mesh screen was placed on the north side of each cage to allow proper ventilation. This prevented any microclimate from forming within the cages and did not interfere with the plant's growth or photosynthetic activity (Demkura et al. 2010, Pers. Obs.). The mesh side of the cage was also oriented to face north and as such positioned away from ambient solar radiation. Treatment plants

were exposed to herbivory, with undamaged control plants. Treatments plants were therefore either exposed to herbivory in the absence of UVB, exposed UVB in the absence of herbivory, or exposed to both UVB and herbivory. There were undamaged control plants as well that were not exposed to UVB.

Plants were sampled during two fixed exposure periods, thus, plants varied in age during sampling periods. The label "young plants" refers to plants that were sampled after two weeks of ambient UVB exposure while "old plants" refers to plants exposed for four weeks (and were thus two weeks older than the plants that were previously sampled). Plant age and UVB exposure are necessarily confounded. Plants sampled after four weeks were beginning to produce flowers; those sampled after two weeks were not. There were 10 plants in each of the above four groups, thus, 40 plants were used for each sampling period.

Exposure to Herbivory & Leaf Tissue Sampling

After the plants in each exposure period treatment were exposed to the appropriate amount of UVB, two adult *Lema daturaphila* (Coleoptera) larvae were placed on a single leaf on plants assigned to the herbivory treatment. The beetle feeds on locally grown *D. wrightii* and is an ecologically relevant herbivore. The adult insects were restricted to feeding on a single leaf using individual mesh bags. Herbivores were allowed to feed for 48 hours. Leaf tissue was harvested an additional 24 hours following the removal of herbivores to ensure induction could occur (Karban & Myers 1989). Upon removing the herbivores, locally damaged leaf tissue was cut from the stem with a razor

blade and immediately flash frozen in liquid nitrogen for preservation and to stop all metabolic processes. Undamaged leaves at the node above and adjacent to the locally damaged leaf were harvested at this time to assess whether there was a systemic effect in induction. Leaf tissue was ground over liquid nitrogen using mortar and pestle and stored at -80° C in labeled 50 ml centrifuge tubes.

Polyphenol Oxidase

PPO activity was measured following the methods of Stout et al. 1998 and Thaler et al. 1996. Roughly 100 mg of flash frozen ground leaf tissue was placed in labeled 1.5 ml microcentrifuge tubes. 1.0 ml of ice cold pH 7 potassium phosphate buffer containing 7% suspended PVPP was added to each sample. Samples were homogenized using the PTFE pestle and periodically cooled over ice. 0.3 ml of 10% Triton X-100 was added to the homogenized samples. Samples were vortexed and subsequently centrifuged at 12,000 rpm for 5 minutes at 4°C. Supernatant containing enzymes was stored over ice or in the refrigerator.

Polyphenol oxidase assays were performed using a spectrophotometer set at wavelength 470 nm. A "blank" was prepared by adding 30 microliters of extraction buffer to a disposable cuvette then 1.0 ml of freshly prepared 2.92 mM caffeic acid in pH8 potassium phosphate buffer. The blank was measured and placed aside in case of need to re-measure.

Samples were prepared for PPO analysis by adding 30 µl of centrifuged plant extract to a disposable cuvette, followed by 1.0 ml of freshly prepared 2.92 mM caffeic

acid in pH 8 postassium phosphate buffer. Readings were taken every second for 30 seconds at 470 nm. Final enzyme activity was reported as the change in absorbance per minute per gram of the original leaf tissue.

Phenolic Extraction & Identification

Phenolics compounds were extracted following Anttila et al. 2010. Briefly, 100 mg of ground flash frozen leaf tissue was transferred to labeled 1.5 ml Eppendorf tubes. 600 µl of 100% methanol (MeOH) was added to the sample, and samples were homogenized using a PTFE pestle for 30s. Samples were subsequently vortexed and centrifuged (10 min, 12,000g), and the supernatant was collected. This was done three times for each sample. Supernatant was pooled and evaporated under a stream of nitrogen flow. Prior to high pressure liquid chromatography (HPLC) analysis, evaporated samples were dissolved in 50% MeOH. Samples were also filtered using 0.45µm nylon membrane discs to ensure there were no particulates.

Samples were analyzed following Keinanen et al. 2001. Briefly, a Hewlett-Packard HP 1100 series liquid chromatograph with a quaternary pump, helium degasser, autosampler, a thermostated column compartment, and a diode array detector was used for the analysis of phenolics. Separations of phenolic compounds were carried out using a 4.6 mm x 7.0 cm Beckman 'Ultrasphere XL' C_{18} column, 3 µl particle size. The solvents used were (A) 0.25% H_3PO_4 in water (pH=2.2) and (B) acetonitrile. The elution system was as follows: 0-5 min, 30% B; 5-10 min, 60% B; 10-15 min, 90% B; 15-20 min, 100% B. The flow rate was 1.2 ml/min with an injection volume of 20 µL. The elutent was

monitored at 230, 305, and 320 nm. Retention times and UV-vis spectra of the compounds of interest were compared with those of authentic reference compounds.

Protein Extraction & Quantification

Protein was extracted by adding 1 mL of cold extraction buffer (50 mM sodium phosphate buffer, pH=7.2, containing 150 mM sodium chloride, 2.0 mM EDTA, 2 mg/ml phenylthiourea, 5 mg/ml diethyldithiocrbamate, 2.0 mM EDTA, 2mg/ml phenylthiourea, 5 mg/ml diethyldithiocarbamate, and 50 mg/ml PVPP) to 100-150 mg of ground flash frozen leaf tissue stored in labeled 1.5 ml Eppendorf tubes. Samples in buffer were homogenized over ice using a PTFE pestle. Samples were then centrifuged (30 min, 13,000g) and supernatant containing protein was stored at -80° C until further analysis.

Total protein quantification was done using the Bradford assay (Bradford 1976). Sample extracts were diluted 1:1 in 0.1 M TRIS, yielding a protein concentration between 0.5 and 0.033 mg/ml. 200 µL of Bradford reagent was added to 10 microliters of each sample in TRIS. The samples were incubated at 595 nm and measured using a multi-detection microplate reader. A calibration curve was determined on the same plate using immunoglobulin standards ranging between 0.033 mg/ml-5 mg/ml in 0.1 M TRIS buffer. Standards and multiplate analysis were performed based on the manufacturer's instructions (Bio-Rad for instructions on microplate analysis preparation and Micro-Tek for the analysis of data on the plate reader).

Protease Inhibitor Quantification

Serine and cysteine protease inhibitors were measured using a microplate assay (Bode et al. 2013). Different types of PINs function against the different protease types expressed within insect guts. Serine PINs are predicted to be more effective against lepidopteran herbivores, and cysteine PINs more effective against coleopteran herbivores (Bode et al. 2013). Serine PIN activity was measured by combining 20 µL of reaction buffer (0.1 M TRIS, ph 7.6), 10 µL of 0.5 mg/ml typsin in 0.1 M TRIS, and 20 µL of sample extract into a microplate. Samples were mixed in the microplate using a pipette, then incubated at 35.7°C for 5 min using a digital electronic incubator. After the initial incubation, 20 µL of 3.1 mg/mL *N*-benzoyl-DL-arginine-b-napthylamide (BANA) in dimethyl sulfoxide (DMSO) was added to each microplate sample and incubated for an additional 20 minutes at 35.7°C. To each sample, 2% hydrochloric acid in ethanol was added to terminate the reaction. Finally, 100 µL of 0.06% p-dimethylaminocinnamaldehyde in ethanol was added to each sample, and the dye reaction developed for 30 minutes at room temperature. Absorbance was recorded afterwards at 540 nm using a microplate reader. Soybean trypsin inhibitor standards were run on the same plate, as well as a positive and negative control (trypsin and no sample, no trypsin and no samples, respectively). A calibration curve was then used to quantify serine PIN concentrations, expressed as nanomoles per mg total protein.

Cysteine PINS concentrations were determined through a similar microplate technique. A 0.25 M sodium phosphate buffer (pH=6.0) with 2.5 mM EDTA and 0.1% b-mercaptoethanol was used as the reaction buffer. Papain (0.25 mg/ml papain in sodium

phosphate buffer, pH=6.0) was used as the protease enzyme. All other conditions, including incubation times and the level of sample to other reactants, were unchanged from the serine PIN method. Again, a negative and positive control were run on each microplate, along with E-64 standards of known concentrations (Sigma). The E-64 standard is an epoxide and commonly used cysteine protease inhibitor (Zhao et al. 1996). These were used to create a calibration curve that was used to calculate final concentrations of cysteine PINS, which were expressed in nanomoles per mg total protein.

Herbivore Performance Bioassay

Herbivore performance was assessed following the techniques outlined in Roth et al. 1994. Excised *D. wrightii* leaves were placed in Aqua Pics ® to maintain turgor. Leaves were coated with various concentrations of rutin and chlorogenic acid in 100% methanol. Concentrations were ecologically relevant and based on low, moderate, and high values found through the HPLC analysis (see results). Pure standards of both compounds were dissolved in 100% methanol, pipetted onto the excised leaves, and allowed to dry prior to herbivore exposure. Liquid extracts of the standards were spread evenly over the leaves to ensure the entire leaf was covered with the phenolic compound of interest. Control leaves were treated with 100% methanol and allowed to dry, as described above.

Lema daturaphila larvae were captured from the field and maintained in a colony fed on *Datura wrightii*. Adult insects were captured from the field, then brought into the

lab and fed using 5th generation backcrossed *D. wrightii* of the same line used in the field experiments. Egg clutches from the following generation were then placed on a *D. wrightii* plant in a separate cage. Each block used in the performance assay used larvae from the same clutch to control for genotypic variation that may lead to differences in performance. Blocks consisted of 3 leaves per each treatment, and there were 5 blocks total (n=105, 15 leaves in each treatment/control). Treatments included low, moderate, and high levels of chlorogenic acid and rutin, as well as control leaves painted with 100% methanol. A single newly molted second instar larvae was placed on each coated leaf and allowed to feed for 5 days. Total biomass gain and relative growth rate (RGR) of the larvae was measured over the 5 day feeding period (Roth et al. 1994). RGR calculated as ln(final weight)-ln(initial weight)/time.

All leaves used in the herbivore performance assays were extracted and analyzed by HPLC to determine final concentrations of rutin and chlorogenic acid. These values included both intrinsic concentration and extrinsic phenolic additions. Extraction and HPLC protocols were identical to those used in the phenolic extraction and quantification detailed previously.

Statistical Analysis

Analyses were performed using R version 3.0.3 with the lme4 and multcomp packages (Hothorn et al 2008, R Core Team 2013, Bates et al. 2014). All analyses were performed on aliquots of tissue from the same leaf, therefore the dependent variables could have been correlated. To account for the possibility of these correlations, a

MANOVA was first used to determine the effects of UVB, herbivory, and plant age on induction, using the c-bind function in R to group the dependent variables (rutin, chlorogenic acid, serine PINS, cysteine PINS, and PPO). Three way ANOVAs were then used for each of the analyses of individual dependent variables (PPO, Rutin, Chlorogenic Acid, Serine PINS, Cysteine PINS) with UVB, herbivory, and plant age as treatment effects. Data for cysteine PINS, serine PINS, and PPO were found to have zeroes in cases where induction of pins did not occur. To improve the homogeneity of variance in the dataset, data were log +1 transformed for these three independent variables. Residuals were found to be normal using a Shapiro-Wilk's test. Local and systemic leaf analyses were performed independently. Post-hoc analyses were performed using Tukey's HSD to assess differences in means across treatments, as well as between local and systemic leaves.

Larval total biomass gain was analyzed using ANCOVA with consumption and initial mass as covariates and total biomass gain as the dependent variable following the methods of Sorensen et al. 2010. RGR was also analyzed with ANCOVA using consumption and initial mass as covariates. Baseline levels of the non treatment compound's effect on herbivore performance were included in the model as well (i.e, baseline rutin in chlorogenic acid manipulated leaves and vice versa). The effect of phenolic concentrations on *L. daturaphila* total consumption was tested using an ANCOVA with initial mass as a covariate.

Results

Effects of Herbivory, UVB, and Plant Age on Induction

In locally damaged tissue, the results of the MANOVA showed that UVB, herbivory, and plant age all had highly significant effects on overall *D. wrightii* induction ($p<0.001$, Table 1). The interaction between UVB and plant age was also significant ($p<0.01$, Table 1), indicating that the differential effects of UVB exposure accumulated over time. However, the interaction between UVB and plant age was not significant for any of the dependent variables when analyzed individually (Table 2).

In the analysis of systemic leaf tissue, plant age was highly significant on induced responses ($p<0.001$, Table 1). Herbivory was significant ($p<0.05$, Table 1). UVB was highly significant ($p<001$, Table 1); although, both local and systemic leaves received equal amount of UVB exposure. UVB therefore did not cause any sort of specific "systemic" effect (Table 1, Figure 2, Figure 3, Figure 4). The interaction between herbivory and plant age was significant as well ($p<0.05$, Table 1). Induced responses in systemic tissue due to herbivore damage therefore varied over time.

Effects of Herbivory & UVB on Individual Phenolic Compounds

The effects of *Lema daturaphila* herbivory and UVB exposure on phenolic concentration differed between the phenolic compounds rutin and chlorogenic acid. In locally damaged tissue, herbivory induced rutin in significantly higher concentrations than undamaged tissue ($p<0.01$, Table 2 Test #1, Figure 2). Plant age was significant ($p<0.001$, Table 2 Test #3), as was the interaction between herbivory and plant age, with

rutin induction decreasing significantly in older plants ($p=0.03$, Table 2 Test #5). UVB did not significantly induce levels of rutin. Herbivory also induced rutin in locally damaged tissue in significantly higher concentrations than systemic tissue ($p<0.01$, Figure 2). The effect from herbivory on rutin concentration in locally damaged leaves changed with plant age as well, again inducing lower concentrations of rutin in older plants (Table 2 Test #5, Figure 2).

UVB and the interaction between UVB and herbivory significantly increased chlorogenic acid in locally damaged tissue compared to undamaged tissue ($p<0.001$, Table 2 Test #9&#11, Figure 2). Herbivory alone did not produce significantly greater concentrations in locally damaged tissue compared to undamaged control leaves ($p=0.142$, Table 2 Test #8, Figure 2). Again, plant age was found to be significant ($p<0.001$, Table 2 Test #10). Similar to rutin, chlorogenic acid was induced in lower concentrations in older plants (Figure 2). The combination of herbivory and UVB also induced chlorogenic acid in significantly higher concentrations in locally damaged leaves compared to systemic leaves ($p<0.01$, Figure 2). This indicates that the priming effect associated with chlorogenic acid was more pronounced in damaged tissue than systemic tissue.

In the analysis of systemic leaves, plant age was found to be significant for both rutin and chlorogenic acid, with lower concentrations of both phenolic compounds again in older plants ($p<0.001$, Table 2 Test #3&#10). UVB had a significant effect on chlorogenic acid concentration in systemic tissue ($p<0.01$, Table 2 Test #9). Local

damaged leaves and systemic leaves responded similarly to UVB exposure in the induction of chlorogenic acid due to having the same level of exposure.

Effects of Herbivory & UVB on Protease Inhibitor Activity

In locally damaged tissue, herbivory had a significant effect on both serine and cysteine protease inhibitor concentrations ($p<0.001$, Table 2 Test #15&#22, Figure 3). Plant age was found to be significant for both serine and cysteine protease inhibitors, with PIN concentrations increasing in older plants independent of UVB exposure ($p=0.031$, $p=0.034$, respectively, Table 2 Test #17&#24). Neither UVB, or the ineteracitons between UVB and herbivory had a significant effect on the accumulation of either type of protease inhibitors.

In systemic tissue, herbivory had a significant effect on the accumulation of cysteine PINS ($p<0.01$), but not serine ($p>0.05$, Table 3). Plant age was significant for both protease inhibitors ($p=0.045$, $p=0.034$, respectively, Table 2 Test #17, Test #24, Figure 3). Mean concentrations were overall greater in older plants for both protease inhibitors. Mean systemic effects were also greater for serine PINS in older plants, again independent of UVB exposure ($p<0.01$, Figure 3).

Herbivory alone resulted in marginal significance in serine PIN induction in locally damaged leaves compared to systemic leaves in young plants, indicating that serine PIN induction was higher in locally damaged tissue than in undamaged systemic tissue for this treatment ($p=0.09$, Figure 3). Herbivory alone was also significant in inducing serine PINS in greater concentrations in locally damaged tissue compared to

systemic tissue in older plants ($p=0.03$, Figure 3). The combination of UVB and herbivory induced marginally greater serine PIN concentrations in locally damaged tissue compared to systemic tissue in old plants only ($p=0.078$, Figure 3). Herbivory alone induced cysteine PINS in significantly higher concentrations in locally damaged leaves compared to systemic leaves for both young and old plants ($p<0.05$, Figure 3). UVB + Herbivory produced significantly higher concentrations of cysteine PINS in locally damaged tissue compared to systemic tissue in young plants ($p<0.05$, Figure 3).

Effects of Herbivory & UVB on Polyphenol Oxidase Activity

In locally damaged tissue, herbivory significantly increased PPO activity ($p<0.001$, Table 2 Test #29). Additionally, the interaction between UVB and herbivory significantly increased PPO activity, ($p<0.01$, Table 2 Test #32, Figure 4). Unlike protease inhibitors and the phenolic compounds, plant age was not significant (Table 2 Test #31, Figure 4). Herbivory, UVB and plant age did not affect polyphenol oxidase activity in systemic tissue (Table 2 Test#29-35, Figure 4). Furthermore, increased PPO induction was specific to damaged tissue and independent of both plant age and exposure to UVB.

Effect of Rutin and Chlorogenic Acid on Lema Daturaphila Performance

Rutin concentration had a significant negative effect on *L. daturaphila* biomass gain ($p<0.01$, Table 3, Figure 5a). Chlorogenic acid had no significant effect on *L. daturaphila* biomass gain (Table 3, Figure 5b). Consumption was not found to be

significant in predicting the amount of biomass variation for both chlorogenic acid and rutin treatments, and was thus excluded from the model. Initial weight was significant for both treatments, and was thus included in both models ($p<0.05$, Table 3, Figure 5a,b). Larvae that were larger initially gained more mass than smaller larvae. Baseline concentrations of the intrinsic phenolic compounds did not affect total gain. Seperate models were run using RGR as the dependent variable as well, however similar results were produced in all cases. The effect of initial mass became nonsignificant, as expected (Table 3).

There were no significant differences in the total leaf area removed (i.e. consumption) between treatments (Table 3, Figure 6). Initial weight was not found to be a significant predictor of consumption. This finding implies that *L. daturaphila* fed at a similar rate across all phenolic treatments.

Discussion

Effect of Plant Age on Induced Responses

Plant age was found to be significant for rutin, chlorogenic acid, serine PINS and cysteine PINS, but not PPO (Table 2 Test #5). The differences in the measured induced traits across plant ages in the present study are difficult to explain, more specifically, the variation across treatments in serine PIN induction (Figure 3). Consistent with previous findings (Kruidof et al 2011), overall PIN production increased over time. Conversely, rutin and overall phenolic concentration decreased with age (Table 2, Figure 2). The interaction between UVB and plant age was not significant for any of the measured induced responses alone, suggesting that the differences found between individual induced responses over time in this study were occurring independently of exposure to UVB (Table 2). This seems likely given that changes in induction over time are not specific to the UVB treatment for any of the dependent variables (Figure 2, Figure 3, Figure 4). Conversely, the results of the MANOVA indicate a significant relationship between UVB and plant age (Table 1). This may be due to obtaining more information from the group of dependent variables than any one alone. This experiment was relatively short term, and an experiment over the entirety of the growing season that accounted for long term differential exposure would help to clarify how induced patterns vary over time.

Changes in induction could be related to ontogenetic stages of the plant. In *D. wrightii,* induced volatiles are greater when growing vegetatively than when flowering (Hare 2010). Although only two weeks separated the "young" and "old" plants in this

study, older plants were producing flowers and the younger plants were not. The findings here thus suggest that older, flowering *D. wrightii* plants lose some capacity to produce high quantities of phenolics, but gain the ability to produce higher quantities of PINS. Inducible traits in *Datura wrightii* may vary independently with plant ontogeny. The structure of the herbivore community varies in space and time on *D. wrightii* and the changes in patterns of induction with plant age will therefore vary with herbivore community (Elle & Hare 2000, Hare & Elle 2002). This suggests that plant age is a complicating factor in field based induction experiments, as the effects of certain treatments can vary dynamically as plants shift from vegetative to flowering states.

Effects of UVB & Herbivory on Phenolics

Although phenolic compounds as a class have been shown to have both UVB protective and antiherbivore properties across a range of systems, few studies have analyzed the effects that individual compounds may have (Izaguirre et al. 2003, 2007)**.** I had predicted that UVB exposure, independent of herbivory, would result in increased levels of rutin and chlorogenic acid, similar to how the compounds are induced in the closely related species *Nicotiana attenuata* (Demkura et al 2010, Dinh et al 2013). The results here demonstrate that the two phenolic compounds respond more independently rather than jointly to UVB exposure and herbivory in this system. This suggets that single compounds can vary in their patterns of induction. Herbivory induced rutin in locally damaged tissue, although exposure to UVB did not have any significant influence on rutin concentration (Fig 2a,b). Conversely, herbivory alone had no effect on chlorogenic

acid induction. UVB exposure, however, caused a significant "priming" effect in the accumulation of chlorogenic acid in locally damaged and systemic tissue (Fig 2c,d). The priming effect may minimize costs of induced responses to specific herbivores or abiotic pressures (Cipollini & Heil 2010, Kruidof et al. 2011). As expected, herbivory had no significant effect on either compound systemically (Table 2, Figure 2). This may be due to the absence of a vascular connection between these leaves (Orians 2005, Sun & Hare unpublished data).

In addition to the disparity in patterns of induction, rutin and chlorogenic acid demonstrated different effects on *Lema daturaphila* performance. Increasing rutin concentration led to diminished *L. daturaphila* performance, yet chlorogenic acid did not have an effect (Table 3, Figure 5a,b). In addition, the percent leaf tissue removed by herbivores did not differ significantly across levels of phenolics and control tissue, indicating that diminished *L. daturaphila* performance may not be due to diminished consumption (Table 3, Figure 6). In this study, *L. daturaphila* fed uniformly across all phenolic concentrations, with no differences between phenolic coated leaves and control leaves (Table 3, Figure 6). As feeding rates are not changing, any diminished effects on *L. daturaphila* performance could therefore physiological in response to phenolic concentration, although further testing would be necessary to confirm.

Several studies have shown phenolic compounds have antiherbivore effects (Haukioja & Niemela 1979, Lindroth & Peterson 1988, Stamp & Yang 1996, Hemming & Lindroth 2000, Ruuhola et al. 2001, Ruuhola et al. 2007, Izaguirre 2007, Anttila et al. 2010). In lima bean, chlorogenic acid had no deleterious effects on 5th instar southern

armyworm performance, although rutin caused some mortality. Rutin also reduced growth rates by decreasing consumption and digestion efficiency in southern armyworm larvae (Lindroth & Peterson 1988). Chlorogenic acid causes depletion in ascorbic acid levels in the generalist *Helicoverpa zea* larvae (Summers & Felton 1994), but had no deleterious effects on southern armyworm performance (Lindroth & Peterson 1988). When added to artificial diet, both chlorogenic acid and rutin inhibited larval growth in the fruit worm, *Heliothis zea*. Later instar larvae, however, show no adverse effects when feeding on these compounds (Isman & Duffey 2011). Antiherbivore properties associated with phenolic compounds therefore vary across plant systems and feeding herbivore.

Rutin and chlorogenic acid can act as protectants from UV exposure as well. In *Nicotiana attenuata*, rutin and chlorogenic acid are induced in response to ambient UVB, while herbivory in the absence of UVB is unable to induce these compounds in levels significantly higher than undamaged control plants (Demkura et al. 2010). Induction of these compounds did not occur in JA-deficient mutant plants, implying a non-JA mediated response (Demkura et al. 2010). Interestingly, Izaguirre et al. (2007) found that chlorogenic acid was induced by *Manduca sexta* oral secretions in *N. attenuata* in the absence of UVB exposure. Patterns of phenolic induction may therefore be variable based on the inducing agent (i.e. the type of herbivore oral secretions used to induce plants), and induction of different phenolic compounds may be herbivore specific. In *D. wrightii,* UVB exposure appears to influence the induction of chlorogenic acid, with an amplified response following herbivory (Figure 2, Table 4). The role of the phenolic compound in direct UVB defense in this system, however, remains unclear.

My findings indicate that chlorogenic acid did not have a deleterious effect against *L. daturaphila* performance (Figure 5). The compound did not have increased induction due to herbivory alone, but was induced from the combination of UVB and herbivory (Figure 2c,d). However, chlorogenic acid may have antiherbivore properties specific to other insect herbivores, and can be induced from herbivory alone in the absence of UVB in a number of other plant systems (Lindroth & Peterson 1988, Summers & Felton 1994, Kranthi et al. 2003, Izaguirre et al. 2007). There are at least ten other insect species that attack *D. wrightii*, including flea beetles, hornworms, leafhoppers, and whiteflies (van Dam & Hare 1998a,b; Elle et al. 1999, Elle & Hare 2000, Hare & Elle 2002). In addition to producing constitutively elevated levels of chlorogenic acid when exposed to ambient UVB, field grown *N. attenuata* also had lower infestation rates of *Tupiocoris notatus* (Dinh et al. 2013). *T. notatus* commonly feeds on local *D. wrightii* as well, thus, it is possible that chlorogenic acid may have antiherbivore properties specific to *T. notatus* in this system.

The tobacco hornworm *Manduca sexta* (Lepidoptera: Sphingidae) is another naturally occurring herbivores on *Datura wrightii* in southern California (Casey 1976). In other systems and independent of other phenolic compounds/conditions, chlorogenic acid concentration has not been negatively correlated with *Manduca sexta* performance (Bi et al. 1997, Traugott 1997, Dinh et al. 2013). The combination of chlorogenic acid with other allelochemicals, however, has been shown to have negative effects on *M. sexta* performance, and effects from the compound can also vary under different thermal regimes (Osier et al. 1996, Stamp & Yang 1996). Under warmer temperature regimes,

rutin, chlorogenic acid, and tomatine were shown to have an effect in delaying *M. sexta* development (Stamp & Yang 1996). Conversely, there was no significant interaction between thermal regime and chlorogenic acid on *Spodoptera exigua* larvae, again demonstrating the potential for herbivore specific effects (Stamp & Osier 1997). In *N. attenuata,* the silencing of two major tobacco phenolamides, which are precursors to rutin and chlorogenic acid, produced from the phenylpropanoid pathway resulted in increased *M. sexta* performance (Kaur et al. 2010). Antiherbivore properties associated with phenolic compounds can vary across both systems and feeding herbivores, and effects can be species specific. When primed from UVB, induced response of chlorogenic acid in *D. wrightii* may be a general response to herbivory, with antiherbivore properties associated with other herbivores in this system. It would be premature to rule out chlorogenic acid related antiherbivore properties in this system without first running herbivore performance studies on other *D. wrightii* native herbivores

Effects of UVB & Herbivory on PPO

The biological effects of several phenolic compounds are dependent on their transformation to quinones by plant PPOs, which may vary across systems and subsequently lead to variation in antiherbivore properties and patterns of induction (Keinanen et al. 2001, Li & Steffens 2002, Demkura et al. 2010). Disruption of the plant cell by feeding herbivores generally releases chlorogenic acid from vacuole in the leaf tissue, and the compound is immediately attacked by peroxidase and polyphenol oxidase (Butt 1981, Felton et al. 1989, Duffey & Felton 1991, Keinanen et al. 2001). The

quinones produced due to oxidation of phenolic compounds by PPOs may possess anti-nutritive effects against insect herbivores, depreciating protein quality in the consumed leaf tissue (Mayer & Harel 1979, Peter 1989, Duffey & Felton 1991). Phenolic compounds can also vary in their effectiveness as a substrate for PPO. In tomato, chlorogenic acid was found to be a good substrate for PPO activity, while rutin was poor (Duffey & Felton 1991).

The oxidation of phenolic compounds from PPO activity can be beneficial against insect herbivory and pathogens (Duffey 1989, Duffey & Felton 1991). Transgenic lines of tomato with an overexpression of PPO oxidized phenolics at a higher rate than wild type control plants and subsequently performed better against infection (Li & Steffens 2002). In the present study, herbivory induced PPO activity in locally damaged tissue, but UVB had no effect on induced PPO (Figure 4). The induction of PPO in response to insect herbivory is in accordance with previous findings in this system, in which treatment with methyl jasmonate resulted in increased levels of PPO activity (Hare & Walling 2006). In addition, the antiherbivore properties in chlorogenic acid have been shown to be largely dependent on oxidation from PPO (Duffey & Felton 1991). The insect herbivores *Heliothis zea* and *Spodoptera exigua* both commonly feed on tomato foliage. Ingested chlorogenic acid in tomato leaf tissue is oxidized by PPO within the insect gut, producing strong antinutritive effects on the feeding larvae (Felton et al. 1989).

PPO activity acts as a marker for JA mediated responses, and it was predicted that UVB alone would induce low levels of PPO activity due to UVB exposure causing JA

accumulation (Izaguirre et al. 2003, Izaguirre et al. 2007, Hare & Walling 2006, Kruidof et al. 2011, Dinh et al. 2013). In the present study, both chlorogenic acid and PPO activity were induced in greater concentrations following UVB and herbivory in comparison to undamaged tissue, and these two responses could therefore be acting synergistically (Figure 2c,d, Figure 4). Chlorogenic acid was not induced from herbivory alone where as PPO activity was in locally damaged tissue (Figure 2c,d, Figure 4). It is possible that the responses are correlated, meaning that chlorogenic acid induced from herbivory alone had been oxidized by the increased PPO enzymatic activity and thus was not detected (Keinanen et al. 2001). If this were true, chlorogenic acid induction from UVB+Herbivory would be expected to be greater than reported as well. I had predicted that chlorogenic acid would be induced in response to UVB due to having a role in defense against UVB exposure. As such, the potential role of chlorogenic acid, and more specifically its transformation into quinones, was not expected based on findings in closely related species (Demkura et. al 2010, Dinh et al 2013). Measuring quinones in addition to chlorogenic acid and PPO activity may have given a better representation of the potential pattern of chlorogenic acid induction.

Effects of UVB & Herbivory on Proteinase Inhibitors

It was initially predicted that in this system, UVB would trigger JA signaling and thus induce low levels of PINS in the absence of herbivory (Kruidof et al 2011). My findings indicate that although herbivory induced serine and cysteine PINS in locally damaged tissue, UVB alone did not induce PINS significantly, and the combination of

UVB and herbivory did not induce a "primed" response (Figure 3). The lack of an effect from UVB on PIN induction in *D. wrightii* is in contrast with the results from UVB exposure experiments using other plant systems (Conconi et al. 1996, Izaguirre et al. 2007, Demkura et al. 2010, Dinh et al. 2013). The effects of UVB on PIN induction have been found to vary across individual plant species tested under different experimental conditions. UVC and UVB from artificial lighting sources had a strong effect on PIN gene expression in tomato (Conconi et al. 1996, Stratmann et al. 2000). Conversely, ambient doses of UVB exposure resulted in increased PIN gene expression following herbivory in damaged tomato and tobacco leaves, but UVB exposure alone was not sufficient in inducing PIN concentrations that differed significantly from undamaged & unexposed control plants (Stratmann et al. 2000, Izaguirre et al. 2003, Demkura et al. 2010, Dinh et al. 2013). Moreover, there is a great deal of variation in results not only across systems, but also within systems using altered experimental protocol.

Mechanistically, ambient UVB exposure can increase JA sensitivity in other systems, thus producing elevated levels of PINS and enhanced expression of trypsin protease inhibitor genes (Demkura et al. 2010). UVB thus "primes" protease inhibitor induction in several plant species, notably tomato and tobacco (Stratmann et al. 2000, Stratmann 2003, Izaguirre et al. 2007, Demkura et al. 2010). Additional field studies on *N. attenuata*, however, found that both UVB exposed and non exposed plants elicited similar levels of PINS following the application of herbivore oral secretions (Dinh et al. 2013). Lower transcript abundances of PIN expression genes in UVB exposed plants are also not directly correlated with lower PIN activity in field grown *N. attenuata*, implying

that post transcriptional modifications may be critical for PIN defense activation (McManus et al. 1994, Dinh et al. 2013). My findings are consistent with PIN activity in field grown *N. attenuata* that used similar experimental condition, in which UVB and non UVB exposed plants did not differ significantly in PIN induction following herbivory (Dinh et al. 2013). When coupled with the results shown in *D. wrightii,* the variation found across multiple systems further indicates that PIN induction cannot be considered a generalized response to UVB exposure, and is highly dependent on both the system and experimental protocol being used.

Role of UVB in Glasshouse vs Field Studies

The glasshouse is opaque to UVB, and studies performed using artificial lighting can obstruct the UVB:PAR ratio found in nature and exacerbate the effects of UVB exposure as a result of altered PAR, which may both plant and herbivore behavior (Caldwell & Flint 1994, Caldwell et al. 2007, Kakani et al. 2003 Demkura et al. 2010, Dinh et al. 2013). More specifically, artificial lighting used under glasshouse/growth chamber conditions can produce UVB radiation that is of higher intensity that would is found under natural ambient exposure. This increases the backscattered component of PAR and affects the depths to which PAR penetrates leaf tissue, which can interfere with photosynthetic processes (Bornman & Vogelmann 1991, Bornman et al. 1994). Tobacco plants grown under artificial UVB exposure, for example, were found to have visible morphological changes such as leaf rolling, diminished growth, and leaf surface glazing. Field plants under natural UVB/PAR regimes, experienced none of these morphological

changes (Dinh et al. 2013). In cotton, prolonged artificial UVB exposure resulted in initial chlorotic patches on interveinal portions of leaves, following by brown spots (Kakani et al. 2003). Artificial lighting can potentially lead to high levels of PAR not realized under field conditions, and subsequently alter the effects of phenolic induction (Kakani et al. 2003). In peas, higher PAR levels resulted in increased phenolic concentrations and a higher rate of UVB absorption after 10 hours of exposure (Strid & Porra 1992). As discussed previously, the disparities between artificial lighting and ambient exposure can drastically alter results, such as in PIN induction in tomoto (Conconi et al 1996, Stratmann et al 2000). These findings emphasize the necessity of using field based studies under natural conditions to ensure ecologically relevant results are obtained.

As UVB primes chlorogenic acid induction in *D. wrightii* and the glasshouse is opaque to UVB radiation, a laboratory based study assessing phenolic compound induction due to herbivory in this system would not have found chlorogenic acid to be induced significantly. Although chlorogenic acid did not have an apparent effect on *L. daturaphila*, the compound has been found to influence herbivore performance in a number of other herbivores that commonly feed on *D. wrightii* as previously mentioned, emphasizing that field based studies using a naturally balanced light spectrum should accompany glasshouse results to ensure results are ecologically relevant. (Osier et al. 1996, Stamp & Yang 1996, Stamp & Osier 1997, Dinh et al. 2013).

Variation in JA Mediated Responses

Although JA was not measured directly in this study, a number of traits that are commonly regulated by JA were (PINS and PPO) (Izaguirre et al. 2003, 2007, Demkura et al. 2010**,** Kruidof et al. 2011). Effects of herbivory and UVB on JA accumulation have been found to be highly variable across systems. In *Arabidopsis thaliana,* UVB exposure increased JA levels directly (Mackerness et al. 1999).). Broccoli has also been shown to induce responses independent from JA due to UVB exposure (Kuhlmann & Muller 2009). On the other hand, ambient levels of UVB had no direct effect on the accumulation of JA in *Nicotiana attenuata,* yet still induced rutin and chlorogenic acid, indicating that these phenolic compounds can be induced independently of JA mediated signaling in tobacco (Keinanen et al. 2001, Kessler & Baldwin 2004, Demkura et al. 2010). UVB exposure increased JA sensitivity in the same system, as UVB exposed plants had significantly higher levels of JA one hour after herbivore oral secretion application compared to plants not exposed to UVB in the same system (Dinh et al. 2013). Similar to what was described for phenolics, PINS and PPO, a large amount of variation in JA induction in response to UVB exposure has been demonstrated across systems, and variation exists within systems as well that vary in experimental protocol, such as artificial lighting compared to field conditions in *N. attenuata,* (Izaguirre et al. 2007, Demkura et al. 2010, Dinh et al. 2013). A synthesis of the literature and the results found here for *D. wrightii* further indicate that both JA dependent and independently mediated responses can be induced in response to UVB exposure, although there appears

to be little consistently across systems regarding specific UVB-herbivory crosstalk patterns of induction.

In *D. wrightii,* the lack of consistent pattern between phenolic compounds, as well as between chlorogenic acid and the other measured JA mediated traits, indicates that UVB exposure may be triggering chlorogenic acid induction independent of jasmonate signaling as herbivory alone was incapable of inducing the compound (Table 2, Figure 2c,d). UVB could thus act as an important defense mechanism against herbivores that do not activate high levels of JA signaling (Dinh et al. 2013). Alternatively, the priming effect demonstrated in chlorogenic acid induction may be a result of UVB exposure increasing jasmonate sensitivity, leading to an exaggerated response following herbivory (Demkura et al. 2010). Given that UVB had no effect on PINS or PPO activity, the role of UVB in the accumulation of jasmonates in this system seems limited. If PPO enzymatic activity had already oxidized chlorogenic acid that had been induced via herbivory, however, then this conclusion would be more concrete.

Conclusions

Much work has been done over the last decade to clarify the interplay between abiotic and biotic stress (Fujita et al. 2006, Izaguirre 2003, Izaguire et al. 2007, Demkura et al. 2010, Dinh et al. 2013, Atkinson & Urwin 2012, Suzuki et al. 2014). Here, I demonstrate that UVB exposure plays a role in the priming of chlorogenic acid, yet has little effect in other JA mediated induced traits in *D. wrightii*. In this system, phenolic compounds can be induced independently of one another. Although chlorogenic acid did

not have a statistically significant effect on feeding *L. daturaphila*, its role against other herbivores found on *D. wrightii* has been documented in a number of studies (Osier et al. 1996, Stamp & Yang 1996, Stamp & Osier 1997, Dinh et al. 2013). UVB may serve as a cost saving mechanism to induce chlorogenic acid as a general defense against herbivory. The phenolic compounds rutin and chlorogenic acid are induced more independently in this system, although there appears to be little consistency across systems and varying experimental protocols based on how UVB regulates individual phenolic compounds and JA mediated responses (Demkura et al. 2010, Dinh et al. 2013, Zavala et al. 2014). I also demonstrated that plant age can be a complicating factor in the analysis of induced responses. Induced responses vary independently over time in this system with phenolic concentration decreasing and PIN concentration increasing. Given the amount of variation demonstrated over multiple studies, these results further indicate that the role abiotic conditions have on induced plant traits may be best analyzed under ecologically relevant, local conditions.

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Figures and Tables

Figure 1. Mean (+SE) UVB transmittance and photosynthetic active radiation in full sun exposure, polyester wrapped cages, and polyethylene wrapped cages. Measurements were taken during the field season over the course of seven days. No significant difference was found between average Full Sun and Polyethylene UV intensity. A significant difference was found between polyester in comparison to the full sun UV intensity mean and the polyethylene UV intensity mean (Tukey's HSD, p<001). No statistical difference was found across treatments for PAR transmittance.

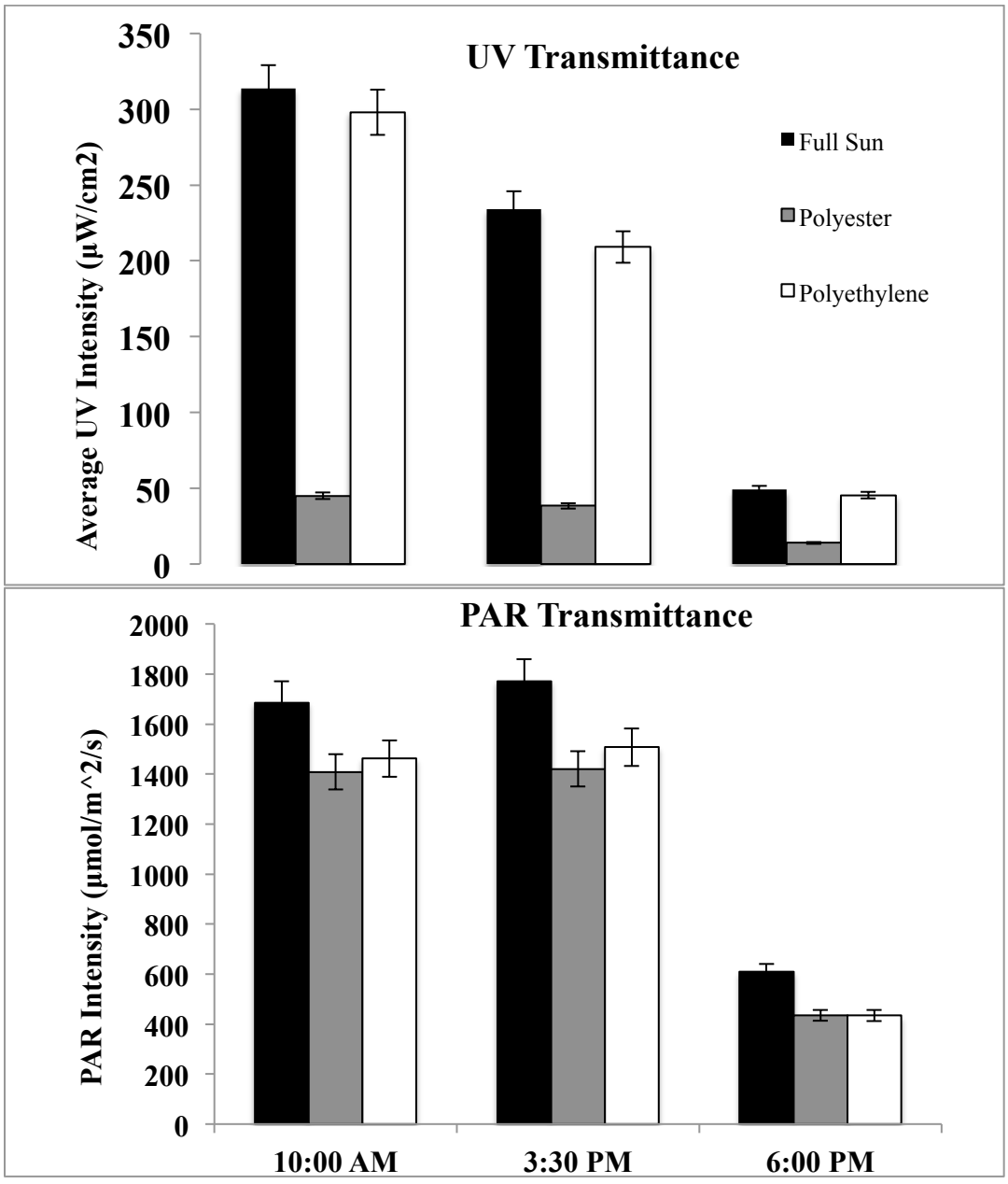
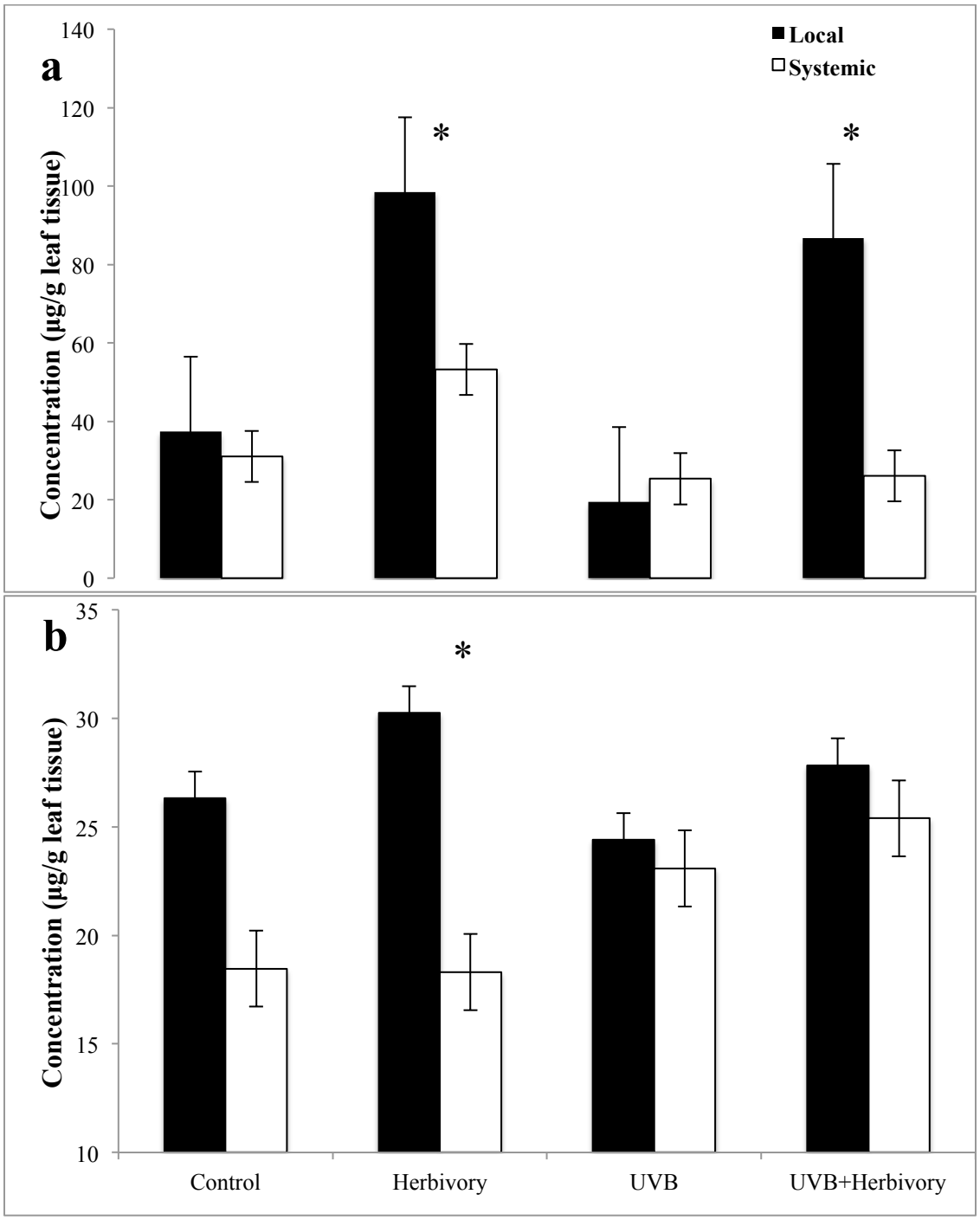


Figure 2. Mean (+SE) phenolic concentrations for field grown *Datura wrightii* plants. Effect of UVB and herbivory on **(a)** rutin concentration in young plants**(b)** rutin concentration in old plants **(c)** chlorogenic acid in young plants **(d)** chlorogenic acid in old plants. Each treatment represents n=10 plants. Asterisks represent significant differences in mean concentrations between local and system leaves for each treatment ($p<0.05$).

Rutin



Chlorogenic Acid

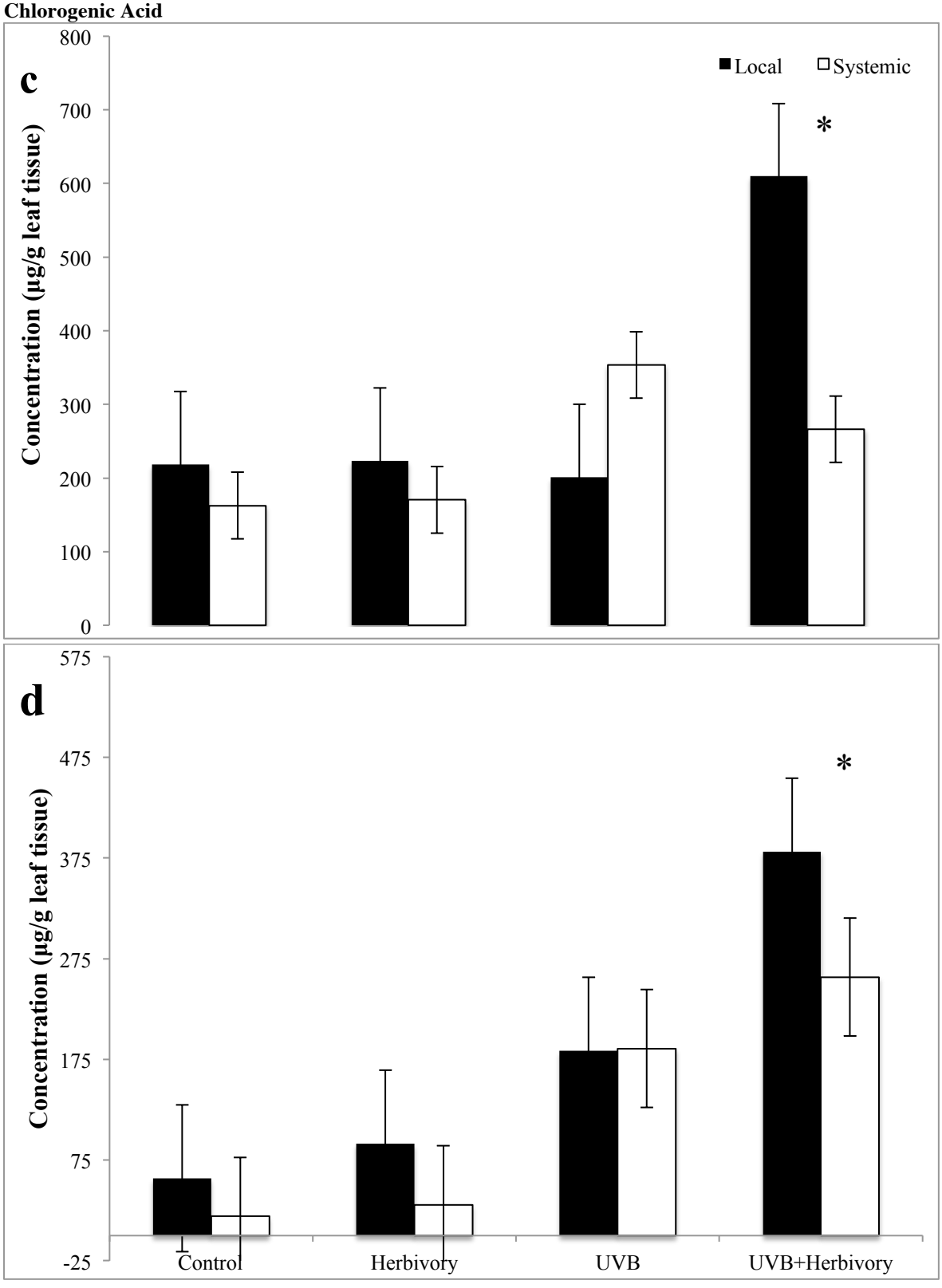
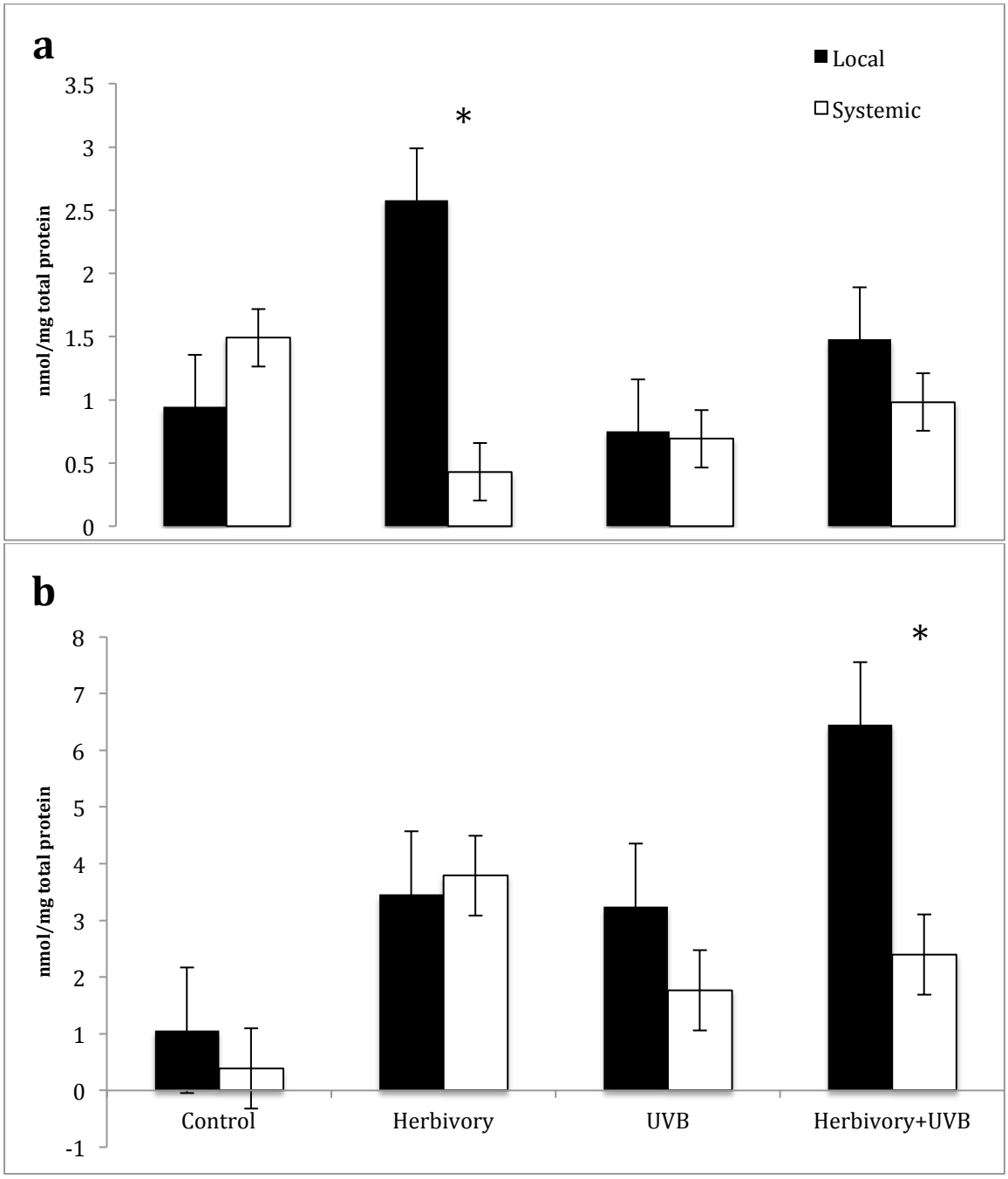


Figure 3. Mean protease inhibitor (+SE) concentrations in field grown *Datura wrightii* plants. Effect of UVB and herbivory on **(a)** serine protease inhibitor concentration in young plants **(b)** serine protease inhibitor concentration on old plants **(c)** cysteine protease inhibitor concentration in young plants **(d)** cysteine protease inhibitor concentration in old plants. Each treatment represents n=10 plants. Asterisks represent significant differences in mean concentrations between local and system leaves for each treatment ($p<0.05$).

Serine Protease Inhibitors



Cysteine Protease Inhibitors

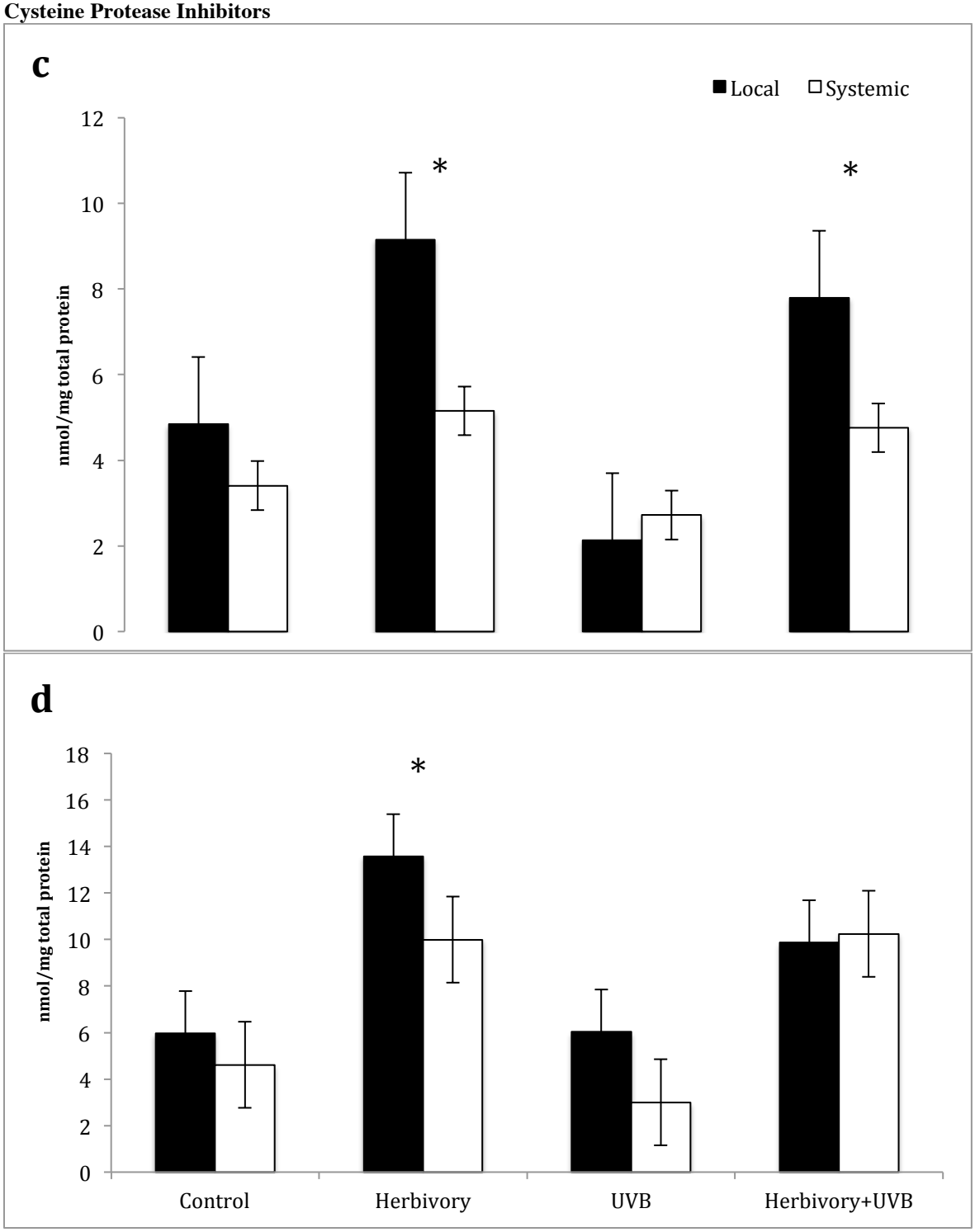


Figure 4. Mean polyphenol oxidase activity (+SE) in field grown *Datura wrightii* plants. There was no significance in plant age, thus averages represent pooled data from both exposure treatments. PPO activity is reported in absorbance rate per minute per gram of leaf tissue.

Polyphenol Oxidase

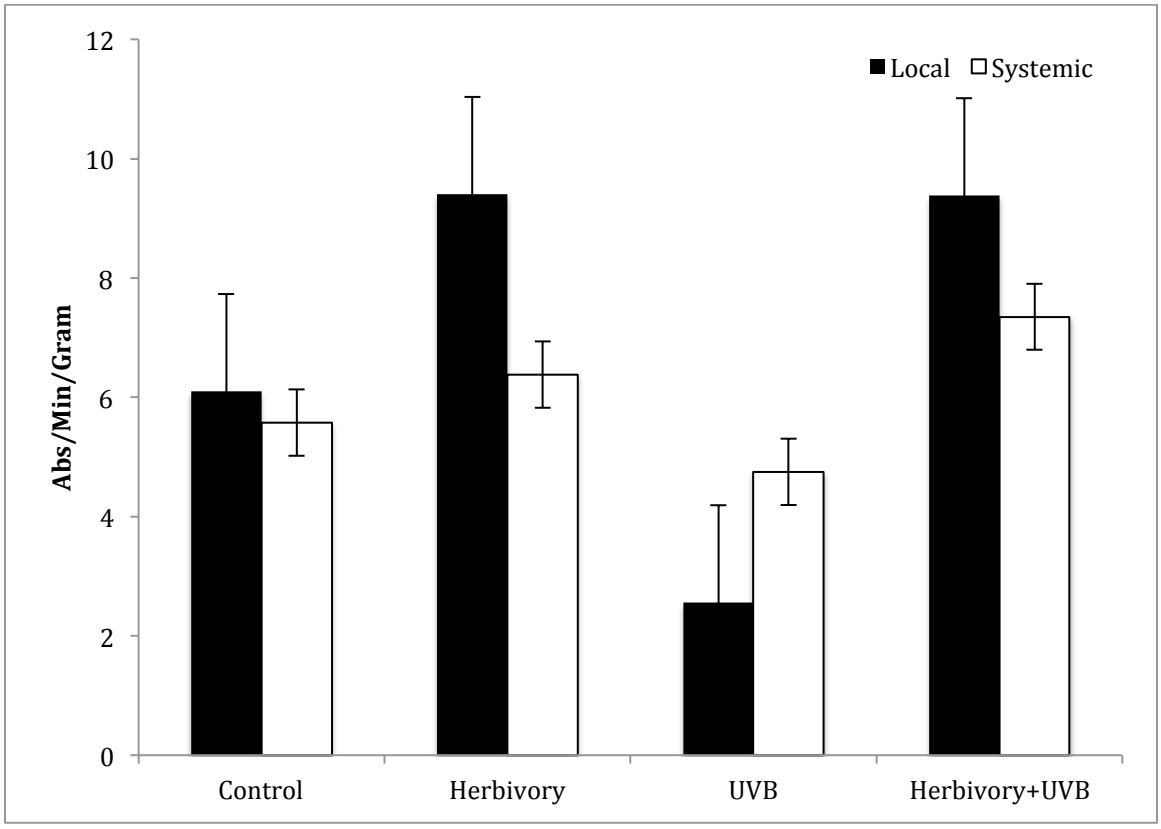


Figure 5. Mean relative growth rate in *Lema daturaphila* across **(a)** rutin and **(b)** chlorogenic acid manipulation treatments. Adults were placed on excised leaf tissue coated phenolic compounds in 100% MeOH of low, moderate, and high phenolic concentrations and allowed to feed for five days. Control leaves were coated with 100% MeOH. Concentrations here represent both intrinsic concentrations and extrinsic additions.

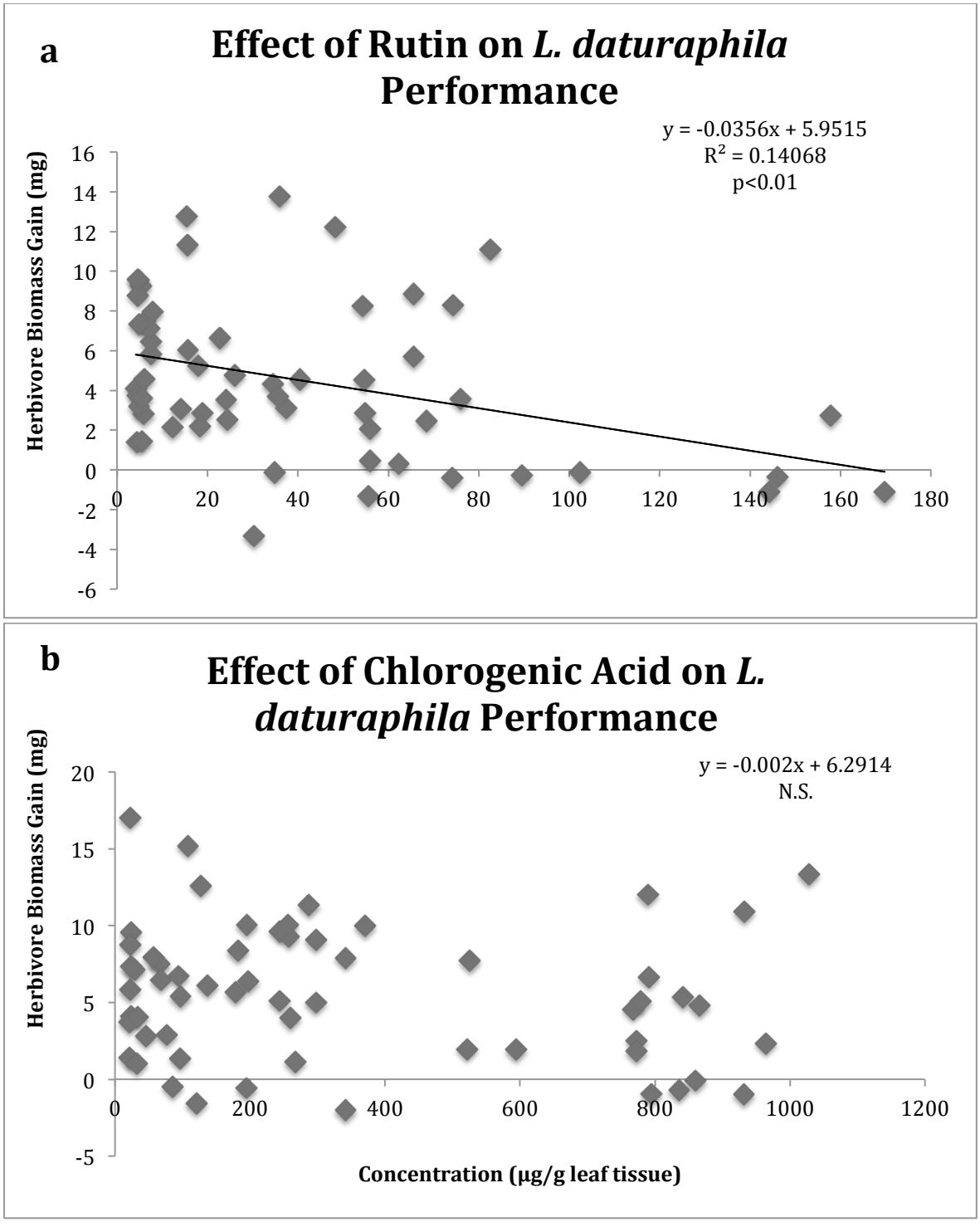


Figure 6: Average rates of consumption across phenolic manipulation treatments. There were no significant differences in area removed across treatments. Each treatment represents 15 leaves.

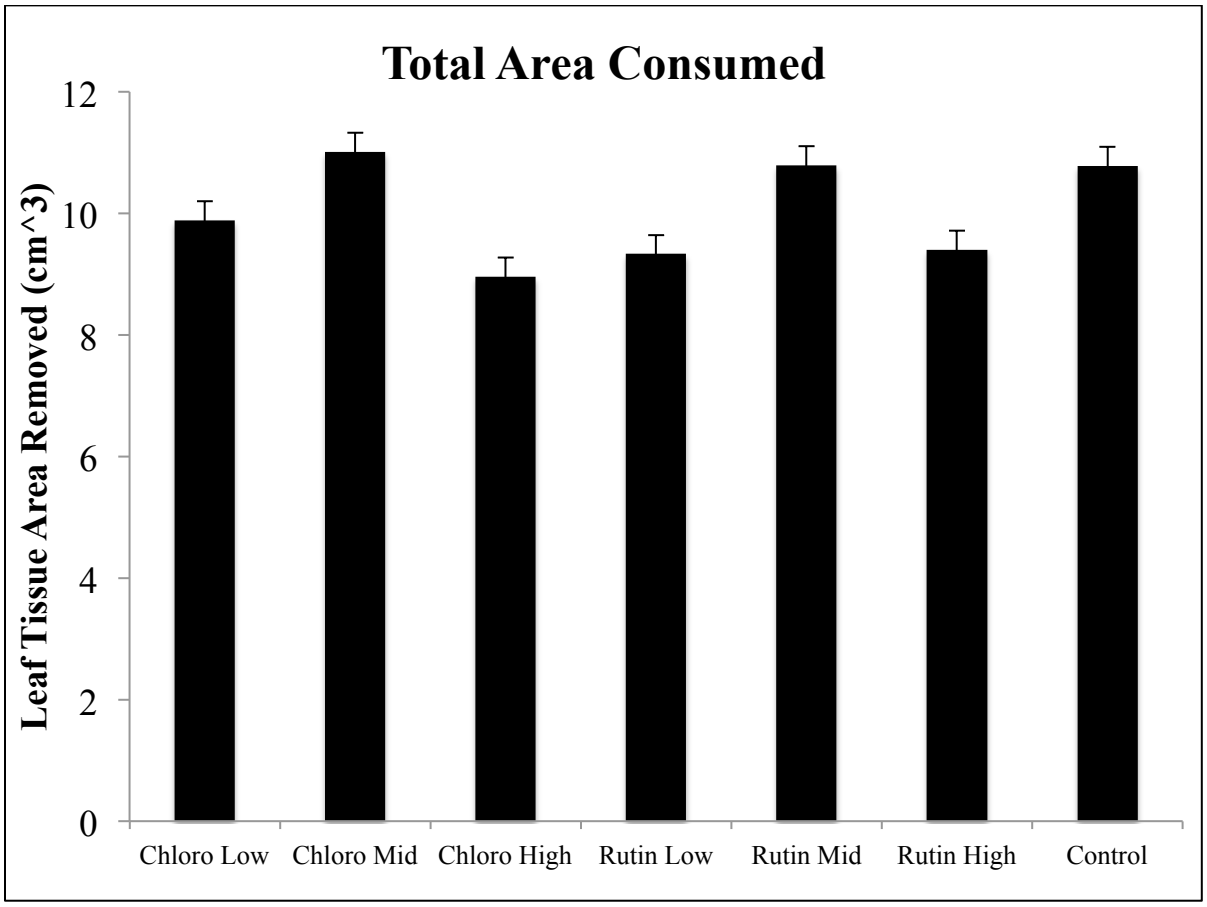


Table 1. Effect of UVB, Herbivory, and plant age on overall induction. The induction variable was created using the cbind function in R to combine the five induced responses listed in Table 1. Removing the three way interaction of herbivory x UVB x Plant Age did not improve statistical significance.

Response	Effect	*p*	*p*
		LOCAL	SYSTEMIC
Induction	Herbivory	**<0.001**	**<0.001**
	UVB	**<0.001**	**0.02**
	Plant Age	**<0.001**	**<0.001**
	Herbivory x UVB	**0.027**	0.817
	Herbivory x Plant Age	0.395	**0.04**
	UVB x Plant Age	**0.019**	0.394
	Herbivory x UVB x Plant Age	0.747	0.525

Table 2. Effect of UVB, Herbivory, and plant age on the accumulation of Rutin, Chlorogenic Acid, Serine PINS, Cysteine PINS, and PPO. Results of the three factorial ANOVA are presented here, with statistically significant relationships in bold. Local designates locally damaged leaves, while systemic refers to the undamaged leaves collected at the above adjacent node.

	Test #	Effect	*p*	*p*
Induced Responses			LOCAL	SYSTEMIC
Rutin	1	Herbivory	**<0.01**	0.201
	2	UVB	0.687	0.436
	3	Plant Age	**<0.001**	**<0.001**
	4	Herbivory x UVB	0.24	0.335
	5	Herbivory x Plant Age	**0.027**	0.238
	6	UVB x Plant Age	0.133	0.223
	7	Herbivory x UVB x Plant Age	0.637	0.487
Chlorogenic Acid	8	Hebivory	0.142	0.76
	9	UVB	**<0.001**	**<0.01**
	10	Plant Age	**<0.001**	**<0.001**
	11	Herbivory x UVB	**<0.001**	0.493
	12	Herbivory x Plant Age	0.494	0.796
	13	UVB x Plant Age	0.25	0.589
	14	Herbivory x UVB x Plant Age	0.983	0.53
Serine PINS	15	Herbivory	**<0.001**	0.12
	16	UVB	0.49	0.591
	17	Plant Age	**0.031**	**0.045**
	18	Herbivory x UVB	0.246	0.445
	19	Herbivory x Plant Age	0.878	0.051
	20	UVB x Plant Age	0.133	0.834
	21	Herbivory x UVB x Plant Age	0.991	0.113
Cysteine PINS	22	Herbivory	**<0.001**	**<0.01**
	23	UVB	0.336	0.799
	24	Plant Age	**0.034**	**0.033**
	25	Herbivory x UVB	0.998	0.63
	26	Herbivory x Plant Age	0.631	0.137
	27	UVB x Plant Age	0.876	0.975
	28	Herbivory x UVB x Plant Age	0.391	0.91
PPO	29	Herbivory	**<0.001**	0.182
	30	UVB	0.055	0.923
	31	Plant Age	0.55	0.274
	32	Herbivory x UVB	**<0.01**	0.916
	33	Herbivory x Plant Age	0.334	0.077
	34	UVB x Plant Age	0.746	0.75
	35	Herbivory x UVB x Plant Age	0.271	0.764

Table 3. Effects of chlorogenic acid coated leaves and rutin coated leaves on *L. daturaphila* performance, measured through both total biomass gain and relative growth rate. Consumption was initially included as a covariate, however, it was not found to be significant and thus excluded from the final model. Baseline concentrations of the nontreatment phenolic compound (rutin for chlorogenic acid treatment and vice versa) were included in the model, and the interaction between the two phenolic compounds was also analyzed.

Response	Effect	*p*
Chlorogenic Acid		
Biomass Gain	Initial Mass	**0.02**
	Chloro Concentration	0.309
	Baseline Rutin	0.238
	Chloro x Rutin	0.129
RGR	Initial Mass	0.498
	Chloro Concentration	0.356
	Baseline Rutin	0.441
	Chloro X Rutin	0.83
Rutin		
Biomass Gain	Initial Mass	**0.029**
	Rutin Concentration	**<0.01**
	Baseline Chlorogenic Acid	0.328
	Rutin x Chloro	0.33
RGR	Initial Mass	0.715
	Rutin Concentration	**0.002**
	Baseline Chlorogenic Acid	0.203
	Rutin x Chloro	0.322

Table 4 Post hoc Tukey's HSD mean contrasts between treatments for both locally damaged and systemic leaves. Plant age has been separated.
Different letters indicate significant letters between means (p<=0.05)

1. Rutin

Local (Young Plants)					
UV	Herbivory	Means			n
UV+	H-	19.468	A		10
UV-	H-	37.39	A		9
UV+	H+	86.682		B	10
UV-	H+	98.476		B	9

Local (Old Plants)					
UV	Herbivory	Means			n
UV+	H-	24.405	A		10
UV-	H-	26.314	A		10
UV+	H+	27.83	A		10
UV-	H+	30.253	A		10

Systemic (Young Plants)					
UV	Herbivory	Means			n
UV+	H-	25.343	A		9
UV+	H+	26.097	A		10
UV-	H-	31.054	A		10
UV-	H+	53.215	A		9

Systemic (Old Plants)					
UV	Herbivory	Means			n
UV-	H-	18.464	A		9
UV-	H+	18.308	A		10
UV+	H-	23.084	A		10
UV+	H+	25.391	A		10

2. Chlorogenic Acid

Local (Young Plants)					
UV	Herbivory	Means			n
UV+	H-	200.939	A		10
UV-	H-	218.373	A		10
UV-	H+	223.389	A		9
UV+	H+	609.655		B	10

Local (Old Plants)					
UV	Herbivory	Means			n
UV-	H-	56.842	A		10
UV-	H+	91.56	A		10
UV+	H-	183.596	A		10
UV+	H+	381.456		B	10

Systemic (Young Plants)					
UV	Herbivory	Means			n
UV-	H-	162.588	A		10
UV-	H+	170.294	A		9
UV+	H+	266.389	A		10
UV+	H-	353.528	A		9

Systemic (Old Plants)					
UV	Herbivory	Means			n
UV-	H-	19.203	A		9
UV-	H+	30.492	A		10
UV+	H-	185.729		B	10
UV+	H+	256.853		B	10

3. Serine PINS

Local (Young Plants)					
UV	Herbivory	Means			n
UV+	H-	0.749	A		10
UV-	H-	0.946	A		10
UV+	H+	1.479	A	B	10
UV-	H+	2.58		B	9

Local (Old Plants)					
UV	Herbivory	Means			n
UV-	H-	1.061	A		10
UV+	H-	3.245	A	B	10
UV-	H+	3.462	A	B	10
UV+	H+	6.447		B	10

Systemic (Young Plants)

UV	Herbivory	Means			n
UV-	H+	0.43	A		9
UV+	H-	0.691	A		9
UV+	H+	0.982	A		10
UV-	H-	1.49	A		9

Systemic (Old Plants)

UV	Herbivory	Means			n
UV-	H-	0.397	A		9
UV+	H-	1.765	A		10
UV+	H+	2.396	A		10
UV-	H+	3.789	A		10

4. Cysteine PINS

Local (Young Plants)

UV	Herbivory	Means			n
UV+	H-	2.136	A		10
UV-	H-	4.842	A	B	9
UV+	H+	7.791		B	10
UV-	H+	9.157		B	9

Local (Old Plants)

UV	Herbivory	Means			n
UV-	H-	5.962	A		10
UV+	H-	6.024	A		10
UV+	H+	9.86		B	10
UV-	H+	13.579		B	10

Systemic (Young Plants)

UV	Herbivory	Means			n
UV+	H-	2.719	A		9
UV-	H-	3.407	A		9
UV+	H+	4.763	A		10
UV-	H+	5.152	A		9

Systemic (Old Plants)

UV	Herbivory	Means			n
UV+	H-	3	A		10
UV-	H-	4.61	A		9
UV-	H+	9.993	A		10
UV+	H+	10.244	A		10

5. PPO

Local (Young Plants)

UV	Herbivory	Means			n
UV+	H-	1.69	A		10
UV-	H-	5.826		B	9
UV-	H+	9.418		B	9
UV+	H+	9.622		B	10

Local (Old Plants)

UV	Herbivory	Means			n
UV+	H-	3.344	A		10
UV-	H-	6.377	A	B	10
UV+	H+	9.145		B	10
UV-	H+	9.388		B	10

Systemic (Young Plants)

UV	Herbivory	Means			n
UV+	H-	4.506	A		9
UV-	H+	5.114	A		9
UV+	H+	5.562	A		10
UV-	H-	6.365	A		9

Systemic (Old Plants)

UV	Herbivory	Means			n
UV-	H-	4.787	A		9
UV+	H-	4.97	A		10
UV-	H+	7.521	A		10
UV+	H+	9.137	A		10