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Research

Methods

Spectroscopic sensitivity of real-time, rapidly induced phytochemical change in response to damage

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Summary

• An ecological consequence of plant–herbivore interactions is the phytochemical induction of defenses in response to insect damage. Here, we used reflectance spectroscopy to characterize the foliar induction profile of cardenolides in *Asclepias syriaca* in response to damage, tracked *in vivo* changes and examined the influence of multiple plant traits on cardenolide concentrations.

• Foliar cardenolide concentrations were measured at specific time points following damage to capture their induction profile. Partial least-squares regression (PLSR) modeling was employed to calibrate cardenolide concentrations to reflectance spectroscopy. In addition, subsets of plants were either repeatedly sampled to track *in vivo* changes or modified to reduce latex flow to damaged areas.

• Cardenolide concentrations and the induction profile of *A. syriaca* were well predicted using models derived from reflectance spectroscopy, and this held true for repeatedly sampled plants. Correlations between cardenolides and other foliar-related variables were weak or not significant. Plant modification for latex reduction inhibited an induced cardenolide response.

• Our findings show that reflectance spectroscopy can characterize rapid phytochemical changes *in vivo*. We used reflectance spectroscopy to identify the mechanisms behind the production of plant secondary metabolites, simultaneously characterizing multiple foliar constituents. In this case, cardenolide induction appears to be largely driven by enhanced latex delivery to leaves following damage.

Introduction

Chemistry plays a dominant role in species interactions, especially between plants and herbivores. Measurement of chemical concentrations important to species interactions provides insight into the mechanisms driving plant allocation of resources in response to herbivory. The underlying factors that drive plant chemical responses to herbivory provide a framework within which we can test ecological and evolutionary theory (Dyer, 1995; Thompson, 1999; Agrawal, 2001; Guisan & Thuiller, 2005; Mooney *et al.*, 2010; Agrawal *et al.*, 2012a).

In particular, a key area of interest for understanding the ecology and evolution of plant–herbivore interactions is inducible plant defenses. Inducible plant defenses are thought to have evolved as a mechanism to minimize the cost of production of defenses, given the unpredictability of herbivore attack (Herm & Mattson, 1992; Karban & Baldwin, 1997). The ability of plants to rapidly mount defensive responses to herbivore damage is important in reconciling variations in plant metabolic tradeoffs, reproductive success, community composition and higher level trophic interactions (Herm & Mattson, 1992; Malcolm, 1995; Malcolm & Zalucki, 1996; Karban & Baldwin, 1997; Thaler *et al.*, 2001; Kessler & Baldwin, 2002; Mooney *et al.*, 2010; Agrawal *et al.*, 2012a).

In general, studies examining the inducibility of plant defenses rely on the sampling of combinations of damaged and undamaged plants over time, or repeatedly sampling the same plant tissue to determine changes in plant chemistry. Both of these methods require destructive sampling of plant tissue for chemical analysis. The former approach relies on the assumption that all plants respond similarly, and necessitates a large and sometimes impractical sample size to appropriately replicate the time points. The latter method relies on the assumption that the damage from tissue collection itself does not elicit further chemical responses. This method also becomes impractical when examining a rapidly induced chemical response, because it requires multiple collections from the same tissue source over a short time period.

Reflectance spectroscopy provides an alternative approach to the investigation of rapid phytochemical changes because the method is noninvasive, relatively inexpensive, fast and can be

applied to living tissue (reviewed in Foley *et al.*, 1998). The use of reflectance spectroscopy to characterize foliar chemistry is based on the influence of illumination on the harmonic vibrations between atoms or among groups of atoms, specifically C–H, N–H and O–H bonds, the main constituents of organic material, at specific wavelengths in the visible, near-infrared (NIR) and shortwave infrared (SWIR). Spectral measurements of organic material collected under uniform and stable illumination provide the basis for the estimation of the chemical composition of a sample (Shenk *et al.*, 1992). The calibration is accomplished by pairing spectra with reliable chemical measurements, from which chemical data are modeled as a function of spectra using multivariate methods. The calibration model is validated by comparing relationships between observed and predicted values to determine the robustness of the model. This calibration model can then be used to predict phytochemical concentrations of the remaining unknown samples on the basis of their spectral signature alone. An often underappreciated benefit of spectroscopy is the ability to collect information on multiple foliar constituents simultaneously from a single spectral measurement.

Common milkweed (*Asclepias syriaca*) is an ideal system to test the ability of spectroscopy to track rapid changes in phytochemistry. Milkweed has been studied extensively to understand the relationships between phytochemical variations and the ecology and evolution of plant–insect interactions (Malcolm, 1991, 1995; Mooney *et al.*, 2010; Agrawal *et al.*, 2012a), with the prime phytochemical focus being cardenolides. Cardenolides are phloem-mobile, steroidal toxins that disrupt cellular ion transport (Malcolm, 1991; Agrawal *et al.*, 2012a), and damage to common milkweed is known to rapidly increase foliar cardenolide concentrations (Malcolm & Zalucki, 1996; Agrawal *et al.*, 2012a). Cardenolides are potent chemical defenses in milkweed plants and are often implicated in the reduction in performance of herbivores specializing on milkweed (Malcolm, 1995; Malcolm & Zalucki, 1996; Zalucki *et al.*, 2001; Agrawal, 2005; Agrawal *et al.*, 2012b). Although most prevalent in the family Apocynaceae, cardenolides are also found in a number of other plant families (Kreis & Müller-Uri, 2010; Agrawal *et al.*, 2012a).

Although cardenolides are an important component of milkweed–insect interactions, multiple plant variables (e.g. nitrogen, C : N, leaf toughness, trichomes and latex) are also known to influence herbivore performance in this system (Agrawal & Fishbein, 2006). For example, in addition to cardenolides, milkweed is also characterized by the exudation of latex in response to damage that negatively affects the feeding of chewing insects by gumming their mouthparts (Dussourd & Eisner, 1987). Moreover, latex can contain cardenolide concentrations more than two orders of magnitude greater than those in leaves (Zalucki *et al.*, 2001). It has been suggested that the increase in foliar cardenolide concentrations in response to damage is caused by the rapid influx of latex, as opposed to localized foliar upregulation of cardenolide biosynthesis or latex-independent transport into the leaf (Zalucki *et al.*, 2001; Agrawal *et al.*, 2012a). However, current methodological approaches make the elucidation of the mechanism responsible for a rapid increase in cardenolides difficult. The ability to nondestructively determine real-time, *in vivo* chemical changes in response to environmental perturbation can greatly enhance our understanding of the relationships among phytochemical variation and species interactions, and the role they play in ecological and evolutionary theory.

Here, we describe a novel approach to quantify rapid phytochemical changes in response to damage. Utilizing recent advances in spectroscopy, we characterize the induction profile of cardenolides in *A. syriaca* in response to damage, noninvasively track these changes through single plants and examine the influence of latex on foliar cardenolide concentrations.

Materials and Methods

Plant culture and experimental design

Seeds of *Asclepias syriaca* L. used in this study were collected from a single seedpod from one milkweed ramet in Madison (WI, USA). Seeds were cold stratified for 4 months, germinated in MetroMix® potting medium (Sun Gro Horticulture, Vancouver, BC, Canada) in 61 × 30-cm^2 flats for 3 wk, and then transferred to individual 500-ml pots containing the same medium. At the time of transfer, plants received a single application of slow-release Osmocote (15 : 15 : 15 N : P : K) fertilizer. Natural light conditions were supplemented with fluorescent lights to create a 16 h : 8 h light : dark cycle in a glasshouse maintained at a constant temperature of 25°C.

Forty days after transfer to individual pots, 87 plants were randomly placed into one of seven groups. The first group consisted of 10 plants on which spectra were collected and the plants were harvested at the start of the study to serve as a control group to measure pre-damage, constitutive chemistry and spectra (time = 0). Of the remaining 77 plants, 57 were mechanically damaged (henceforth referred to as 'damaged') to simulate herbivory and to stimulate an induction response. Damage was inflicted on one leaf of the uppermost fully expanded leaf pair of each plant, ensuring that leaves were of a similar developmental stage. Damage consisted of the creation of four holes using a 6-mm hole punch, was uniform among plants and was inflicted almost simultaneously (< 1 min).

The 57 damaged and 20 undamaged plants were categorized into six groups. Five groups consisting of 14 plants each (10 damaged plus four undamaged) were sampled at specific post-damage time points (0.25, 1, 24, 72 and 124 h post-damage, based on Malcolm & Zalucki, 1996) to construct the cardenolide induction profile of *A. syriaca*. Therefore, spectra were collected on leaves of the first group of 14 plants at 15 min following damage and were immediately harvested and flash frozen in liquid nitrogen, freeze–dried and stored in a −20°C freezer. Each set included four undamaged plants as controls, as these plants should not have shown a notable induction response in either the chemistry or spectral profile. The second set of 14 plants (10 damaged and four undamaged) was sampled and harvested at 1 h, and so forth, for each collection period up to 124 h. The final group of seven plants, all of which were damaged, were measured using spectroscopy throughout the study at each time period, but were

not harvested. This group of unharvested plants was used to assess the consistency of measurements through time, as all other sets of plants used for spectroscopy were harvested following measurement, and was intended to demonstrate the ability of spectroscopy to nondestructively track cardenolide changes *in vivo* in *A. syriaca*.

To determine the influence of latex on the ability of milkweed to rapidly elevate cardenolide levels, we modified the petioles of one leaf of the uppermost fully expanded leaf pair on a separate group of plants ($n = 5$) to reduce latex flow. Latex flow was inhibited by removing the bottom two-thirds of the petiole of the leaf, immediately before inflicting damage, using blunt forceps (Zalucki *et al.*, 2001). We then damaged both leaves from this leaf pair and repeatedly collected reflectance measurements in a manner similar to that described above, excluding the 124-h collection. For these plants, cardenolide concentrations were estimated using spectroscopy, as detailed later.

Spectral collections

Leaf reflectance was measured using a high-spectral-resolution ASD FieldSpec 3 Full-Range (350–2500 nm) spectroradiometer (Analytical Spectral Devices, Boulder, CO, USA). All measurements were taken from the leaf adaxial surface using a leaf-clip assembly attached to a plant probe with an internal, calibrated light source. Reflectance was measured on three different areas of the leaf, with five spectra averaged per leaf location, and the spectra from the three areas were averaged to determine the mean leaf reflectance. In addition to the live plant material, we collected spectra on purified digitoxin, a cardenolide and the standard for analytical determination of cardenolide concentration (see later).

Cardenolide analyses

Concentrations of cardenolides needed to calibrate with ASD reflectance spectra were measured following Agrawal (2004, 2005), as modified from Brower *et al.* (1972) and Nelson (1993) to accommodate the use of a microplate reader. This spectrophotometric approach uses the absorbance of a chromophore created from the reaction between the butenolide portion of cardenolides and 2,2′,4,4′-tetranitrodiphenyl (TNDP). Each extracted and TNDP-reacted sample is compared with a blank, an extracted sample without the addition of TNDP, and the difference in absorbance is calibrated to an external standard curve. Comparison of the analysis of cardenolides using high-pressure liquid chromatography (HPLC) and the spectrophotometric approach has produced highly correlated results (Rasmann *et al.*, 2009).

The damaged and undamaged leaves harvested from the plants were prepared for analysis as follows. Lyophilized, pulverized plant material (50 ± 5 mg) was extracted in 1.9 ml of 95% ethanol, vortexed, sonicated for 10 min in a 65°C water bath and then centrifuged at 1398 ***g*** for 5 min. Two 45-μl aliquots of each extracted sample were added to a 96-well plate to provide one active sample and one blank. To the blanks, we added 90 μl of 95% ethanol and, to the active samples, we added 90 μl of 0.15% TNDP in 95% ethanol. We then added 70 μl of 0.1 M aqueous NaOH to each well to catalyze the colorimetric reaction. In addition, each plate contained six concentrations of digitoxin (0.001–1 mg ml^{-1}; Sigma-Aldrich), with each concentration replicated three times, to construct a standard curve. The plates were read at 620 nm on a SpectraMax 340 microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 23 min. Differences between the absorbance of active samples and blanks were calibrated against the digitoxin standard curve measurements of cardenolide concentrations. Each sample was run twice and then averaged to produce a single value for each plant.

Information on additional leaf traits and vegetation indices was determined from the same reflectance measurements as used in cardenolide predictions. Leaf nitrogen and leaf mass per area (LMA) were determined using existing models relating leaf reflectance to these variables from Serbin *et al.* (2012). We also included several vegetation indices commonly used in the analysis of plant physiological status via leaf optical properties to determine whether these indices covaried with cardenolide levels. The photochemical reflective index (PRI), sensitive to changes in light use efficiency, was calculated using the relative differences between wavelengths 570 and 531 nm (Gamon *et al.*, 1997). The normalized differential vegetation index (NDVI), a measure of plant vigor, was calculated using the relative differences between wavelengths 800 and 680 nm (Sims & Gamon, 2003). The normalized differential water index (NDWI), a measure of plant water content, was calculated as the relative difference between wavelengths 857 and 1241 nm (Gao, 1996).

Statistical analyses

Models to predict leaf cardenolide concentrations based on one spectrum per leaf (2001 reflectance measurements covering 400–2400 nm) employed partial least-squares regression (PLSR) (Wold *et al.*, 1984, 2001). In cases in which predictor variables are highly correlated, as with hyperspectral data, classical regression techniques produce unreliable coefficients, because collinear predictor variables contribute similar information to the response variable (Grossman *et al.*, 1996). In contrast with normal regression techniques, PLSR reduces a large number of collinear predictor variables into relatively few, uncorrelated latent variables, and has become the standard for chemometric analyses (Bolster *et al.*, 1996; Asner & Martin, 2008; Atzberger *et al.*, 2010; Asner *et al.*, 2011; Serbin *et al.*, 2012). The number of latent variables extracted was determined iteratively through reduction of the predicted residual sum of squares (PRESS) statistic (Chen *et al.*, 2004). The final set of extracted latent variables is combined into a linear model predicting cardenolide concentrations. Examination of residuals of the predictive model showed that one plant was consistently an outlier, and thus was excluded from the study to improve model predictive ability.

Model verification was performed through the evaluation of 1000 random subsets of the full dataset using a 70–30 split of the data for calibration and validation (Serbin *et al.*, 2012). We calculated the coefficient of determination (R^2), root-mean-square error (RMSE) and other model diagnostics on the validation dataset. This analysis generated a distribution of R^2, RMSE and

bias from the multiple permutations of the data and allowed for the assessment of uncertainty in model prediction and the determination of model stability across numerous permutations of the calibration data. We determined the strength of the contribution of PLSR loadings by wavelength using variable important to the projection (VIP) selection (Wold *et al.*, 1984, 2001). This analysis measured the importance of an individual predictor variable in explaining the variation in the response and predictor variables; larger weightings confer greater value of contribution by specific wavelengths to the predictive model (Wold *et al.*, 2001; Chong & Jun, 2005).

To determine the effects of damage treatment, time and their interaction on the cardenolide responses of plants used to build the calibration models, we used an analysis of variance (ANOVA) following the model $Y_{jk} = D_j + T_k + CO_{jk} + e_{jk}$. In this model, D is damage level j, T is time level k and e_{jk} represents the error term. To determine the effect of latex on cardenolide induction in response to damage on the petiole-modified plants, we used a similar ANOVA as earlier, except that leaf notching replaced damage. Relationships among cardenolide concentrations and other leaf chemicals and vegetation indices were evaluated using Pearson correlations. Statistical analyses were performed in either JMP 9.0 (SAS Institute Inc., Cary, NC, USA) or R (www.r-project.org).

Results

Predictive models produced using PLSR accurately characterized fresh leaf cardenolide concentrations in *A. syriaca*, with average R^2, RMSE and bias values of 0.851, 0.221 μg mg^{-1} and 0.007, respectively (Fig. 1a–c). The distribution ranges for R^2, RMSE and bias reported also suggest that the model predicting cardenolides is relatively stable, as all models produced a high R^2 (range, 0.54–0.95), RMSE values (range, 0.11–0.36 μg mg^{-1}) between 5% and 15% of the mean and minimal bias (range, −0.16 to 0.18). The range of cardenolides reported in the plants used in this study, determined by standard chemical analysis, ranged from 1.0 to 3.1 μg mg^{-1}, and was closely matched by the range of cardenolides predicted via spectroscopy: 0.9–3.3 μg mg^{-1} (Fig. 1d). Calibration coefficients for raw spectra are reported in Supporting Information Table S1. Foliar water status, determined by NDWI, was not affected by damage treatment in plants used to build the calibration ($t = -0.21$, $P = 0.82$) or in plants with notched petioles ($t = 0.67$, $P = 0.41$). Petiole notching, however, visibly reduced latex flow to damaged areas on leaves (J. Couture, pers. obs.).

Substantial variation existed in reflectance among damaged and undamaged leaves and across collection periods (Fig. 2a). Overall, the evaluation of the strength of the PLSR loadings by wavelength, determined using VIP selection, indicated the highest contribution to the model of cardenolide concentrations in the general areas of major peaks from purified digitoxin, especially in the visible and NIR regions (Fig. 2b). The general pattern of variation in reflectance of damaged, relative to undamaged, leaves was similar for plants used either to build the model or repeatedly sampled (Fig. 2c,d). Relative variation in reflectance was most pronounced within 24 h and generally largest in the visible and SWIR regions (Fig. 2c,d). Relationships between foliar traits that might co-vary with cardenolides and cardenolide concentrations themselves were nominal (Table 1).

Foliar cardenolide concentrations varied significantly among collection times and treatments (Table 2). Cardenolide concentrations increased over the first 24 h in response to damage and then decreased to levels similar to constitutive, undamaged plants (Fig. 3). Estimates of cardenolide concentrations derived from spectra for the seven unharvested, but damaged, *A. syriaca* plants matched the induction profile of the harvested plants, in which concentrations were determined by standard chemical analysis

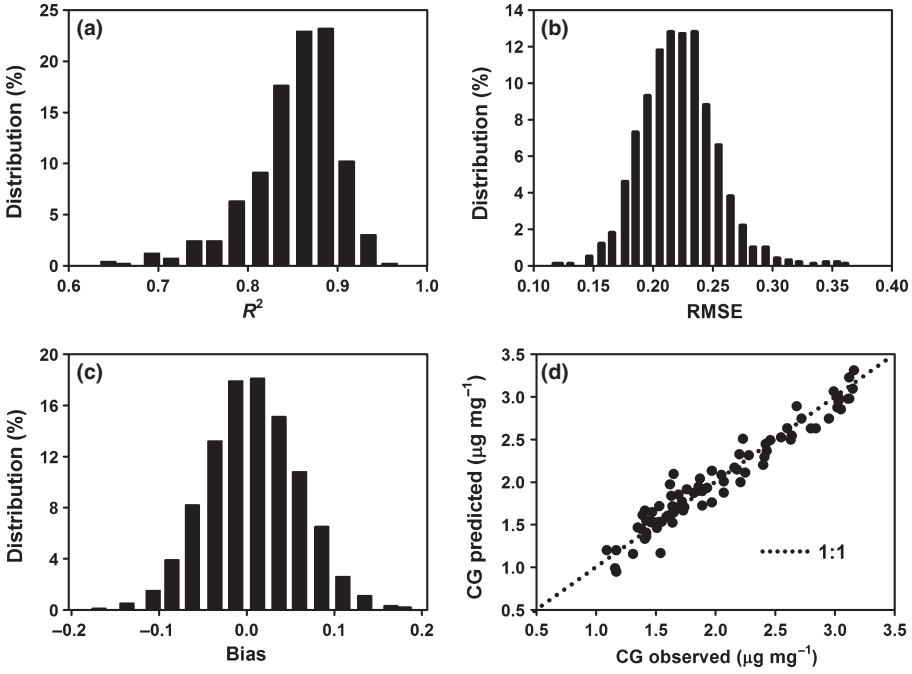

Fig. 1 Error distribution of (a) R^2, (b) root-mean-square error (RMSE) and (c) bias for validation data generated via cross-validation using 1000 random subsamples of the data for calibration (70%) and validation (30%). (d) Observed vs predicted values of cardenolide (CG) concentrations of *Asclepias syriaca*.

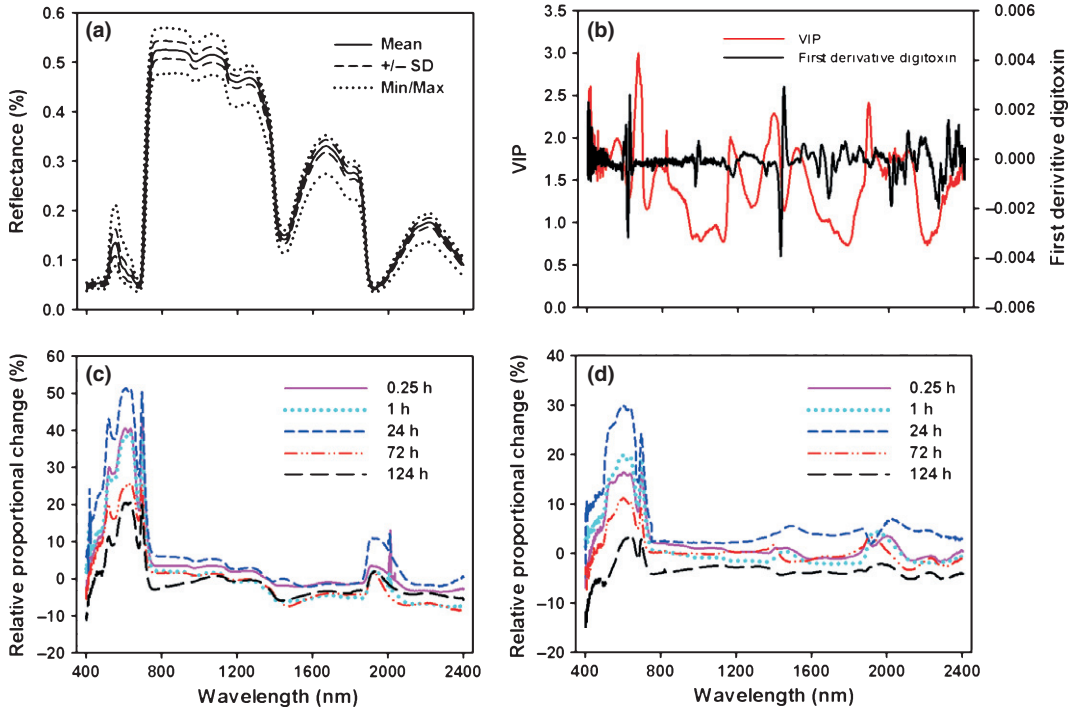

Fig. 2 (a) Average, ± SD, and minimum and maximum *Asclepias syriaca* leaf reflectance. (b) Variables important to the projection (VIP) plot showing the relative strength of the contribution of partial least-squares regression (PLSR) loadings by wavelength to the model predicting cardenolide concentrations (red line) overlaid on the first derivative of the reflectance spectra of purified digitoxin (black line). (c) Relative variation in reflectance of leaves at 0.25, 1, 72 and 124 h post-damage for plants used to build the predictive model relating leaf reflectance and cardenolide concentrations. (d) Relative variation in the reflectance of leaves at 0.25, 1, 72 and 124 h post-damage from repeatedly sampled plants.

Table 1 Pearson correlations among foliar levels of cardenolides, nitrogen, leaf mass per area, phytochemical reflective index, normalized differential vegetation index and normalized differential water index

	Cardenolides	Nitrogen	LMA	PRI	NDVI	NDWI
Cardenolides	1.00					
Nitrogen	**−0.313**	1.000				
LMA	**−0.324**	**−0.558**	1.000			
PRI	0.180	**0.493**	**−0.502**	1.000		
NDVI	−0.200	0.193	−0.044	0.207	1.000	
NDWI	**0.306**	−0.168	−0.029	0.001	−0.202	1.000

LMA, leaf mass per area; NDVI, normalized differential reflective index; NDWI, normalized differential water index; PRI, photochemical reflective index. Significant relationships ($P < 0.05$) indicated in bold.

Table 2 Two-way ANOVA examining the effects of damage (undamaged vs damaged) and time on foliar cardenolide concentrations of *Asclepias syriaca* of plants used to build the calibration model

Treatment	df	*F*	*P*
Damage	1, 78	77.7	< 0.001
Time	5, 78	28.6	< 0.001
Damage × time	5, 78	6.5	< 0.001

Numerator and denominator degrees of freedom (df: numerator, denominator) were calculated by the Satterthwaite approximation.

(open circles in Fig. 3). Importantly, this shows that estimates of cardenolide concentration based exclusively on reflectance spectroscopy of intact live leaves exhibit the expected trends based on the analytical chemistry of harvested samples.

Comparison of intact and modified leaves, whose petioles were notched to reduce latex flow, revealed that the enhanced expression of foliar cardenolides in response to damage was mostly eliminated in leaves in which latex flow was reduced (Table 3, Fig. 4). This result suggests a strong relationship between cardenolide concentrations and latex, and further suggests that latex exudation is a probable mechanism for the increase in leaf-level cardenolides in response to tissue damage.

Discussion

We successfully characterized phytochemical variation in milkweed in response to damage using reflectance spectroscopy. Our approach follows on from numerous recent advances in chemical spectroscopy in which multivariate modeling techniques, such as PLSR, are increasingly being utilized to estimate foliar biochemical, nutritional and morphological traits (Gillon *et al.*, 1999; Petisco *et al.*, 2006; Asner & Martin, 2008; Kleinebecker *et al.*, 2009; Asner *et al.*, 2011; Serbin *et al.*, 2012). Most studies predicting foliar chemistry, however, use dried and ground leaf material. Although spectroscopy on dried leaf material saves time and the cost of processing and chemically analyzing samples, it is not possible to repeatedly sample the same leaf. As a

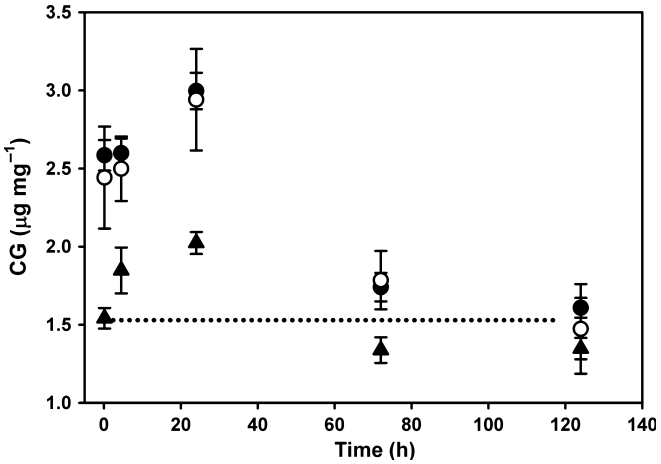

Fig. 3 Cardenolide (CG) induction profile of *Asclepias syriaca* collected at 0.25, 1, 24, 72 and 124 h post-damage. Damaged (closed circles) and undamaged (closed triangles) values were generated from the predictive model. Repeat measured (open circles) values were generated by repeatedly collecting reflectance from the subset of seven plants damaged at the same time as those used to build the predictive model, but not themselves included in model building. Constitutive (dotted line) is the average cardenolide concentration, determined using the predictive model, of the 10 plants harvested before damage. Values are means ± SD.

Table 3 Repeated-measures ANOVA of the effect of petiole modification and time on foliar cardenolide concentrations of *Asclepias syriaca*

Treatment	df	*F*	*P*
Petiole modification	1, 59	64.8	< 0.001
Time	4, 59	41.1	< 0.001
Petiole modification × time	4, 59	19.7	< 0.001

Numerator and denominator degrees of freedom (df: numerator, denominator) were calculated by the Satterthwaite approximation.

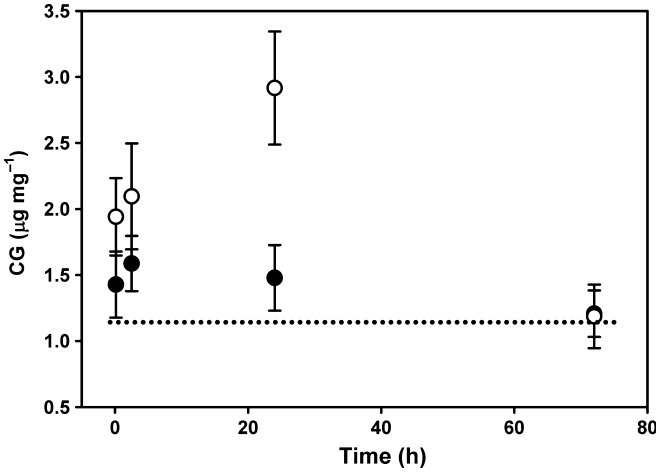

Fig. 4 Cardenolide (CG) induction profile of *Asclepias syriaca* collected at 0.25, 1, 24 and 72 h post-damage in leaf pairs in which one leaf had its petiole modified to reduce latex flow (closed circles) and the other petiole remained intact (open circles). Constitutive (dotted line) is the average cardenolide concentration of leaves before damage. Values are means ± SD.

consequence, reflectance spectroscopy on green leaves offers great promise for the characterization of plant responses to perturbation.

Our findings are similar to those of Ebbers *et al.* (2002), who were able to accurately characterize the concentrations of specific terpenoids and phenolics from fresh leaves of *Eucalyptus*. In our study, water absorption features (1450 and 1950 nm) partially obscured some spectral features of a purified cardenolide, digitoxin. Nevertheless, we were able to predict cardenolide concentrations of *A. syriaca*, with some of the wavelengths of importance occurring in areas identified for the purified digitoxin standard. Model coefficient loadings strongly contributing to cardenolide predictions, determined using VIP analysis, appeared in general areas of major features of purified digitoxin, the analytical standard, in particular in the visible and SWIR regions, and on the shoulders of the major water absorption features (1450 and 1950 nm, Fig. 2b). Specifically, key spectral features associated with cardenolide concentrations appeared at wavelengths of *c.* 670, 819, 1159, 1389, 1494 and 1890 nm.

The alignment of spectral features of purified digitoxin with PLSR coefficient loadings was not exact, but this result was not surprising as digitoxin, although a cardenolide, is not known to be present in *A. syriaca*. The value in this analysis is comparative: digitoxin is a commonly used cardenolide standard with strong similarities to the cardenolides in *A. syriaca*. Cardenolides found in the genus *Asclepias* are different from digitoxin (e.g. stereochemistry) and vary among themselves (e.g. differences in the number of hydroxyl groups on the steroid nucleus) in chemical characteristics (Malcolm, 1991; Agrawal *et al.*, 2012a). These variations are probably responsible for differences in digitoxin spectra and VIP values in Fig. 2(b). Ultimately, the detection of cardenolides can be complicated by water absorption features, multiple forms of the compound of interest and other leaf morphological characteristics (e.g. leaf wax cuticular structure or trichomes). The ability to predict the phytochemical composition of fresh leaves represents an important step in the accurate estimation of real-time changes in plant metabolism in response to biological perturbation and environmental variation (Serbin *et al.*, 2012).

In response to damage, leaves of *A. syriaca* can rapidly increase their cardenolide concentrations, and we observed that cardenolide levels peaked after 24 h in response to mechanical damage. Different types of damage elicit different cardenolide induction profiles in *A. syrica* depending on damage type (i.e. herbivory vs simulated herbivory), herbivore feeding guild (i.e. chewing vs sucking) and herbivore identity within feeding guilds (Agrawal *et al.*, 2012a). Foliar tissue removal generally elicits an influx of latex, and has been suggested to be the reason for the rapid elevation of cardenolide levels in response to damage, as milkweed latex can contain cardenolide concentrations orders of magnitude larger than those of foliar tissue (Zalucki *et al.*, 2001). Moreover, localized cardenolide biosynthesis is less likely to occur as quickly in response to damage as does increased latex flow to a damaged area (Agrawal *et al.*, 2012a). By notching leaf petioles and reducing latex flow into the leaf, we observed that the induction of foliar cardenolide concentrations was mostly eliminated. Such an

analysis was facilitated by the ability to measure cardenolides nondestructively (i.e. using reflectance spectroscopy), whilst simultaneously measuring and manipulating multiple factors. Although a more detailed understanding of the genetic and enzymatic upregulation of cardenolide biosynthesis is needed to reveal the underlying cause of the increased cardenolide expression in milkweed leaves following damage, our findings are consistent with the conclusion that the rapid induction of foliar cardenolides in response to damage is probably driven by latex influx (Zalucki *et al.*, 2001; Agrawal *et al.*, 2012a).

The correlations of several plant traits and commonly used vegetation indices derived from reflectance spectroscopy (i.e. PRI, NDVI and NDWI) with cardenolide concentrations were relatively low, which indicates that our method appears to be sensitive to cardenolide concentrations rather than other widely measured characteristics that are also correlated with plant performance. The plant trait exhibiting the strongest relationship with cardenolide concentration was LMA (−0.324), potentially suggesting a trade-off between the allocation of leaf carbon to growth or metabolite synthesis. Overall, LMA varied among all collection periods for damaged plants ($F = 5.7$, $P = < 0.001$), but was not statistically significantly different in the first three collection periods post-damage (data not shown). Moreover, the inclusion of leaf mass and LMA as covariates in the ANOVAs of cardenolide concentrations did not alter the significant effects of time or treatment on total cardenolide amounts (data not shown). The minimal relationship of cardenolides with foliar nitrogen and LMA, and other commonly used reflectance-derived vegetation indices (Table 1), demonstrates that our ability to detect the rapid increase in foliar cardenolide production over a short (< 24 h) time period is not a byproduct of the relationships among other plant variables.

A key component of this study is the ability of spectroscopy to provide chemical and morphological information regarding multiple plant constituents simultaneously with a single spectral measurement. We were able to generate foliar nitrogen and LMA with the same spectrum as used to determine cardenolides. Although the calibrations used to determine foliar nitrogen and LMA were generated from models built using *Populus* spp. leaves (Serbin *et al.*, 2012), the measurements reported (1.2–5.2% leaf nitrogen and 48.7–94.8 LMA) fall within the values reported in the literature for *A. syriaca* (Nagel & Griffin, 2004; Zehnder & Hunter, 2007). The ability to simultaneously determine changes in multiple plant constituents on fresh, rather than harvested, leaves undoubtedly provides a more complete understanding of the physiological changes in plants in response to herbivory.

By repeatedly sampling reflectance from the same leaf, we successfully tracked changes in phytochemical concentrations, thereby reducing the need for progressive harvesting of foliage over the course of an induction study. Although not identical, the reflectance patterns of leaves used to build the model and leaves repeatedly sampled were similar, suggesting that changes in leaf optical properties varied in a predictable manner. To our knowledge, this is the first study demonstrating the ability of spectroscopic measurements to follow a rapid chemical induction *in vivo* in response to foliar damage. Few studies have explored the ability of leaf optical properties to nondestructively characterize real-time changes in plant chemical properties (Bilger *et al.*, 1989; Gamon & Surfus, 1999). We have built on these previous studies by demonstrating the utility of leaf optical characteristics, in our case reflectance spectroscopy, to describe variations in plant responses to foliar damage.

The rapid induction of plant defenses in response to herbivory has received considerable attention as a mechanism by which plants can avoid the costs of producing defenses whilst maintaining their resistance benefits (Karban & Baldwin, 1997). As such, the inducibility of defenses has been used to help elucidate theories in plant ecology and evolution (Herm & Mattson, 1992; Rasmann & Agrawal, 2009). Our results demonstrate a unique application of existing technologies in spectroscopy to characterize phytochemical variation in response to damage. Our findings highlight the potential of these techniques for the study of plant–environment interactions, specifically because of the rapid and repeatable determination of multiple plant traits simultaneously.

Our study demonstrates the capacity to estimate cardenolide concentrations in milkweed using reflectance spectroscopy, but we recognize possible limitations in our measurement approach for the calibration estimates of cardenolide concentrations. Our chemical assay relies on the measurement of the absorbance of a chromophore produced from the reaction between the butenolide portion of cardenolides and TNDP. Although this method is highly correlated with cardenolide concentrations produced via more sensitive chemical assays, such as HPLC (Rasmann *et al.*, 2009), TNDP can also react with foliar components other than cardenolides, including ketones, pregnane glycosides and plant pigments (Malcolm *et al.*, 1989). In addition, our analytical approach does not discern among individual cardenolides; thus, we are unsure of the sensitivity of our model to the prediction of specific cardenolides. Ultimately, the comparison of models using estimates produced via multiple methodologies will demonstrate whether more sensitive chemical assays used for calibration data are needed to reduce residual error in predictive models and to demonstrate the ability of our approach to predict individual cardenolides. However, our results are within the range of total cardenolide concentrations reported in the literature, and replicate the expected cardenolide induction profile through time as a consequence of simulated herbivory seen by Malcolm & Zalucki (1996).

Our analysis was based on plants from one population, and so the range of cardenolide prediction of our model does not completely capture the full range of variability in cardenolides across all genotypes of *A. syriaca* in northern North America (Bingham & Agrawal, 2010; Vanette & Hunter, 2011). As such, our model probably breaks down at cardenolide concentrations outside the range sampled in our study; however, the cross-validation results, combined with the nominal relationships of cardenolides with foliar nutrient and morphological traits, suggest that our model is reliable within the range of cardenolide concentrations measured here, and is robust against variation in plant nutritive status and physical characteristics. The inclusion of a wider concentration range of cardenolides from a variety of genotypes, and species, of milkweed, together with more sensitive chemical information,

would probably increase model precision at lower concentrations. Regardless of these known limitations, we have demonstrated the effectiveness and ability of repeatedly measured spectroscopic data to accurately characterize phytochemical variation in fresh milkweed leaves.

Our work demonstrates that ecologically relevant secondary metabolites can be quantified and tracked successfully in fresh foliage using reflectance spectroscopy in the visible, NIR and SWIR wavelengths. In addition, we have shown that variation in these compounds in response to environmental perturbations can be detected *in vivo* through the resampling of the same leaf. Although based solidly in sound analytical chemistry, the expansion of research relating hyperspectral data and multivariate modeling to a broad array of secondary metabolites and physical traits can provide novel insights into the ecology and evolution of plant–herbivore interactions by rapidly and simultaneously measuring multiple plant characteristics.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Coefficients produced from the partial least-squares regression (PLSR) model predicting cardenolide concentrations using wavelengths of 400–2400 nm

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