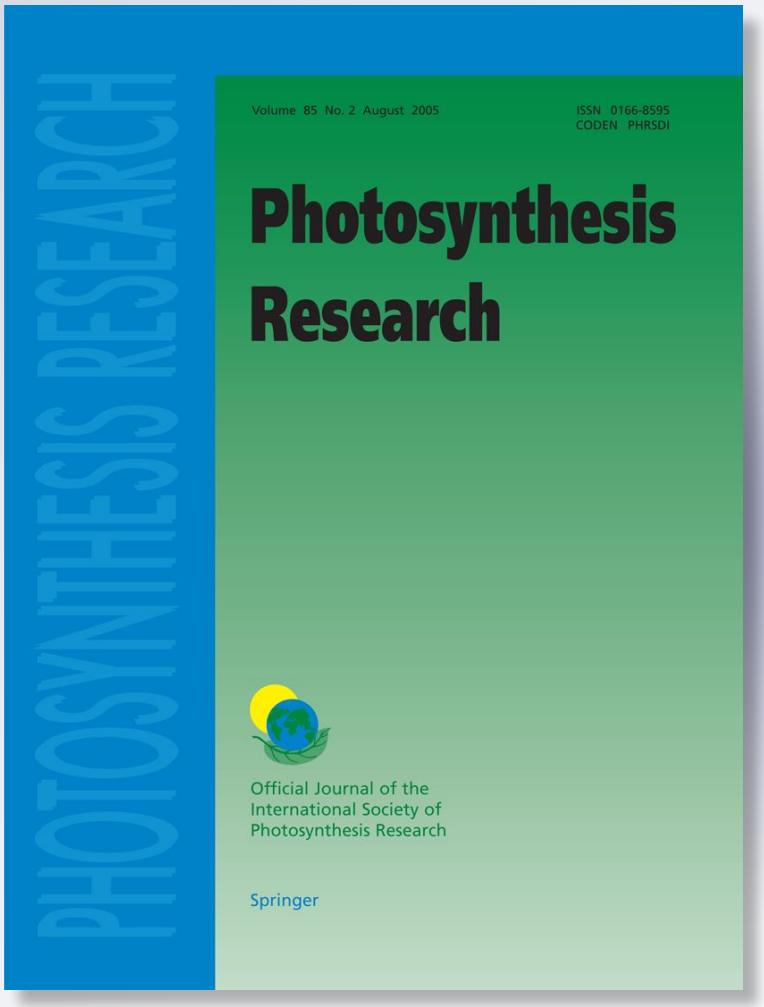
Herbivory of wild Manduca sexta *causes fast down-regulation of photosynthetic efficiency in* Datura wrightii: *an early signaling cascade visualized by chlorophyll fluorescence*

Greg A. Barron-Gafford, Uwe Rascher, Judith L. Bronstein, Goggy Davidowitz, Brian Chaszar & Travis E. Huxman

Photosynthesis Research
Official Journal of the International Society of Photosynthesis Research

ISSN 0166-8595

Photosynth Res
DOI 10.1007/s11120-012-9741-x



Springer

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REGULAR PAPER

Herbivory of wild *Manduca sexta* causes fast down-regulation of photosynthetic efficiency in *Datura wrightii*: an early signaling cascade visualized by chlorophyll fluorescence

Greg A. Barron-Gafford · Uwe Rascher · Judith L. Bronstein · Goggy Davidowitz · Brian Chaszar · Travis E. Huxman

Received: 4 November 2011 / Accepted: 18 April 2012

Abstract Plants experiencing herbivory suffer indirect costs beyond direct loss of leaf area, but differentially so based on the herbivore involved. We used a combination of chlorophyll fluorescence imaging and gas exchange techniques to quantify photosynthetic performance, the efficiency of photochemistry, and heat dissipation to examine immediate and longer-term physiological responses in the desert perennial *Datura wrightii* to herbivory by tobacco hornworm, *Manduca sexta*. Herbivory by colony-reared larvae yielded no significant reduction in carbon assimilation, whereas herbivory by wild larvae induced a fast and spreading down-regulation of photosynthetic efficiency, resulting in significant losses in carbon assimilation in eaten and uneaten leaves. We found both an 89 % reduction in net photosynthetic rates in herbivore-damaged leaves and a whole-plant response (79 % decrease in undamaged leaves from adjacent branches). Consequently, herbivory costs are higher than previously estimated in this well-studied plant–insect interaction. We used chlorophyll fluorescence imaging to elucidate the mechanisms of this down-regulation. Quantum yield decreased up to 70 % in a small concentric band surrounding the feeding area within minutes of the onset of herbivory. Non-photochemical energy dissipation by the plant to avoid permanent damage was elevated near the wound, and increased systematically in distant areas of the leaf away from the wound over subsequent hours. Together, the results underscore not only potential differences between colony-reared and wild-caught herbivores in experimental studies of herbivory but also the benefits of quantifying physiological responses of plants in unattacked leaves.

Keywords Herbivory · *Datura wrightii* · *Manduca sexta* · Down regulation · Mutualism · Antagonism

Abbreviations

F_0	Ground fluorescence of dark-adapted leaf
F_m	Maximum fluorescence of dark-adapted leaf
F_s	Steady-state fluorescence of light adapted leaf
F'_m	Maximum fluorescence of light-adapted leaf
F'_0	Ground fluorescence after a saturating pulse, in the absence of actinic light
F_v	Variable fluorescence of dark-adapted leaf $F_v = (F_m - F_0)$
F_v/F_m	Maximum quantum yield of photosystem II of dark adapted leaf
ΔF	Variable fluorescence of light adapted leaf $\Delta F = F'_m - F_s$
$\Delta F/F'_m$	Effective quantum yield of light adapted leaf
NPQ	Non-photochemical quenching NPQ $= [(F_m - F'_m)/F'_m]$
qN	Coefficient of non-photochemical quenching $q\text{N} = [(F_m - F'_m)/(F'_m - F'_o)]$
qP	Coefficient of photochemical quenching $q\text{P} = [(F'_m - F_s)/(F'_m - F'_0)]$

G. A. Barron-Gafford (✉) · B. Chaszar · T. E. Huxman
B2 Earthscience, University of Arizona, Tucson, AZ 85721, USA
e-mail: gregbg@email.arizona.edu

G. A. Barron-Gafford · J. L. Bronstein · T. E. Huxman
Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ 85721, USA

U. Rascher
IBG-2: Plant Sciences, Institute of Bio- and Geosciences, Forschungszentrum Jülich, Leo-Brandt-Str., 52425 Jülich, Germany

G. Davidowitz
Department of Entomology, University of Arizona, Tucson, AZ 85721, USA

Published online: 11 May 2012

Introduction

Mutualistic interactions between species lead to both species accruing greater fitness than if they were not involved in the association. Such relationships have been found to be ubiquitous in nature (Janzen 1985). However, the ecology and evolution of mutualisms are shaped not only by the benefits that they confer, but also by the diverse costs involved (Bronstein 2001). A fundamental question is the biological means by which one species prevents its partner from extracting excessive costs, shifting mutualism toward unilateral benefit or antagonism (Holland et al. 2004). Occasionally, an interspecific association can be considered a mutualism during one life-history stage but as an antagonism in another. In one well-studied example, certain herbivores feed upon nectar at and, incidentally, pollinate the same plant they oviposit upon during that same visit. These multifaceted associations offer particular advantages for studying the physiological ecology of the intersection between pollination and herbivory (Adler and Bronstein 2004; Bronstein et al. 2006).

The association between the North American desert perennial *Datura wrightii* (Solanaceae) and the tobacco hornworm *Manduca sexta* (Sphingidae) provides a model system for studying the mutualism-antagonism interface (Bronstein et al. 2009). During the adult life stage, the interaction with *M. sexta* is mutualistic, in which *D. wrightii* provides copious amounts of nectar for the *M. sexta* ($\sim$60 µl, Riffell et al. 2008). *D. wrightii* benefits by receiving outcrossed pollination, which has been shown to significantly increase the percentage of flowers that set fruit and average number of seeds per fruit (Bronstein et al. 2009). *M. sexta* larvae however, can completely defoliate an entire *D. wrightii* by their final instar (McFadden 1968), resulting in a clear antagonistic component of the *D. wrightii—M. sexta* interaction. Assessing indirect costs of herbivory above and beyond the physical loss of leaf area is particularly important for understanding the balance of costs and benefits within this complex interaction.

Quantifying changes in plant carbon gain through photosynthesis is an especially valuable means of evaluating both indirect and direct costs of herbivory. Plants may respond to the antagonistic effect of herbivory by reducing photosynthetic uptake, resulting in fewer resources available to invest in reproductive structures, defensive compounds, or attracting and rewarding subsequent pollinators (Bronstein et al. 2006). Within this study, we made continuous measurements of the physiological status of *D. wrightii* experiencing *M. sexta* herbivory with the aim of quantifying the degree to which herbivory negatively affected an individuals' physiological capacity for photosynthetic energy conversion and carbon gain, beyond the removal of leaf tissue.

Generally speaking, previous studies using traditional measures of net photosynthesis have shown that herbivory can induce a reduction, no net change, or an over-compensatory stimulation of photosynthesis (von Caemmerer and Farquhar 1984; Meyer and Whitlow 1992; Meyer 1998; Zangerl et al. 2002; Turnbull et al. 2007; Delaney 2008), depending on the guild of insect involved, site of herbivory, age of leaves eaten, and the extent of the damage induced. Though measurements made using traditional gas exchange equipment are highly repeatable, they are somewhat limited in only delivering a single net value of CO_2 uptake for large sections of leaves (e.g., 6 cm^2 in commonly used gas exchange systems). This can be especially problematic if the entire measurement area is an order of magnitude larger than the area affected by herbivory.

Chlorophyll fluorescence techniques have been increasingly used in addition to gas exchange approaches to provide additional information on the efficiency of photochemistry, non-photochemical energy dissipation, and overall photosynthetic performance (Maxwell and Johnson 2000; Zangerl et al. 2002; Nabity et al. 2009). Such paired measurements yield information on the extent to which PSII is using absorbed energy and the degree to which PSII is being damaged by excess light. With chlorophyll fluorescence imaging, spatial measures of leaf function can be used to quantify the spatio-temporal regulation of quantum efficiency, rates of electron transport, and the level of photoinhibition a plant is experiencing due to environmental stresses across an entire leaf. Use of chlorophyll fluorescence imaging has increased in recent years for rapidly detecting indirect perturbations in leaf metabolism before any effects on growth and development are detected (Zangerl et al. 2002; Barbagallo et al. 2003; Aldea et al. 2005; Aldea et al. 2006; Tang et al. 2006; Delaney 2008; Nabity et al. 2009).

Here, we simultaneously quantify how net photosynthesis of *D. wrightii* responds to *M. sexta* herbivory in eaten leaves and leaves from adjacent branches that never came into contact with the herbivore. We do not know the degree to which carbon assimilation might be reduced as a result of herbivory in this association, nor do we understand the spatio-temporal dynamics of any potential responses in photosynthesis in adjacent leaves as a result of internal plant signaling. This study made use of both classic and developing plant ecophysiological techniques to (1) quantify any immediate changes in leaf photosynthetic performance in *D. wrightii* in response to the onset of herbivory by *M. sexta*, (2) examine spatio-temporal variations in leaf net photosynthesis, quantum efficiency and/or photoinhibition through repeated measures on the same

leaf through time, and (3) quantify the indirect costs of herbivory on photosynthetic performance in adjacent leaves that never come into contact with the herbivore.

Because the *D. wrightii/M. sexta* relationship is considered a model system for plant–insect interactions (Bronstein et al. 2006), research is often conducted both in the laboratory and in field. One important feature of this overlap in research settings is the frequent use of both colony-reared and wild-caught larvae. The former derive from individuals that have been bred in the laboratory on artificial diets for hundreds of generations (D'Amico et al. 2001); the latter are native to the southwestern US habitat where the host plant occurs. To date, no comparison has been made of how host plants respond to these two populations. Thus, a fourth goal of this study was to compare the ecophysiological consequences of *D. wrightii* consumption by colony-reared and wild-caught *M. sexta*.

Materials and methods

Growth of plant material and rearing of insects

Datura wrightii Regel (Solanaceae) seeds were collected from plants located on the Santa Rita Experimental Range south of Tucson, Arizona, USA (SRER; 31°35′N lat, 110°53′W long). Seeds were separated from their fruits, scarified, washed, and placed in a cold treatment for 10 days before sowing. Seeds were sown in two-gallon pots containing a 3:2 (v/v) ratio of Sunshine Professional Growing mix (Vancouver, British Columbia, Canada): vermiculite. Pots were then placed in a controlled environment growth chamber (Conviron E7/2, Controlled Environments Ltd, Winnipeg, Manitoba, Canada) with an irradiance of ~1,100 μmol m^{-2} s^{-1} and average day/night temperatures of 30/22 °C over a 12-h day/night cycle. A 1:20 Hoagland's nutrient solution was delivered to all plants once every week (Hoagland 1920) to insure that the plants did not experience nutrient deficiency. All measurements were performed on fully expanded leaves that had experienced neither any herbivory nor any detectable physical damage, and none of the plants had begun producing buds.

Two populations of *Manduca sexta* L. (Sphingidae) larvae were used within this study. One population had been raised in a laboratory colony for more than 240 generations (Sanes and Hildebrand 1976; Tolbert et al. 1983; D'Amico et al. 2001). These larvae, hereafter referred to as colony larvae, had been fed a standard artificial diet (as described in Davidowitz et al. 2003) in previous generations, but were fed solely *D. wrightii* foliage from the day of hatching. The second population of *M. sexta* was wild larvae collected from *D. wrightii* plants within the SRER, where the seed-stock had been collected the previous year. Both colony and wild larvae were raised on foliage removed from *D. wrightii* grown exclusively for supplying foliar food. Larvae were used for our study after they had reached their third instar.

Immediate and lagged photosynthetic uptake responses to herbivory in eaten and uneaten leaves

The immediate and longer-term responses of *D. wrightii* photosynthetic uptake in response to herbivory by colony and wild larvae were quantified using a portable photosynthesis system (LI-6400, LI-COR Biosciences, Nebraska, USA) outfitted with a 30 × 20 mm measurement cuvette with a controllable light source. Conditions within the cuvette were set to match those of the entire growth chamber, such that the day/night cycles experienced by the leaf were the same as those experienced by the entire plant. Two fully-unfurled leaves of like age and size were chosen from different branches within each plant for sampling. One of these leaves was randomly selected to experience controlled herbivory by a larva, while the other leaf from an adjacent branch, hereafter referred to as the undamaged leaf, never came into contact with a larva but was used to quantify any within-plant changes in photosynthetic uptake. Controlled herbivory never crossed the mid-vein of the leaf's axial side, i.e., between the petiole and the cuvette, and was conducted by supporting the larva such that it was able to grip onto the leaf and crawl around each leaf's perimeter while eating the foliage. In this way, any damage to the plant was induced by herbivory and not by the weight of the insect hanging on the leaf. Importantly, all damage occurred outside of the measurement cuvette, so any reductions in leaf area were not, themselves, responsible for the quantified reductions in leaf performance within the cuvette. Near-continuous logs of photosynthetic uptake, stomatal conductance, and transpiration were taken from at least 30 min before herbivory and for the subsequent 60 h.

Photosynthetic response curve measurements on both the eaten and undamaged leaves were conducted to quantify internal (within the leaf) limitations to photosynthesis induced by herbivory. Photosynthetic CO_2 response curves (A-C_i) were calculated by measuring net photosynthesis (A) in response to a range of intercellular CO_2 concentrations ($[C_i]$). External ambient CO_2 concentrations ($[C_a]$) were delivered by the measurement instrument in 10 steps, decreasing from 1,800 to 50 μmol mol^{-1}, while the photosynthetic photon flux density (light; *PPFD*) was maintained at a saturating value of 1,500 μmol m^{-2} s^{-1}. Photosynthetic light response curves (A-*PPFD*) were developed by measuring the response of A to varying *PPFD* at saturating atmospheric $[CO_2]$ of 1,000 μmol mol^{-1}. We reduced *PPFD*

in 10 steps from 1,500 to 0 μmol m^{-2} s^{-1}. All response curve measurements were recorded at each value after photosynthesis had equilibrated. The *A*-C_i and *A*-*PPFD* curves were measured on the same leaf before and 3 h after herbivory on each of five replicate plants. Subsequent information on the maximum *A* rates within these curves (A_{max}) and biochemical limitations on *A* (Vc_{max}, J_{max}) were obtained using the processes described by Long and Bernacchi (2003). The relative stomatal limitation to photosynthesis (*RSL*), was calculated from *A*-C_i curves by the method of Farquhar and Sharkey (1982) using the equation: $R = [1 - (A_g/A_o)] * 100$, where A_g is the rate of net photosynthesis at the ambient growth [C_a] ([400 ppm]) within the growth chamber and A_o is the net photosynthetic rate when [C_i] equals the *operational*, growth [C_a] ([400 ppm]). Theoretically, when [C_i] equaled [C_a], A_o was the rate of *A* that would have occurred had stomatal conductance been limitless. *RSL* therefore produces an estimate of the relative reduction in photosynthesis attributable to CO_2 diffusion between the atmosphere and the site of carboxylation in the leaf.

Quantitative imaging of chlorophyll fluorescence

Images of chlorophyll fluorescence were acquired at 2 min intervals using an imaging chlorophyll fluorometer (Mini-PAM, Heinz Walz GmbH, Effeltrich, Germany). A single leaf was fixed within the optical plane of the imaging system such that a 3 × 2 cm area of the leaf, equal to the standard photosynthesis measurement cuvette detailed above, was measured at a resolution of 640 × 512 pixels. After 30 min of dark adaptation, initial fluorescence when all PS II reaction centers were open (F_o) was measured, and then maximum fluorescence (F_m) was measured at the end of a saturating light pulse (intensity ~2,800 μmol m^{-2} s^{-1}). Light sufficient to drive photosynthesis (actinic light, PPFD = 1,000 μmol m^{-2} s^{-1}) was then applied, and ground fluorescence (F_s) was measured throughout the next few hours. Maximum light-adapted fluorescence (F'_m) was acquired every 2 min in response to a saturating light pulses superimposed on the actinic light. Three measures of F'_m were acquired prior to introducing the *M. sexta* larva to the plant. Measures of F_s and F'_m were acquired throughout herbivory and for the subsequent 1.5 h after the insect was removed from the leaf. These chlorophyll fluorescence measurements were repeated on five plants.

From these measurements, the instrument calculated the following parameters describing the state of photochemistry of light reactions. Maximum quantum yield of photosystem II (F_v/F_m) of the dark-adapted leaf was calculated by the instrument as $F_v/F_m = (F_m - F_o)/F_m$, where F_v (variable fluorescence) equals the fluorescence increase induced by the saturation pulse (Kitajima and Butler 1975). Effective quantum yield (Φ_{PSII}) of the light-adapted leaf was calculated as $\Delta F/F'_m = F'_m - F_s/F'_m$ (Genty et al. 1989; Maxwell and Johnson 2000). $\Delta F/F'_m$ provides an estimate of the effective portion of absorbed quanta used in PSII reaction centers. *q*P is defined as the coefficients of photochemical fluorescence quenching and is a measure of the fraction of still-open PSII reaction centers. *q*P was calculated as $[(F'_m - F_s)/(F'_m - F'_0)]$ (Bilger and Schreiber 1986; Maxwell and Johnson 2000). Non-photochemical quenching (NPQ), calculated as: $NPQ = (F_m - F'_m)/F'_m$, is induced under conditions when the photosynthetic apparatus cannot use all of the absorbed light energy for photochemistry. The magnitude of NPQ is an indicator of the stress severity and measures changes in heat dissipation relative to the dark-adapted state (Bilger and Björkman 1990; Maxwell and Johnson 2000). The coefficient of non-photochemical quenching (*q*N) was calculated as $qN = [(F_m - F'_m)/(F'_m - F'_0)]$ and was used as a very sensitive indicator of stress induced limitations (Bilger and Schreiber 1986; Walz 2006). *q*N accounts for the fact that not only variable fluorescence (induced upon reaction center closure), but also dark-level fluorescence (all centers open) can be quenched non-photochemically, primarily by heat dissipation stimulated during illumination (Maxwell and Johnson 2000; Walz 2006). The calculation of *q*P and *q*N require the parameter F'_0 that is experimentally obtained after a dark red light pulse is applied on previously light adapted leaves. In our measurement protocol, which involved long time series, such a measurement was not possible and therefore we estimated F'_0 from F_0, F_m, F'_m, and F_v according to Eq. 1 (Oxborough and Baker 1997).

$$F'_0 = \frac{F_0}{\frac{F_v}{F_m} + \frac{F_0}{F'_m}} \quad (1)$$

In order to quantify the spatial variation in each leaf's photosynthetic function through time in response to herbivory, we used the software associated with the fluorometer (ImagingWIN, Heinz Walz GmbH, Effeltrich, Germany) to acquire values of all of the aforementioned variables from every point along a transect "drawn" on the leaf image (Fig. 5). Within this experiment, five transects were drawn across each of five replicate leaves from different plants to gain insight into the photosynthetic function of leaves prior to, during, and following controlled herbivory.

Results

Controlled herbivory resulted in both a direct cost to the plant in terms of loss of leaf area for CO_2 assimilation through photosynthesis and in indirect costs in terms of

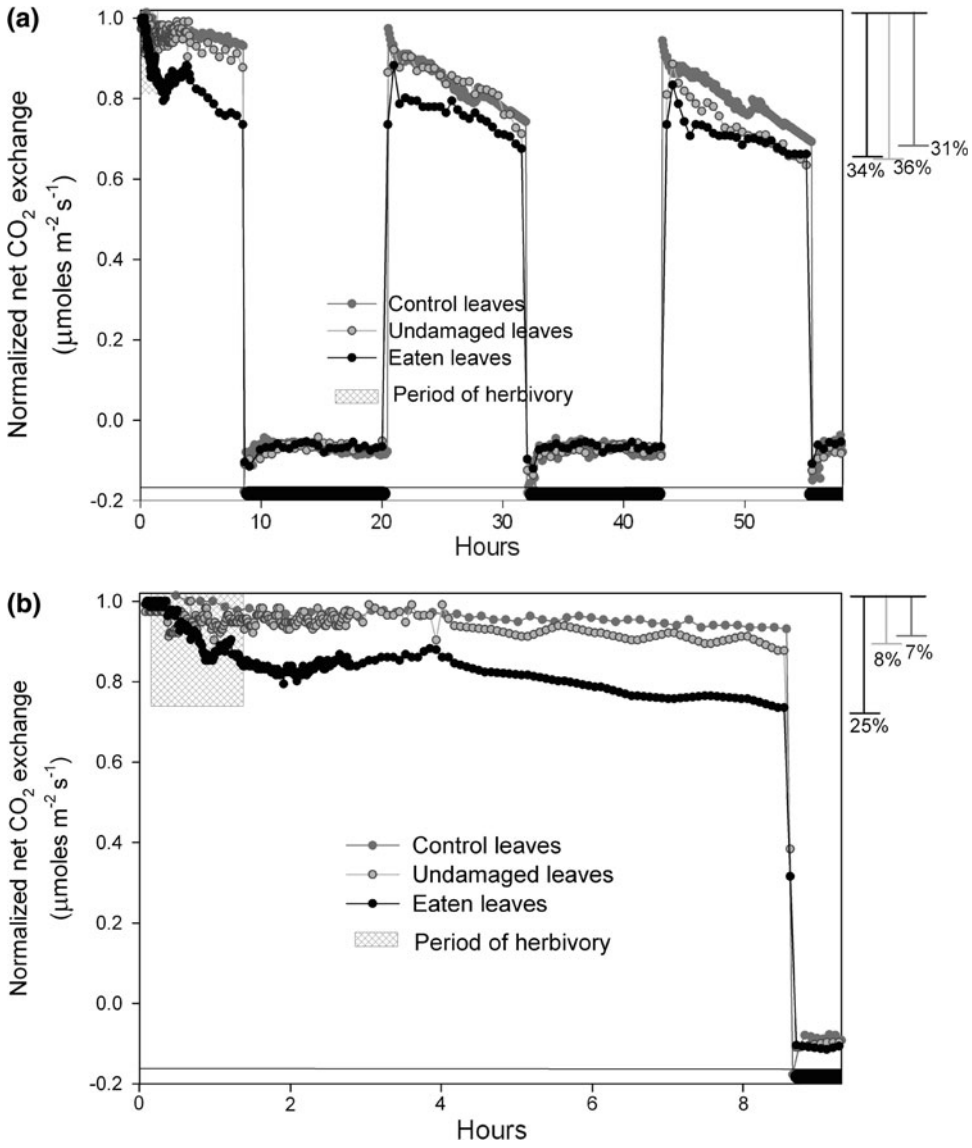

Fig. 1 Measures of net CO_2 exchange (positive = photosynthesis; negative = respiration) in leaves experiencing herbivory by colony-raised *Manduca sexta* larvae, alongside simultaneous measures of photosynthetic rates in uneaten leaves from adjacent branches illustrate that **a** after 3 days post-herbivory there were no significant reductions in photosynthesis relative to the uneaten controls. **b** Closer examination of the first day pre- and post-herbivory illustrate that there was a transient significant reduction in photosynthetic rates in eaten leaves, but not in uneaten leaves. The *bars* below each graph represents light conditions: *white bars*, *light periods*; *black bars*, *dark periods*

reductions in net photosynthetic uptake within the remaining leaf area, including leaves from adjacent branches that did not experience any contact with the herbivore. Average loss of leaf area was 3.4 cm^2, which represented an average loss of 36.2 ± 2.3 % of the original leaf for all replicates. Reductions in photosynthesis within the remaining portion of the leaf varied depending on the type of larvae involved (colony-reared vs. wild) and the proximity to the damage (eaten leaf versus uneaten leaf on an adjacent branch). Average net photosynthetic rates (A) in control plants and in treatment plants before herbivory averaged 21.4 ± 3.1 µmol m^{-2} s^{-1}. Because we were comparing average changes in A in response to herbivory, rather than quantifying plant-to-plant variation, we normalized A rates within each plant relative to that plant's pre-herbivory rate. At the end of a 60 h period following herbivory by a colony-raised larva, A was reduced an average of 34 % in eaten leaves (Fig. 1a). Reductions in A were not significantly different between undamaged leaves from adjacent branches and the leaves of control plants, which experienced reductions of 36 % ($P = 0.353$) and 31 % ($P = 0.286$), respectively. Initial declines in A after herbivory, however, were greater in leaves that experienced herbivory than in undamaged leaves or control plants (Fig. 1b). In contrast, herbivory by wild-caught larvae resulted in substantially greater reductions in A, of an average of 89 and 79 % after 3 days in eaten leaves and those that never experienced herbivory, respectively (Fig. 2a). Dramatic reductions in both the eaten and undamaged leaves could be seen after only 8 h, in which A were already reduced by an average of 73 and 37 %, respectively (Fig. 2b).

To examine the underlying biochemical limitations to A seen in response to herbivory by wild larvae, we

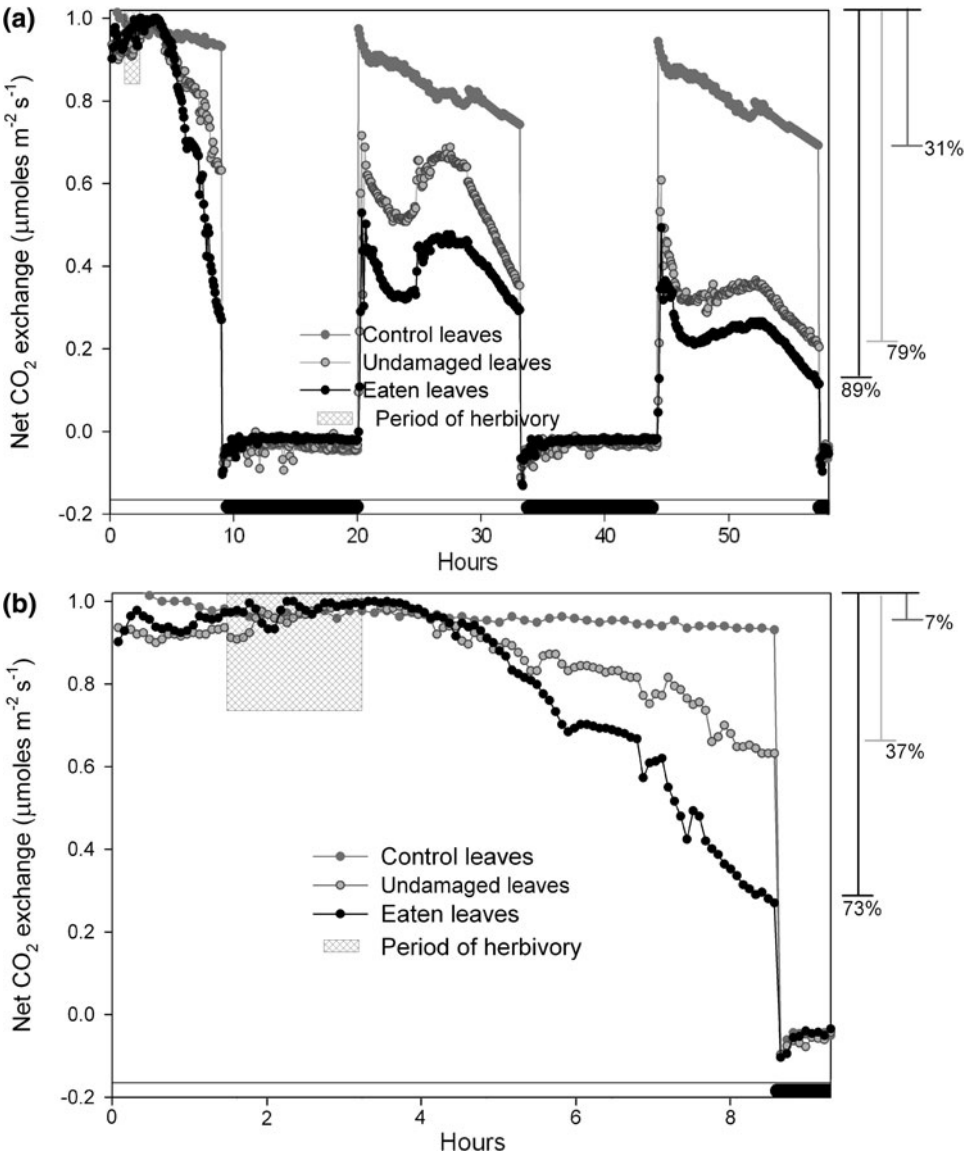

Fig. 2 Measures of net CO_2 exchange (positive = photosynthesis; negative = respiration) in leaves experiencing herbivory by wild-caught *Manduca sexta* larvae, alongside simultaneous measures of photosynthesis in uneaten leaves from adjacent branches illustrate the **a** detection of significant reductions in photosynthesis in eaten and uneaten leaves on treatment plants relative to uneaten controls 3 days post-herbivory. These results illustrate not only a down-regulation in physiological performance in leaves that experienced the herbivory, but also a reduction throughout the plant. **b** Closer examination of the first day pre- and post-herbivory illustrate that significant reductions in photosynthetic rates in eaten and uneaten leaves on treatment plants were detectable within a few hours. The *bars* below each graph represents light conditions: *white bars*, *light periods*; *black bars*, *dark periods*

analyzed the response of A to variations in CO_2 concentrations (A-C_i curves) and light levels (*A-PPFD* curves). Subsequent analysis through A-C_i curves and chlorophyll imaging were only conducted on those plants experiencing herbivory from wild-caught larvae because of the relatively limited response of photosynthetic performance to herbivory by colony-reared insects. Actual maximum photosynthetic rates under saturating light and [CO_2] (A_{max}) averaged 28.9 ± 2.1 µmoles m^{-2} s^{-1}. Though there was an average 18 % reduction in A_{max}, as determined within a A-C_i curve, 3.5 h after herbivory, A_{max} values were not significantly different between the eaten and undamaged leaves either pre- (Fig. 3a) or post-herbivory (Fig. 3b). Vc_{max}, a proxy for maximal carboxylation rate of Rubisco, as determined from the initial slope of the A-C_i curve, averaged 61.8 and 53.4 pre-herbivory in undamaged and eaten leaves, respectively. There was no significant change in undamaged leaves, but a 12.6 % increase post-herbivory in the eaten leaves. J_{max}, an indicator of the maximum rate of electron transport used in the regeneration of Ribulose-1,5-bisphosphate, the molecule with which CO_2 reacts during photosynthetic carbon fixation, averaged 46.2 ± 9, but increased an average of 14 % ($P = 0.022$) and 32 % ($P < 0.0001$) post-herbivory in undamaged and eaten leaves, respectively. We also did not detect a change in the relative stomatal limitation to photosynthesis in eaten leaves pre-versus post-herbivory % ($P = 0.793$; Fig. 3a vs. b insets). Normalized A_{max} values as determined within the *A-PPFD* curves were not significantly different between undamaged and eaten treatment leaves pre-herbivory, but were reduced to an average of 11 and 44 %, respectively, illustrating a significant difference between the maximum

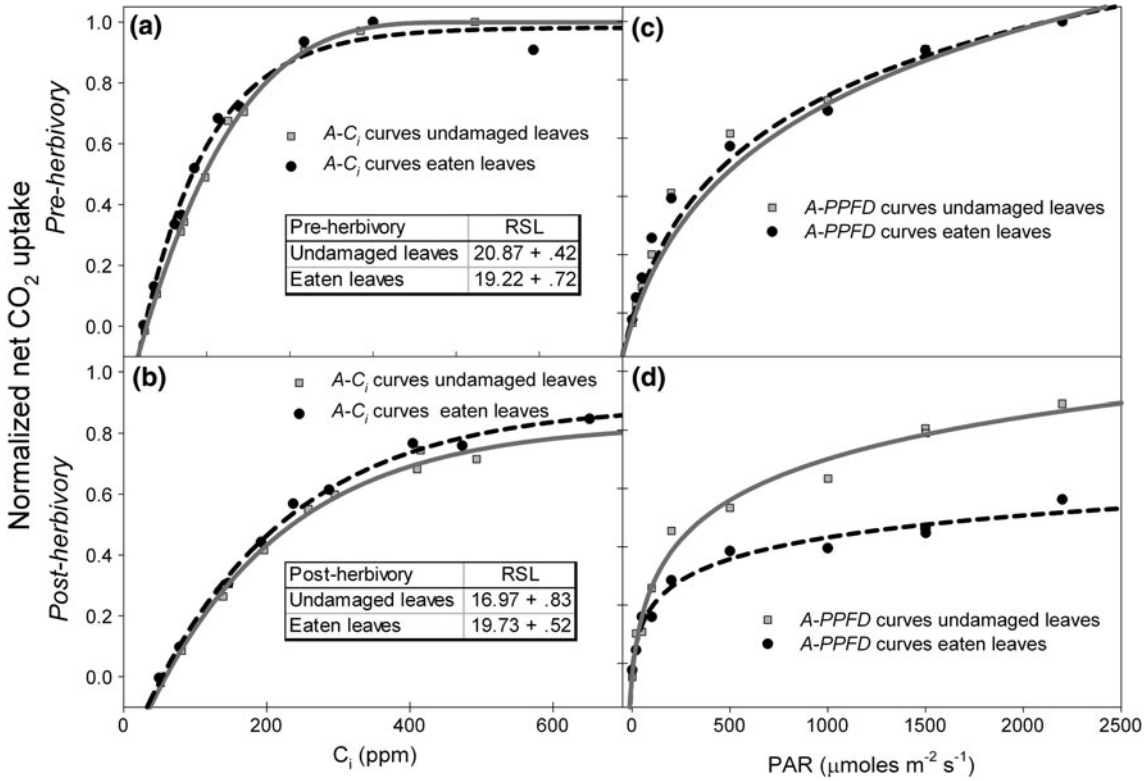

Fig. 3 Measures of net photosynthesis normalized on a per-plant basis (A) in leaves that experienced herbivory by wild-caught *Manduca sexta* larvae, alongside simultaneous measures in uneaten leaves from adjacent branches. Responses of A to varying CO_2 concentrations (A-C_i) **a** pre- and **b** 3 h post-herbivory illustrate that there were significant reductions in maximum photosynthesis, but no real changes in the relative stomatal limitations (RSL; *insets*) to net photosynthetic uptake in response to herbivory. Responses of A to varying irradiance (A-$PPFD$) in eaten and uneaten leaves **c** pre- and **d** 3 h post-herbivory illustrate significant reductions in maximum photosynthesis, the light saturation point, and convexity of the fitted nonrectangular, hyperbolic response curve, particularly in eaten leaves in response to herbivory

photosynthetic capacity of the leaves post-herbivory ($P < 0.0001$; Fig. 3c, d). Similarly, there was a 7.7 % ($P = 0.036$) and 44.2 % ($P < 0.0001$) reduction in the undamaged and eaten leaves, respectively, from the pre-herbivory light saturation point from average of 1,935 ± 254 μmol m^{-2} s^{-1}. Furthermore, we detected a significant 48 % ($P < 0.0001$) and 81 % ($P < 0.0001$) increase in the convexity of the fitted A-$PPFD$ curves in the undamaged and eaten leaves, respectively.

To examine the immediate impact of feeding on the photosynthetic machinery, we used chlorophyll fluorescence imaging to quantify the spatial responses of photosynthetic performance of the leaf through time to herbivory by wild *M. sexta*. Transects were drawn within the data-processing software of the fluorometer that allowed for repeated gathering of spatial transects of typical chlorophyll fluorescence parameter data. An example of these transects and cumulative areas pre- and immediately post-herbivory are shown in Fig. 4. The most significant reductions in the first hours after herbivory in both photochemical parameters [quantum yield ($\Delta F/F'_m$) and the coefficient of photochemical fluorescence quenching (qP)] and non-photochemical parameters [non-photochemical energy dissipation/quenching (NPQ) and the coefficient of non-photochemical quenching (qN)] occurred within the 2.5 mm perimeter around the herbivory wound (Figs. 5, 6). Immediate and longer-term influences of herbivory on these parameters in the distant portion of the leaf are shown in the inset bar graphs. Several zones were detected with respect to proximity to the herbivory wound. In the area immediately adjacent to the wound (the zone of no recovery; zone I), we detected a significant decrease in Φ_{PSII}, near-zero qP, maximum NPQ, and a significant reduction of qN, and we found no pattern through time within any of the parameters. Within zone II, there was a systematic increase through time in the occurrence of NPQ (Fig. 6a), but no pattern through time in the measure of $\Delta F/F'_m$ (Fig. 5a). $\Delta F/F'_m$ and qP remained reduced from the pre-herbivory, undamaged state by an average of 43 % within this zone, even after nearly 1.5 h (Fig. 5). Within zone III, $\Delta F/F'_m$ and qP remained depressed by approximately 20 %, having reached this reduced level of recovery within minutes (Fig. 5).

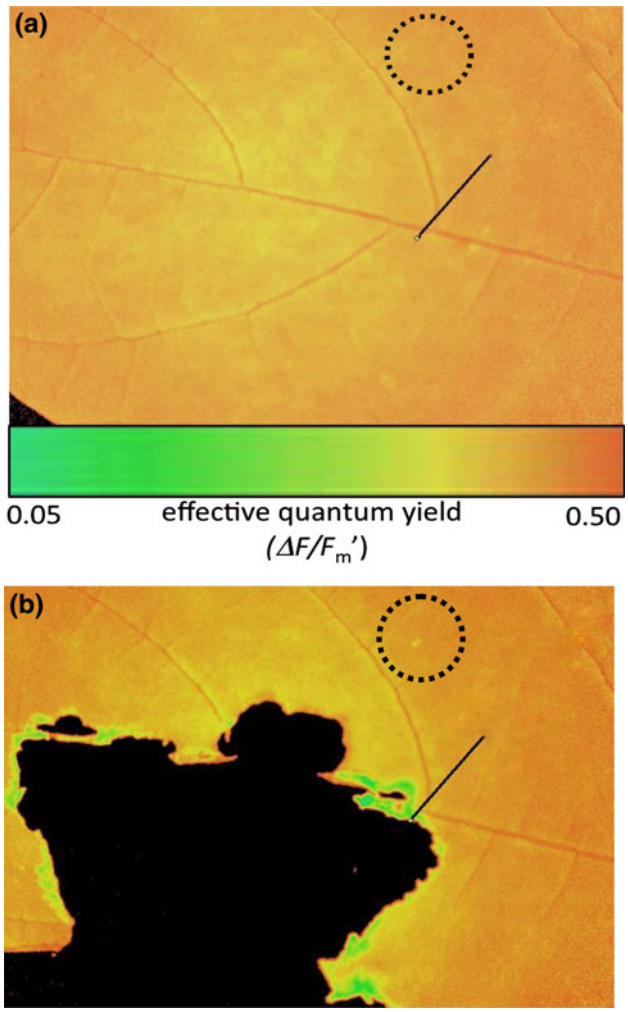

Fig. 4 False-color images of effective quantum yield ($\Delta F/F'_m$) of a *Datura wrightii* leaf **a** prior to and **b** 1 min after the induction of controlled herbivory. The image represents a 20 × 30 mm portion of the entire leaf. The black line moving away from the wounded area toward the edge of the fluorescence image is an example of a "transect" drawn digitally on the leaf for analyses in data-processing within the fluorometer software. The *circle* represents a 100 mm^2 cumulative area of interest, typical of a conventional fluorometer, from which an average "distant" measurement was taken. Data from these transect lines and area of interest regions are shown in Figs. 5 and 6. The *black area* in this image corresponds to non-leaf area, so the increase in *black area* from **a** to **b** illustrates leaf area lost to herbivory

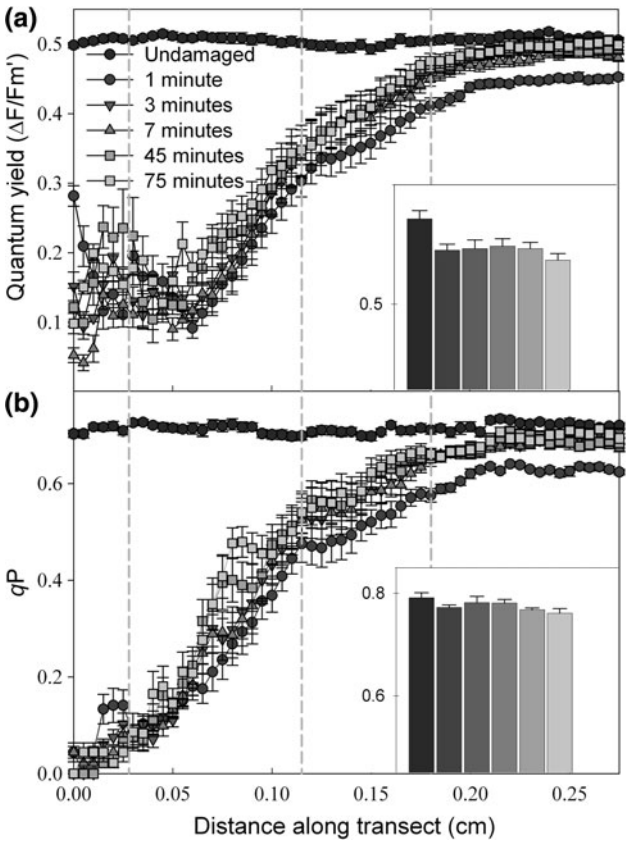

Fig. 5 Spatio-temporal changes in photochemical quenching parameters across a transect line drawn from the point of herbivory (0 on the *x*-axis). *Dashed lines* separate four "Zones" surrounding the herbivory wound of varied plant response. **a** The quantum yield ($\Delta F/F'_m$) of light reactions of photochemistry and **b** the proportion of open PSII reaction centers [*q*P] for the area of the leaf most immediately proximal to the herbivory wound. *Insets*: Changes in the quantum yield and *q*P in a cumulative area of interest in a distant portion of the leaf that did not experience direct herbivory (circle area in Fig. 4). *Error bars* represent one standard error of the mean ($n = 5$ leaves)

Within the non-photochemical parameters, however, a systematic increase NPQ and *q*N continued through time beyond the pre-herbivory state (Fig. 6), indicating a potential spreading of the effects of damage into these portions of the leaf. Even though NPQ and *q*N both quantify non-photochemical energy dissipation, in our case NPQ and qN were not simply correlated, which points toward non-photochemical quenching processes that not only affect maximum fluorescence but also ground fluorescence. Possible mechanisms may be sustained photodamage and non-photochemical energy dissipation.

Discussion

Within this study we quantified immediate and longer-term direct and indirect losses of CO_2 assimilation potential by *Datura wrightii* in response to herbivory by *Manduca sexta* in a controlled setting. These costs of herbivory are particularly intriguing within this system because the interaction within one life-stage of the insect confers a net benefit, as the adult is a very effective pollinator. During the larval stage, however, the interaction with this same species is antagonistic, with the costs manifested as direct leaf area loss and reduced capacity for photosynthetic assimilation in the remaining tissue. Determining the net effect of *M. sexta*'s actions on *D. wrightii* depends on precise measurements of both the benefit and cost components of the interaction (Bronstein et al. 2006). While the benefits of pollination have been relatively well-studied in this system (Bronstein et al. 2009), the costs of herbivory have not.

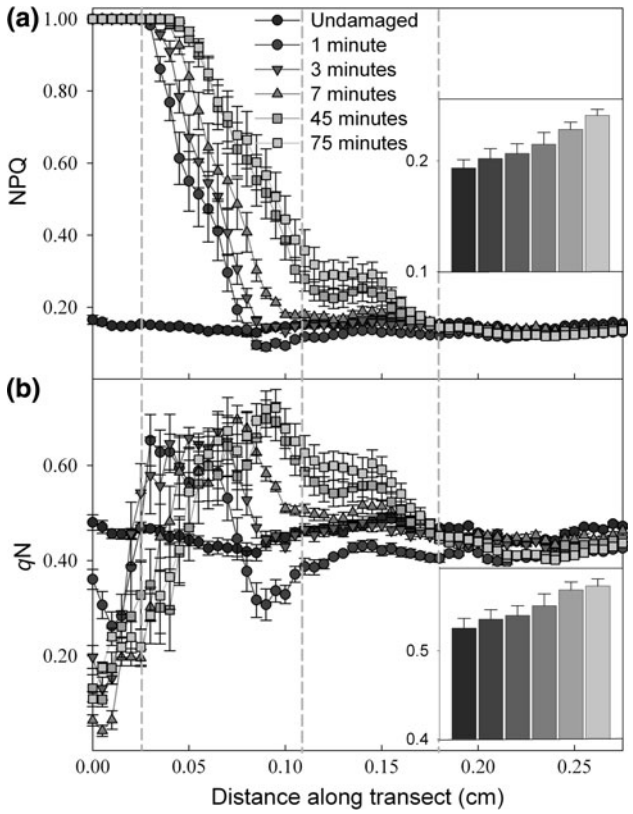

Fig. 6 Spatio-temporal changes in non-photochemical quenching parameters across a transect line drawn from the point of herbivory (0 on the *x*-axis). **a** Rates of non-photochemical quenching (NPQ) and **b** the coefficient of non-photochemical quenching [*q*N] for the area of the leaf most immediately proximal to the herbivory wound. *Insets*: Changes in NPQ and *q*N in a cumulative area of interest in a distant portion of the leaf that did not experience direct herbivory (*circle area* in Fig. 4). *Error bars* represent one standard error of the mean ($n = 5$ leaves)

In this context, we used a combination of classic and developing plant ecophysiological techniques to thoroughly analyze indirect costs of herbivory on CO_2 assimilation. While previous studies have combined both traditional gas exchange and fluorescence imaging (Zangerl et al. 2002; Aldea et al. 2005; Tang et al. 2006), to date, no other studies have simultaneously quantified these metrics in leaves experiencing herbivory and leaves from adjacent branches and tracked a spreading of any plant responses. It is beyond the scope of this study to speak to variations in response to herbivory with leaf position, as we normalized this point by randomly selecting measurement branches within each individual and selecting like leaves. We began by comparing colony-reared and wild-caught larvae to quantify the differential effects that these populations might induce in terms of plant responses to herbivory-induced damage. This was important because the majority of studies of *M. sexta*, a model system in insect biology, rely on colony-reared individuals; studies with wild-caught individuals are relatively rare.

Variation in photosynthetic responses to herbivory by colony-reared versus wild-caught larvae

By simultaneously measuring leaves experiencing herbivory and undamaged leaves on different branches of the same plant, we were able to quantify the indirect costs of reduced rates of net photosynthesis (*A*) in the eaten leaves and the spreading of this down-regulation of *A* throughout the plant. Initial measures illustrated a significant decline in *A* within the first hours of the leaves eaten by colony-reared insects, but reductions of *A* in undamaged leaves from adjacent branches were no more than those of leaves on control plants that never experienced herbivory. After 3 days, all reductions in *A* were statistically similar among these eaten, undamaged, and leaves from control plants, suggesting that any damage induced by herbivory from colony larvae (*i*) was transient, (*ii*) was restricted within the leaf that was eaten, and (*iii*) resulted in no spreading of down-regulation of *A* throughout the plant. The decline of *A* in all leaves throughout the 3 day measurement period was likely due to aging or acclimation of the leaves to the experimental conditions.

Herbivory by wild larvae, however, induced significant immediate and longer-term reductions in *A* in both eaten and undamaged leaves, leaving the resulting leaf area photosynthesizing at an average of 11 and 21 %, respectively, of their pre-herbivory rates. It is particularly intriguing that the response to herbivory, in both eaten and undamaged leaves, from *M. sexta* collected from the field (within a natural *D. wrightii* population) was so different than from herbivory by those taken from a long-established laboratory colony used widely throughout the US (D'Amico et al. 2001). One possible explanation is that having been raised nearly 250 generations in a laboratory, removed from a diet of *D. wrightii*, has changed the chemical composition of the saliva, and therefore the response of *D. wrightii* in response to their feeding. It is well known for *M. sexta* that the chemical composition of the larval saliva triggers a cascade of specific plant reactions (Wu and Baldwin 2009). In fact, application of herbivore saliva alone can have dramatic effects on plant defensive responses to herbivory, more so than the mechanical damage created by herbivory (Felton and Eichenseer 1999; Halitschke et al. 2001). Recent study comparing colony and field populations of *M. sexta* have shown that colony populations experienced reduced fitness (survival and fecundity) when grown on *Proboscidea louisianica* but similar survival when grown on tobacco (*Nicotiana tabacum*), suggesting a mixed response to herbivory on natural host plants (Diamond et al. 2010). An intentional longer-term re-introduction of colony-reared *M. sexta* to a diet of *D. wrightii* over the course of multiple generations could shed light on the evolution of the

chemical biology underlying the responses of *D. wrightii* to feeding by wild larvae. A whole-plant analysis of the upregulation of the wound-signal molecule, jasmonic acid, should be carried out in response to herbivory (McCloud and Baldwin 1997; Henkes et al. 2008; Paschold et al. 2008) by a suite of larvae that have experienced a range of reintroduction time. Regardless, the results of this study suggest that the source of the insect (colony-reared vs. field-caught) to be used in any plant–insect experiment may be as important in determining plant physiological responses as any treatment factors.

Immediate changes in leaf photosynthetic rates in response to herbivory

In the leaves that experienced herbivory by wild larvae, *A* declined to an average of only 27 % of pre-herbivory rates within the first 8 h after herbivory. These decreases will yield a significant limitation in the capacity of these plants, beyond the loss of leaf area. As such, assimilating significantly less carbon will directly limit carbon-based resources used for plant defenses against future attack, as well as resources invested in attracting and rewarding future pollinators. These reductions in *A* do not appear to be driven by increased stomatal limitation (Fig. 3a, b insets), as might have been expected given the foliar lesions that would allow for extreme water loss (Tang et al. 2006), as there was no change in the relative stomatal limitation between pre- and post-herbivory. Maximum photosynthetic rates under saturated light and CO_2 conditions, however, were reduced nearly 50 %. Quantifying *A* responses to changes in light elucidated some underlying physiological and biochemical limitations to *A* that may have resulted in the significant down-regulation in *A*. We found that herbivory significantly reduced the light saturation point, that is, the light-level beyond which increased irradiance produces no increase, and possibly even a decline in *A*. Similarly, there was a significant increase in the convexity in the nonrectangular-hyperbola response curve of *A* across a range of light levels. All of these internal changes to the photosynthetic capacity of the remaining leaves suggest that they were less able to respond to physiological stresses induced by excess heat or light after experiencing herbivory, the two most likely conditions the plants are likely to experience in their native range.

Spatial variations in photosynthetic rates through time suggest a spreading of the down-regulation of photosynthetic efficiency within the eaten leaf

Within eaten leaves, there was quantifiable spatial variation in photosynthetic performance parameters, and the spatial patterns of these changes varied through time. We found significant reductions in $\Delta F/F'_m$, near-zero *q*P, maximal NPQ, and reduced *q*N within the area immediately adjacent to the site of herbivory damage, indicating that photosynthetic performance within this zone was permanently damaged. Just outside of this area, there was a zone of minimal recovery, within which $\Delta F/F'_m$ returned to about 40 % of pre-herbivory rates but rates of NPQ continued to increase systematically through time. Further away, we could identify a third concentric zone in which $\Delta F/F'_m$ returned to an average of 75 % of pre-herbivory rates, but NPQ, again, continued to increase systematically through time at the same point in space as the leaf was forced to dissipate an increasing percentage of excited electrons non-photochemically because photochemical quenching pathways were obviously still limited. Similar localized increases in NPQ near the site of a wound have been shown in other studies (Tang et al. 2006). Measures of these same parameters in a distant portion of the leaf illustrated that after 1.5 h, estimates of photochemical quenching ($\Delta F/F'_m$ and *q*P) were also significantly reduced and means of non-photochemical quenching (NPQ and *q*N) were significantly increased.

Taken together, these results indicate that there was a fundamental difference in the physiological regulation of the response to herbivore damage related to the distance from the feeding wound. We observed a correlated behavior between the photochemical energy pathway (expressed as $\Delta F/F'_m$ or *q*P) and non-photochemical energy dissipation (NPQ and *q*N) in proximity of the feeding. At the transition between zone II and III, the two pathways, however, were clearly decoupled. *q*N, in particular, showed strong spatio-temporal variations, indicating internal physiological disruptions to the means by which the eaten leaf processed excited electrons and dissipated excessive absorbed photon energy. Furthermore, these detrimental disruptions to photosynthetic performance spread away from the wound to distant portions of the leaf that had not experienced direct contact with the herbivore. This is due to either an effect of long-distance signal transduction that influences photochemistry or the fact that unbalanced non-photochemical energy dissipation itself acts as one part in the a signal cascade. These observations may have an analogy to spreading of the heat signal in leaves that also is linked to great variations in photosynthetic parameters (Grams et al. 2010). Furthermore, these disruptions to photosynthetic performance spread away from the wound to distant portions of the leaf that had not experienced direct contact with the herbivore. Though these same parameters were not measured in the adjacent leaves, we did quantify changes in *A* in these uneaten leaves. After 8 h of daylight *A* was reduced to only 63 % pre-herbivory rates, and average *A* was only 21 % of pre-herbivory rates after 3 days.

Conclusions

Within this study we quantified direct and indirect losses of CO_2 assimilation potential by *D. wrightii* in response to herbivory by *M. sexta* in a controlled setting. We found that the degree of response was significantly influenced by the origin of the larvae used in the study: colony-raised insects induced minimal longer-term reductions in *A*, whereas herbivory by wild larvae caused fast down-regulation of photosynthetic efficiency, a possible early-signaling cascade that was visualized by chlorophyll fluorescence imaging. Furthermore, by simultaneously tracking CO_2 uptake rates (*A*) in leaves from adjacent branches that never experienced herbivory, we were able to confirm the spreading of these indirect costs of herbivory to other portions of the plant. Such reductions in *A* are significant for the plant, as reduced carbon uptake will yield fewer resources for growth, development or sustaining of reproductive structures, and construction costs of defensive compounds. Future studies will incorporate these results into a broader reconsideration of the balance between mutualism and antagonism within this interaction (Aber et al. 1989).

Acknowledgments We thank Abreeza Zegeer for greenhouse assistance and Katherine Grieve Rascher, Bryan Helm, and Kristen Potter for assistance in conducting measurements or providing larvae. This study was primarily supported by the National Science Foundation grants to J.L.B., G.D. and T.E.H. (DEB 0316205 and 0522431) and to U.R. (INT-0340609). Additional support was provided by the Philecology Foundation of Fort Worth Texas and the Forschungszentrum Jülich GmbH.

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