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Seasonal Patterns of Leaf Photosynthesis after Insect Herbivory on Common Milkweed, Asclepias syriaca: Reflection of a Physiological Cost of Reproduction, not Defense?

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ABSTRACT.—After defoliation occurs leaf photosynthetic rate (Pn) can increase, decrease or remain unchanged relative to control uninjured leaves depending on the plant species. With common milkweed *Asclepias syriaca* (Asclepiadaceae) we conducted 27 experiments with insect herbivory or mechanical tissue removal to examine whether *A. syriaca* leaf Pn reductions were correlated with the occurrence of gross cardenolide induction and/or with reproductive phenology. Using spectrophotometry, positive cardenolide induction was detected in only one study when Pn impairment was detected with gas exchange data from injured *A. syriaca* leaves, while negative or no cardenolide induction was detected in the other five *A. syriaca* studies with Pn impairment. The occurrence of Pn impairment after partial leaf defoliation did have a seasonal pattern which correlated with *A. syriaca* reproductive phenology: little or no Pn impairment occurred on leaves of pre-flowering or maturing seed pod plants, while moderate to severe leaf Pn impairment occurred on leaves of flowering and early seed pod formation plants. Our results fail to support either constitutive cardenolide levels or gross cardenolide induction trade-offs to be reflected in injured leaf Pn impairment; however, our results could be explained by a resource or hormone trade-off between investment into reproduction with maintaining leaf photosynthesis after herbivory. Specifically, we suggest that a physiological 'cost of reproduction' is increased susceptibility to Pn impairment after herbivory injury on a leaf. Future studies will need to examine whether resource or hormonal regulation trade-offs cause this proposed physiological trade-off between reproduction and photosynthesis.

INTRODUCTION

Across species, plant response to injury has been characterized as highly variable. This variability depends on such factors as what tissue is injured, the type of injury, how much tissue is injured, the timing of injury during a growing season, the tolerance capacity of the plant to injury and the environment in which the plant lives (Pedigo *et al.*, 1986; Maschinski

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and Whitham, 1989; Welter, 1989; Peterson and Higley, 1993, 2001; Trumble *et al.*, 1993; Delaney and Macedo, 2001; Garcia and Ehrlén, 2002). Herbivory can have negative effects (Marquis, 1984; Belsky, 1986; Bergelson and Crawley, 1992; Coley and Barone, 1996), no detectable effect (McNaughton, 1983; Bergelson *et al.*, 1996) or even increases in rare cases (Maschinski and Whitham, 1989; Paige, 1999) on plant growth or fitness.

This variation in effect of herbivory on plants is in part due to various plant compensatory mechanisms to reduce the impact of herbivory, such as delayed leaf senescence, reallocation of assimilates, overproduction of leaves, changes in canopy architecture and compensatory photosynthesis of injured or adjacent uninjured leaves (Chapin, 1991; Peterson and Higley, 1993, 2001; Trumble *et al.*, 1993). Understanding how biotic agents affect plants is important, but understanding is still limited about the mechanisms involved (Higley *et al.*, 1993; Peterson and Higley, 2001). An improved understanding of how herbivores influence the physiology of their host plants will allow more insights about the ecology and evolution of plant-herbivore interactions (Higley *et al.*, 1993; Delaney and Macedo, 2001). One physiological insight that has been studied concerns trade-offs between secondary (*e.g.*, chemical defense allocation) and primary (*e.g.*, photosynthesis) plant physiological responses after herbivory on a plant.

Plant chemical defenses against herbivores can be constitutive (Gershenzon, 1994; Strauss *et al.*, 2002), induced after an initial bout of herbivory (Karban and Myers, 1989; van Dam *et al.*, 1993; Heil and Baldwin, 2002) or both (Malcolm and Zalucki, 1996). The concept of a cost of defense on plant fitness has been extensively examined (Simms and Rausher, 1987; Simms, 1992; Gershenzon, 1994; Bergelson and Purrington, 1996; Baldwin, 1998; Heil and Baldwin, 2002; Strauss *et al.*, 2002). Many defense compounds are physiologically expensive to produce constitutively (Gershenzon, 1994) and induced defenses can be expensive when the defense compound(s) requires a limiting resource to the plant (Baldwin, 1998; Heil and Baldwin, 2002).

Yet, what is the currency of the trade-off to explain the cost of constitutive or induced chemical defenses? Physiological possibilities include costs of synthesis, storage, transport and maintenance, such that a plant has to allocate more of limiting resource (*e.g.*, energy or nitrogen) to a constitutive or induced chemical defense that could otherwise be allocated to plant maintenance, growth, survival or reproduction (Gershenzon, 1994). Numerous studies have also sought to understand how plant photosynthesis is affected by herbivory injury (Welter, 1989; Peterson and Higley, 1993; Zangerl *et al.*, 2002; Tang *et al.*, 2006). One possible proximate source of a trade-off with the cost of defense could be with primary metabolism (*e.g.*, photosynthetic or respiration rates) trading-off with chemical defense induction (Baldwin and Ohnmeiss, 1994; Zangerl *et al.*, 1997; Zangerl *et al.*, 2002), or due to hormonal regulation (Kessler *et al.*, 2004). A physiological trade-off between defense and photosynthesis could then be manifested in plant relative growth rate, new leaf production, compensatory ability, survival and reproduction (Karban and Myers, 1989; Peterson and Higley, 1993; Trumble *et al.*, 1993).

Another factor that may be important in understanding a plant's physiological response to herbivory is seasonal variation based on a plant's reproductive phenology (Chiarello and Gulmon, 1991). It is known that the time of herbivory injury (early, mid or late season) on a plant influences the injury severity on plant physiology (Mercader and Isaacs, 2003; but *see* Macedo *et al.*, 2006), as well as growth, yield and fitness (Maschinski and Whitham, 1989; Higley, 1992; Garcia and Ehrlén, 2002; Knight, 2007). This is due to specific aspects of a plant's source/sink relationships across its phenology (Chiarello and Gulmon, 1991; Mercader and Isaacs, 2003). At times when sink demand is highest (such as flower growth or

seed maturation), it is predicted that plant capacity to compensate following herbivory is lowest (Pedigo *et al.*, 1986; Pedigo and Rice, 2006). Thus, it can be inferred that a plant would be most vulnerable to physiological (including photosynthetic) impairment following herbivory injury (Chiarello and Gulmon, 1991; Higley, 1992; but *see* Macedo *et al.*, 2006).

STUDY SYSTEM

We chose common milkweed, *Asclepias syriaca* (Asclepiadaceae), to examine a possible trade-off between photosynthesis with defense or reproduction for several reasons. First, several specialized defoliating insect herbivores feed on *A. syriaca* (Dussourd and Eisner, 1987; Delaney and Higley, 2006). Second, many milkweeds (Asclepiadaceae) like *A. syriaca* and members of Apocynaceae have a milky white latex that has been suggested to serve as a mechanical defense against herbivores (Dussourd and Eisner, 1987, Malcolm, 1991) and many species in these two plant families constitutively contain cardenolides (Malcolm, 1991, 1995). Cardenolides make milkweeds distasteful or toxic to mammalian herbivores and some generalist insect herbivores (Malcolm, 1991), and vein cutting has been suggested to be an insect countermeasure to bypass or deactivate induced defense responses to injury for several plant groups like milkweeds (Dussourd and Denno, 1991). Third, Malcolm and Zalucki (1996) demonstrated significantly positive cardenolide induction one day after manually imposed partial leaf defoliation on *A. syriaca* and have substantial additional data to support this claim (Malcolm, pers. comm.; Zalucki, pers. comm.). Fourth, *A. syriaca* produces several large inflorescences that can lead to multiple, large seed pods. As such, *A. syriaca* invests heavily into reproduction over a period of several weeks during which several herbivores can feed on it.

Finally, early studies often detected Pn impairment on partially defoliated *Asclepias syriaca* leaves (Delaney *et al.*, 2008), but not always (Delaney and Higley, 2006). Thus, to understand why Pn impairment occurs in injured *A. syriaca* leaves, an explanation must cover both when Pn impairment does and does not occur after defoliation injury. Finally, inflorescences can take two weeks to form and an additional 2 wk to bloom, then plants take 2 or more wk preparing to produce seed pods and seed pods grow and mature for several additional weeks by the end of the growing season (Kaul *et al.*, 1991). This allows several potentially reproductively important phenological times to be examined in relation to Pn impairment. We hypothesized that a physiological cost of defense would be best indicated by a coupling between the Pn impairment and gross cardenolide defense induction from manual or insect defoliation on *A. syriaca* leaves. A physiological cost of reproduction would be best explained if Pn impairment on defoliated *A. syriaca* leaves is tightly coupled with plants investing in flowering or seed production.

METHODS

We collected gas exchange data from 27 *Asclepias syriaca* field experiments from established stands of this perennial species during nine summers from 1994–2002 in Nebraska. These experiments involved *A. syriaca* injured leaves (insect and/or manually imposed partial leaf defoliation treatments) compared to uninjured control leaves, while more complex experiments contained multiple leaf injury treatments (manual and/or insect). The earliest seasonal study started on 9 May in 2001 with pre-flowering stems, and the latest study was 28 Aug. also in 2001 (Table 1). Therefore, multiple experiments were conducted with plants at each reproductive phenology stage (Table 2). The experiments that we conducted tested whether the leaf Pn activity of injured (manual and/or insect defoliation) *A. syriaca* leaves decreased (Pn impairment) relative to uninjured control leaf

TABLE 1.—Information is presented for all experiments about manually imposed and/or insect defoliation treatments on individual leaves. The estimated range of insect herbivory (% leaf area removed from a leaf), herbivores involved* and manual defoliation treatments are shown

Study start date	Location	Plant stage	% Herbivory	Herbivore	% Manual
May 9, 2001	Arbor Lake	Stem Emergence	.	.	50
May 18, 2001	9-Mile Prairie	Stem Emergence	.	.	hole #, diam
May 24, 2001	UNL EC Prairie	Stem Emergence	2–10	DP	.
May 28, 2001	UNL EC Prairie	Stem Emergence	10–50	DP	.
Jun. 2, 2000	UNL EC Prairie	Inflorescence Formation	.	.	25, 50
Jun. 6, 2002	UNL EC Prairie	Inflorescence Formation	.	.	25, 75
Jun. 12, 1997	9-Mile Prairie	Inflorescence Formation	.	.	25, 50
Jun. 16, 2001	9-Mile Prairie	Blooming	10–80	DP	.
Jun. 25, 2002	UNL EC Prairie	Blooming	.	.	25, 75
Jul. 5, 2000	9-Mile Prairie	Blooming	25–50	EE	.
Jul. 12, 2002	UNL EC Prairie	Blooming	.	.	25, 75
mid-Jul., 2001	9-Mile Prairie	Blooming	.	.	50
Jul. 16, 2002	UNL EC Prairie	Blooming	.	.	25, 75
Jul. 29, 1994	9-Mile Prairie	Post-Flowering	25–50	EE	25–50
Aug. 1a, 2001	9-Mile Prairie	Post-Flowering	20–80	EE	.
Aug. 1b, 2001	9-Mile Prairie	Post-Flowering	25–50	EE	.
Aug. 2, 1994	9-Mile Prairie	Post-Flowering	20–80	EE & TT	.
Aug. 7, 1997	9-Mile Prairie	Post-Flowering	25–50	EE	.
Aug. 12, 2000	UNL EC Prairie	Early Seed Pods	30–90	EE	.
Aug. 16, 2000	UNL EC Prairie	Early Seed Pods	30–80	EE, DP, & EA	.
Aug. 18, 1995	9-Mile Prairie	Early Seed Pods	25–50	EE	.
Aug. 19, 1999	9-Mile Prairie	Early Seed Pods	25–50	EE	.
Aug. 23, 1996	9-Mile Prairie	Maturing Seed Pods	25–50	EE	.
Aug. 24, 2001	9-Mile Prairie	Maturing Seed Pods	10–35	EE & CI	33, 50, 66
Aug. 26, 1996	9-Mile Prairie	Maturing Seed Pods	25–50	EE	.
Aug. 27, 1998	9-Mile Prairie	Maturing Seed Pods	25–50	EE	.
Aug. 28, 2001	9-Mile Prairie	Maturing Seed Pods	10–50	EE & CI	33, 50, 66

* CI = *C. inopinatus* (unexpected cycnia moth larvae); DP = *D. plexippus* (monarch butterfly larvae); EA = *Estigmene acrea* (salt marsh tiger moth larvae); EE = *Euchaetes egle* (milkweed tiger moth larvae); TT = *Tetroapes tetrophthalmus* (milkweed beetle adults)

Pn mostly at 1 d post-injury (Table 2). Note that the primary focus here is whether injured *A. syriaca* leaf Pn impairment initially occurs. Other results concerning duration of leaf Pn recovery, mechanisms of Pn impairment (inferred from other gas exchange parameters) and relationship between percent leaf tissue lost from a leaf with leaf Pn on remaining tissue will be published in companion papers (*e.g.*, Delaney *et al.*, 2008).

For insect partial leaf defoliation treatments, herbivores were placed on a leaf at a density sufficient to allow a sufficient percentage (≥10%) of a caged leaf's area to be removed over

TABLE 2.—Experimental design information is presented (RCB- randomized complete block, CRD-complete randomized design). Repeated measured (RM) were also involved in some experiments. Pre-injury Pn was kept as a covariate when $P < 0.15$ in an analysis. ''Day(s)'' refers to the measurement interval(s) after injury occurred, and ''DF'' refers to degrees of freedom used in an analysis

Date	Design	DF	Block	Day(s)	RM	Covariate	Analysis
May 9, 2001	Split-Plot	1,38	1 Stem	1–5	.	Yes	mixed ANCOVA
May 18, 2001	RCB	1,84	2 Stems	1	.	Yes	mixed ANCOVA
May 24, 2001	RCB	1,11	2 Stems	1	.	.	mixed ANOVA
May 28, 2001	RCB	1,16	2 Stems	1	.	Yes	mixed ANCOVA
Jun. 2, 2000	RCB	2,40	12 Stems	2 h;4 d	Yes	.	RM ANOVA
Jun. 6, 2002	RCB	3,20	4 Stems	1	.	Yes	mixed ANCOVA
Jun. 12, 1997	CRD	1,8	.	1 & 4	Yes	.	RM ANOVA
Jun. 16, 2001	RCB	1,28	2 Stems	1	.	Yes	mixed ANCOVA
Jun. 25, 2002	RCB	3,16	4 Stems	1	.	Yes	mixed ANCOVA
Jul. 5, 2000	Split-Plot	3,87	3 Stems	2	.	Yes	mixed ANCOVA
mid-Jul., 2001	RCB	2,71	18 stems	3	Yes	Yes	RM mixed ANCOVA
Jul. 12, 2002	RCB	4,15	5 stems	1	.	Yes	mixed ANCOVA
Jul. 16, 2002	Split-Plot	4,14	3 stems	1	.	Yes	mixed ANCOVA
Jul. 29, 1994	CRD	3,15	.	1	.	.	ANOVA
Aug. 1a, 2001	RCB	12	4 stems	1	.	.	*t*-test
Aug. 1b, 2001	RCB	8	3 Stems	1	.	.	*t*-test
Aug. 2, 1994	CRD	2,13	3 Stems	2;4;13	Yes	.	RM mixed ANOVA
Aug. 7, 1997	CRD	1,4	.	1	.	.	*t*-test
Aug. 12, 2000	RCB	2,12	3 Stems	1	.	Yes	mixed ANCOVA
Aug. 16, 2000	RCB	3,20	4 Stems	1	.	Yes	mixed ANCOVA
Aug. 18, 1995	CRD	6	.	1	.	.	*t*-test
Aug. 19, 1999	CRD	1,9	.	1	.	Yes	mixed ANOVA
Aug. 23, 1996	CRD	9	.	1	.	.	*t*-test
Aug. 24, 2001	RCB	5,33	2 Stems	1	.	.	mixed ANOVA
Aug. 26, 1996	CRD	6	.	1	.	.	*t*-test
Aug. 27, 1998	CRD	8	.	1	.	.	*t*-test
Aug. 28, 2001	RCB	6,31	2 Stems	1	.	.	mixed ANOVA

a 24 h period (Table 1). We used a mesh cage to confine an insect(s) to the treatment leaf, and sealed the cage with tape. Uninjured leaves also received a mesh cage for 24 h to control for any cage effects in experiments where insect herbivory was a treatment. Uninjured control leaves did not have a mesh cover when only manual defoliation leaf treatments were in an experiment. Based on experiments with *Asclepias syriaca* and other milkweeds (*A. incarnata*, *Cynanchum laeve*) leaf cages did not significantly reduce leaf Pn compared to uncovered leaves, perhaps because cages allowed sufficient (>90%) light penetration to the leaf surface so that covered leaves received saturating light intensities (Peterson *et al.*, 1998; Delaney, 2003). Manually imposed partial leaf defoliation treatments were imposed by scissors or by hand, where non-midrib leaf area was clipped or ripped off the leaf (without damaging the midrib) based on visual estimation. We present data from studies where the amount of tissue loss from an individual leaf was medium (50%) or high (66–75%), since we already know that Pn impairment was rarely detected in low (25–33%) treatments throughout the growing season with the milkweeds reported here (Delaney and Higley, 2006). Experimental design variation across experiments is summarized (Table 2).

In 1994, a closed-system infrared gas analyzer (Model 6200, LI-COR, Lincoln, NE) was used to collect Pn measurements (μmol CO_2 m^{-2} s^{-1}) in 2 experiments (Table 1, 2). In all other years (1995–2002), an open-system infrared gas analyzer (Model 6400, LI-COR, Lincoln, NE) was used to measure leaf Pn. The LI-6400 was equipped with a red-blue light source in the measurement chamber to provide a consistent light intensity for a leaf when in the chamber and so did not rely on ambient light conditions like the LI-6200. All experiments involved saturating Photosynthetically Active Radiation (PAR) levels $>$ 1,400 μmol m^{-2} s^{-1}, similar to bright summer days that *Asclepias syriaca* plants experience in the field based on quantum sensor readings (Delaney, 2003). The LI-6400 included a CO_2 mixer that was set at a constant concentration of 400 μmol CO_2 (mol air)$^{-1}$ to allow leaf Pn to stabilize more quickly and to prevent CO_2 diffusion through gasket seals from the chamber to outside air. Leaf photosynthesis measurements were collected from 1300 to 1700 h.

After Pn measurements were taken, injured and control treatment leaves were removed from the stem, flash frozen in liquid nitrogen and stored at −20 C. To measure the cardenolide levels of the leaves, we followed a spectrophotometric technique of Brower *et al.* (1972) that used a cardenolide concentration dependent color change reaction caused by tetranitrodiphenol (TNDP) under highly basic conditions, though we used 530 nm wavelength for our measurements. Leaves were removed from cold storage, and each entire leaf was individually ground up in liquid nitrogen. Non midrib leaf tissue was used for cardenolide analysis in partial leaf defoliation and control leaf treatments. The ground leaf tissue was allowed to dry in a 70 C drying oven for 24 to 48 h so the tissue was fully dried. A constant quantity (100 mg) of dried leaf tissue was measured and placed into a vial with 10.0 ml of ethanol (90%) to extract cardenolides from the leaf tissue and the vials were placed into a 70 C water bath for 1 h. When $<$ 100 mg of dry mass leaf tissue was available (some injured leaves), then proportionally less ethanol was added to the vial.

After the vials were removed from the water bath, they were centrifuged at 1300 *g*-force for 5 min. One cuvette with 2.0 ml ethanol (90%) and 1.0 ml 1 M NaOH was used to zero the spectrophotometer before readings were taken. Two cuvettes were prepared from each sample vial. The control cuvette contained 0.67 ml sample supernatant, 1.33 ml of TetraNitroDiPhenol (TNDP) and 1.0 ml of ethanol (90%). The sample cuvette contained 0.67 ml sample supernatant, 1.33 ml ethanol (90%) with TNDP (0.15%) and 1.0 ml of 1 M NaOH. The cuvettes were set for 45 min as the reaction stabilized. Absorbance measurements were collected at 530 nm based on preliminary data from digitoxin standard absorbance curves (unpublished data). Subtracting out the control cuvette absorbance ($Absorbance_{sample}$ − $Absorbance_{control}$) for each tissue sample allowed chlorophyll and TNDP background absorbance to be removed, and the difference indicated cardenolide concentration for that sample. The absorbance of uninjured control leaves served as the reference level for comparison with insect and/or manual defoliation leaf treatments to infer whether cardenolide induction occurred. The cardenolide, digitoxin, was used to generate standard concentration curves.

Each experiment included a fixed injury factor with two or more treatments. If an experiment only had two treatments, no pre-injury values to serve as covariates and a completely randomized design, then we used two-sample *t*-tests to compare the manually imposed or actual insect Pn (and cardenolides in some experiments) of partially defoliated treatment leaves to uninjured leaf treatment leaves (Table 2). Proc Mixed in SAS was used to analyze all ANOVA and ANCOVA, where results with type III SS were used with mixed-model ANOVA and ANCOVA (SAS Institute, 2001). LSD post-hoc tests were used to separate injury treatment means when $\alpha = 0.05$ for ANOVA or ANCOVA.

TABLE 3.—The results are shown for 10 *Asclepias syriaca* cardenolide induction response studies to leaf injury. In the TrtCard and TrtPn columns, it is indicated whether injured leaf Pn, or gross cardenolide concentration, increased (Pos), was not statistically distinguishable from (-), or decreased (Neg) occurred after leaf injury relative to control uninjured leaves. Predictions for cardenolide induction are shown in the far right column

Date	Plant	Injury	df	F_{Pn}	TrtPn	$F_{Cardenolide}$	TrtCard	Expect
5/9/01	*A. syriaca*	Manual	1,14	0.1	-	6.8*	Neg	- or Neg
5/18/01	*A. syriaca*	Manual	2,33	5.5*	Neg	2.2	-	Pos
5/24/01	*A. syriaca*	*D. plexippus*	1,11	1.7	-	5.3*	Neg	- or Neg
5/28/01	*A. syriaca*	*D. plexippus*	1,16	$t = 1.4$	-	0.3	-	- or Neg
6/16/01	*A. syriaca*	*D. plexippus*	1,23	8.3**	Neg	2.3	-	Pos
7/05/00	*A. syriaca*	*E. egle*	3,74	6.9**	Neg	3.1*	Neg	Pos
8/1/01a	*A. syriaca*	*E. egle*	12	$t = 2.8$*	Neg	0.1	-	Pos
8/1/01b	*A. syriaca*	*E. egle*	8	$t = 4.3$**	Neg	2.7*	Pos	Pos
8/24/01	*A. syriaca*	*E. egle*	5,33	6.2***	Neg	7.6***	Neg	Pos
8/24/01	*A. syriaca*	Manual			-		-	- or Neg
8/28/01	*A. syriaca*	*E. egle*	6,31	1.1	-	0.8	-	- or Neg
8/28/01	*A. syriaca*	Manual			-		-	- or Neg

* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$

To show whether injured leaves had statistically significantly lower leaf Pn than uninjured leaves for presentation of results across the growing season, we used SAS to calculate the mean Pn difference ($mean_{injured} - mean_{uninjured}$) between injured and uninjured leaf Pn with a 95% confidence interval around this mean difference (SAS Institute, 2001). If the 95% confidence interval around this mean difference did not overlap zero, then this reflected that injured leaf Pn was significantly lower than uninjured leaf Pn at $P < 0.05$. However, if the confidence interval of this mean difference did cross zero, then this reflected that injured leaf Pn was not statistically distinguishable from uninjured leaf Pn at $P > 0.05$.

RESULTS

There were 10 studies with *Asclepias syriaca* with data collected on cardenolide concentration estimates in addition to Pn data after manually imposed and/or insect defoliation (Table 3). In six out of ten studies, we detected Pn impairment from leaf defoliation. Of the six studies with Pn impairment after leaf defoliation injury, we detected marginally significant negative cardenolide induction in two studies and no cardenolide induction in three studies. In a 6th study with Pn impairment after injury on 3 Aug. 2001b (Table 3), a contrast ($t_{11df} = 2.79$, $P = 0.018$) indicated that insect defoliated leaves in general had significant positive cardenolide induction compared to uninjured leaves, though none of the individual treatment levels were statistically different than the uninjured leaf treatment. In the four studies where Pn impairment was not detected on defoliated leaves, two studies did not detect cardenolide induction and two studies detected negative cardenolide induction (Table 3).

For actual partial insect defoliation, injured leaf Pn compared to uninjured leaf Pn, either decreased slightly or had no statistically significant decrease across several studies that occurred from mid-Aug. to the end of Aug. (Fig. 1). However, injured leaf Pn decreased moderately relative to uninjured leaf Pn in the few studies from late May through mid-Jul. and injured leaf Pn experienced the most severe decreases in studies from late Jul. through

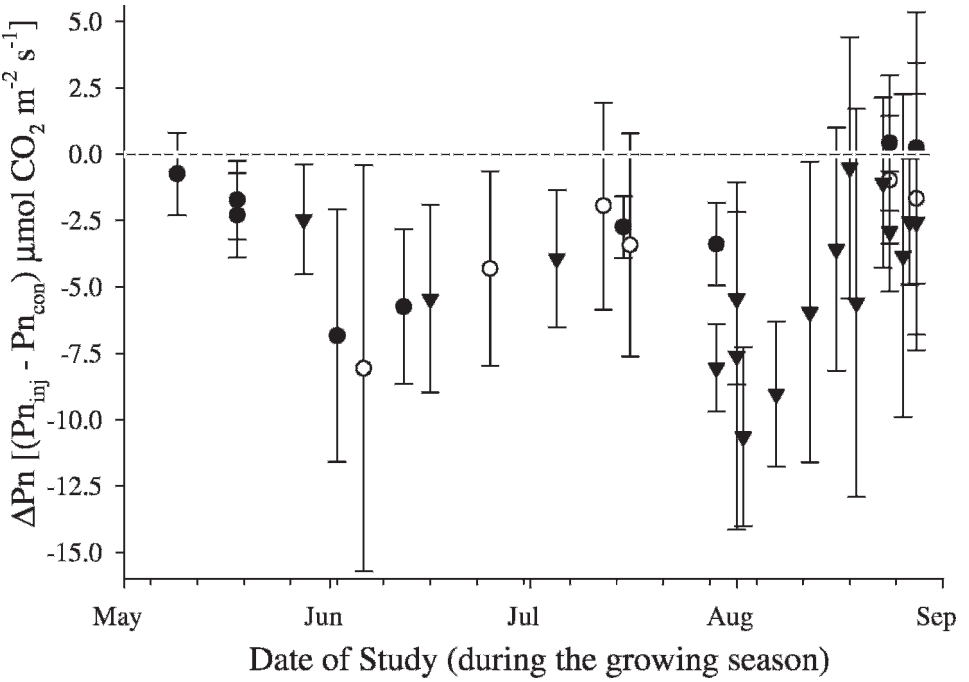

FIG. 1.—The results shown are a mean ± 95% confidence interval of 1–2 d post-injury Pn difference (injured leaf − uninjured leaf) across most of the growing season for *Asclepias syriaca* leaves after experiencing insect (mostly by *Euchaetes egle* or *Danaus plexippus* larvae) herbivory (▼), 50% manual defoliation on the injured leaf (●), or 75% manual defoliation on the injured leaf (○). The studies presented here were conducted between 1994–2002 (Table 1, 2). If the confidence interval does not cross zero, then statistically significant Pn impairment occurred with $P < 0.05$. In experiments with repeated measures, the data from first measurement period was used for presentation in this figure (though Pn reductions are detected up to 5 d post-herbivory; Delaney *et al.*, 2008)

early Aug. With manually imposed partial leaf defoliation (50 and/or 75%), statistically significant Pn reduction on injured leaves compared to uninjured leaves did not result in the first May study, the last two Aug. studies, two studies in the middle of Jul. and two studies in late Aug. Otherwise, leaves with manually imposed partial leaf defoliation (50–75%) had slight to moderate Pn decreases from late May to late Jul., where Pn impairment severity often reached the impairment severity caused by insect herbivory (Fig. 1).

In terms of *Asclepias syriaca* reproductive phenology, stems emerging from an existing rhizome began to do so in early May, with an emphasis on shoot (stem and leaf) growth. During early to late May, one study involving *Danaus plexippus* larval partial leaf defoliation resulted in a small but significant degree of injured leaf Pn impairment (Fig. 1). With two pre-flowering manually imposed partial leaf defoliation experiments, the earliest study did not detect a statistically significant reduction in injured leaf Pn while second study detect slight but significant Pn impairment from holes caused by cork borers (Fig. 1). During early to mid Jun., stems began to produce inflorescences which took 1 to 2 wk before blooming began. The most severe Pn decreases occurred from manually imposed partial leaf

defoliation in three studies during this pre-flowering period (Fig. 1). There were no studies on stems producing inflorescences that involved insect defoliation.

Inflorescences tended to bloom from mid-Jun. through mid-Jul., usually for a period lasting 2–3 wk, depending on when inflorescence initially began to form. On leaves of flowering stems, insect partial leaf defoliation resulted in decreased injured leaf Pn in two studies (Fig. 1). Four manually imposed partial leaf defoliation studies (50%) also resulted in significant leaf Pn impairment, though two studies with higher manual leaf defoliation (75%) did not result in statistically detectable Pn reductions on blooming plants. During mid Jul. through early Aug., inflorescences had senesced and dropped off, and in early Aug. reproductive stems began to produce small seed pods. It is during this post-flowering and early seed pod period that the most severe leaf Pn decreases occurred in five insect partial leaf defoliation experiments, while small but significant Pn impairment was detected in one manual defoliation experiment (Fig. 1). From mid to late Aug., reproductive *Asclepias syriaca* stems typically had multiple maturing seed pods and stem/leaf senescence occurs from late Aug. into Sep. Three insect partial leaf defoliation studies detected statistically significant Pn impairment during this period, but with large confidence intervals (95%) with all three studies that showed large variation in individual leaf Pn responses to insect feeding (Fig. 1). Six other studies involving herbivores did not detect significantly reduced leaf Pn on injured leaves compared to uninjured leaves during this period. Neither of two manually imposed partial leaf defoliation studies detected statistically significant Pn impairment during late August.

DISCUSSION

Before discussing how our experimental results relate to *Asclepias syriaca* reproductive phenology, it is useful to consider the timing over which our experiments were conducted. We do this because the small sample sizes of herbivore treatments in some of our experiments were quite small, and so would have little power when not detecting significant Pn reductions. However, our first experiments that we conducted in late Jul. and early Aug. in 1994 detected significant Pn reductions of a large magnitude in herbivore leaf treatments with sample sizes as small as n = 4. Thus, mid/late Aug. experiments conducted in 1995 and 1996 used small herbivore treatment sample sizes (n = 4 to 5) with the expectation of detecting significant Pn reductions; our surprise came when significant Pn reductions were not detected. To add to our confusion, small sample size experiments in mid-Jun. (n = 5) and early Aug. (n = 3) in 1997 again detected significant and large Pn reductions from herbivory treatments. A large amount of variation was already known to exist in how Pn was affected after insect defoliation across plant species including on injured leaves (Welter, 1989; Peterson and Higley, 1993), with increased leaf Pn (Welter, 1989; Meyer, 1998; Oleksyn *et al.*, 1998), no change in Pn (Welter, 1989; Mercader and Isaacs, 2003; Peterson *et al.*, 2004; or very little change—Aldea *et al.*, 2006), or decreased leaf Pn (Welter, 1989; Oleksyn *et al.*, 1998; Peterson *et al.*, 1998; Zangerl *et al.*, 2002; Mercader and Isaacs, 2003; Peterson *et al.*, 2005; Delaney and Higley, 2006).

We also know that environmental conditions influence whether Pn reduction occurs and the magnitude of such reduction after manually imposed tissue removal from *Asclepias syriaca* leaves (Delaney, 2003). In a field experiment, *A. syriaca* leaves did not experience Pn reductions after injury when plants received relief from water stress and/or competition with other plants, while leaves on stressed plants had the largest Pn reductions after injury (Delaney, 2003). These results may seem obvious as leaves on water stressed sunflower plants, *Helianthus annuus*, were also prone to Pn reductions after injury, but not when on

unstressed plants (Haile and Higley unpublished data). With soybean, *Glycine max*, leaves on unstressed plants experienced the largest Pn reductions on injured leaves compared to leaves on water stressed plants (Haile and Higley, 2003). Plant nutritional, water, and light status are known to influence plant growth responses after herbivory (Hawkes and Sullivan, 2001); it is possible that these factors may interact with plant reproductive investment to also influence responses to herbivory. Finally, the same amount of tissue loss spread out among more holes on a leaf led to Pn reduction from *Trichoplusia ni* herbivory on *Arabidopsis thaliana* compared to when the tissue loss was in only one or two larger holes (Tang *et al.*, 2006), where the specific mode of feeding even among defoliators can be important on whether herbivory reduces injured leaf Pn (Peterson *et al.*, 1998).

Our results with *Asclepias syriaca* are different from other reported species because significant Pn reductions were often, but not always, detected (Delaney and Higley, 2006; Delaney *et al.*, 2008). We had initially suspected that Pn impairment might be related to a physiological cost of defense, particularly related to *A. syriaca* constitutive or induced cardenolide defense investment. When we had studies without Pn reductions after leaf injury, we realized that an explanation would have to address why Pn reductions do and do not occur in common milkweed after tissue consumption by herbivores. It was at this point that we considered that a cost of reproduction could be an alternative hypothesis to account for frequent Pn reductions on injured leaves after herbivory. Early season herbivory studies were especially needed, since although manually injury studies can be performed anytime, we also knew that *A. syriaca* leaf Pn was more sensitive to Pn reductions from insect herbivory than manual tissue removal (Delaney *et al.*, 2008). However, few defoliating herbivores attack *A. syriaca* before flowering (pers. obs.), so it wasn't until we found *Danaus plexippus* larvae in May 2001 that we were able to perform early season herbivory studies on pre-flowering plants. It also was not until Jul. 2000 that we had TNDP (tetranitrodiphenol) synthesized and could use the spectrophotometric technique of Brower *et al.* (1972) to measure gross cardenolide concentrations of samples from leaves where photosynthesis measurements were collected.

Our ten *Asclepias syriaca* studies with both photosynthesis and gross cardenolide concentration measurements most strongly rule out a resource based trade-off between leaf constitutive cardenolide investment with causing leaf Pn reductions after injury. Multiple studies did not detect significant Pn reductions, inconsistent with a constitutive cardenolide resource trade-off with leaf photosynthesis after herbivory. This was because the cardenolides were always present, and so would always be expected to result in Pn reductions when herbivores breach this plant defense. Also, if we extend this argument with other milkweed species, we have detected Pn reductions after herbivory (Delaney and Higley, 2006) in very low constitutive cardenolide species like *A. tuberosa*, *A. incarnata*, and *Cynanchum laeve* (Malcolm, 1995). Thus, our results with *A. syriaca* and other milkweeds suggest that injured leaf Pn reduction after herbivory is not caused from a resource trade-off with constitutive cardenolide levels.

Another possible cost of defense could be reflected in a trade-off between positive cardenolide induction whenever Pn reductions occur with *Asclepias syriaca*. This explanation makes more sense since the trade-off would be related to the limited immediate use of resources either for defense induction or to maintain photosynthesis after injury. With a defense induction trade-off, Pn impairment would be expected whenever cardenolide induction occurs, and no Pn reduction on injured leaves when cardenolide induction is not detected. Our results with ten studies found essentially no coupling between gross cardenolide induction with the occurrence of Pn reductions after herbivory on a leaf. In

addition, results with other milkweeds (*A. tuberosa*, *A. incarnata* and *Cynanchum laeve*; also hemp dogbane, *Apocynum cannibinum*) have detected Pn reductions, but usually without significant positive cardenolide induction (unpublished data). These results with other milkweeds further suggest that Pn reduction and cardenolide induction are uncoupled. Note we did not detect gross cardenolide induction (overall increase in cardenolide concentration); we cannot rule out the possibility that an individual cardenolide(s) is induced that results in a resource trade-off with photosynthesis reduction after herbivory. Also, our cardenolide induction results do not address the hypothesis that Pn impairment could reflect a hormonal regulation trade-off, where a hormone like jasmonic acid could upregulate leaf wound responses while simultaneously downregulating photosynthesis (Metodiev *et al.*, 1996; Kessler *et al.*, 2004). What we can say from our results is that if such a defense-photosynthesis hormonal trade-off exists, we usually did not detect expected cardenolide induction (Malcolm and Zalucki, 1996; pers. comm.) after leaf herbivory.

HERBIVORY AND LEAF PN: A COST OF REPRODUCTION?

The 27 field experiments over nine summers (to our knowledge, the most extensive plant-insect ecophysiology data set) to examine *Asclepias syriaca* leaf Pn responses to insect and manually imposed herbivory were necessary to observe a seasonal pattern in whether Pn impairment occurred after leaf injury. We suggest that a consideration of plant reproductive phenology is necessary to help understand variation in injured *A. syriaca* leaf Pn responses. This is because leaf Pn impairment was not statistically detectable on remaining tissue of a partially defoliated leaf in several studies, particularly when leaves were on newly emerging stems or on stems with maturing seed pods. Note that though some of our studies may not have detected Pn reductions after herbivory due to small sample sizes and thus low statistical power, four late season studies and the earliest season study had very small differences in leaf Pn between control and herbivore treatments (Fig. 1). As such, these studies probably would not have detected Pn reductions in herbivory treatment leaves with larger sample sizes, and we infer that Pn impairment was not detected after leaf injury in at least these five studies. In contrast, leaf Pn reduction was moderate or severe when leaves were on flowering, post-flowering and early seed-pod forming stems; the few exceptions were with manually imposed tissue removal, which tends to be less likely to cause Pn impairment of tested milkweeds (Delaney and Higley, 2006; Delaney *et al.*, 2008).

We suggest that despite our studies being conducted across several years, we detect a pattern where leaves on reproductive stems (flowering to seed pod formation) are more sensitive to herbivory that is reflected in more likely Pn reduction after injury. Without a consideration of *Asclepias syriaca* phenology with our studies, we would have concluded from our first two experiments in 1994, or the two experiments in 1997, that leaves experience Pn impairment after partial leaf defoliation. However, if we instead had first conducted our three studies from 1995 and 1996, we would have concluded that leaves do not experience Pn impairment following leaf herbivory. Only by conducting several studies were we able to establish a pattern which suggested photosynthetic impairment was most likely following leaf injury on plants investing in reproduction. From our seasonal pattern results we infer that higher Pn impairment sensitivity on reproductive plants after herbivory reflects a possible physiological cost of reproduction.

Leaf Pn can serve as a useful assay to indicate the level of stress a plant is experiencing, or to show how well a plant handles a stressor like herbivory at a specific time (Peterson and Higley, 1993). By collecting leaf photosynthetic responses at multiple times, this allows one to demonstrate that at a specific time(s)/reproductive stage that a plant either is more or

less vulnerable to a stress like herbivory. Reproductive phenology is known to be important for understanding plant response to stress, as plants can vary in sensitivity to stresses over a growing season (Chiarello and Gulmon, 1991; Pedigo and Rice, 2006). With agricultural plants and yield (or 'fitness' in natural settings), plants may not suffer reductions outside of sensitive time periods for a plant's resource allocation pattern of photosynthate to reproductive sinks.

For example, defoliation of canopy leaves during seed-pod filling can reduce the flow of photosynthate to seed-pod growth, resulting in yield decreases in soybean, *Glycine max* (Higley, 1992). However, defoliation occurring outside of seed-pod filling on soybeans tends not to result in reduced yields (Haile *et al.*, 1998). This is because soybean plants need a Leaf Area Index (LAI) > 3.5 to be able to intercept most (95%) of photosynthetically active radiation that reaches a plant (Higley, 1992). Thus, in an agricultural setting soybean is likely to experience yield reductions only when defoliation reduces LAI < 3.5 right before or during seed-pod defoliation (Haile, 2001). Studies in natural systems have also shown that plant responses to injury can vary based on time injury during a growing season (*e.g.*, early, middle and late season) for leaf Pn (Mercader and Isaacs, 2003; this study; but *see* Macedo *et al.*, 2006), and growth rate and fitness responses (Maschinski and Whitham, 1989; Garcia and Ehrlén, 2002; Knight, 2007).

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