

Differential Physiological Responses of Dalmatian Toadflax, *Linaria dalmatica* L. Miller, to Injury from Two Insect Biological Control Agents: Implications for Decision-Making in Biological Control

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ABSTRACT Successful biological control of invasive weeds with specialist herbivorous insects is predicated on the assumption that the injury stresses the weeds sufficiently to cause reductions in individual fitness. Because plant gas exchange directly impacts growth and fitness, characterizing how injury affects these primary processes may provide a key indicator of physiological impairment—which then may lead to reductions in fitness. The objective of this study was to use physiological methods to evaluate how the invasive weed, *Linaria dalmatica* L. Miller (Dalmatian toadflax), is affected by two introduced biological control agents within different injury guilds: the stem-boring weevil, *Mecinus janthinus* Germar, and the defoliating moth, *Calophasia lunula* Hufnagel. All studies with *M. janthinus* were conducted under field conditions at two sites in Montana in 2003 and 2004. For *C. lunula* evaluations, a total of five greenhouse studies in 2003 and 2004 were used. One field study in 2003 and two studies in 2004 also were conducted. Variables measured included net CO₂ exchange rate, stomatal conductance, and transpiration rate. Results from both field sites revealed that the primary physiology of Dalmatian toadflax was deleteriously affected by *M. janthinus* larval injury. There were no significant differences among treatments for any of the gas exchange variables measured in all eight experiments with *C. lunula*. Our results indicate that insect herbivores in two distinct injury guilds differentially affect Dalmatian toadflax physiology. Based on the primary physiological parameters evaluated in this study, *M. janthinus* had more impact on Dalmatian toadflax than *C. lunula*. With such information, improved risk-benefit decisions can be made about whether to release exotic biological control agents.

KEY WORDS *Calophasia lunula*, *Mecinus janthinus*, herbivory, photosynthesis, plant gas exchange

BIOLOGICAL CONTROL OF WEEDS through the intentional introduction of nonindigenous herbivorous insects has reached a crossroads, both in terms of research and application. The research community has long acknowledged the potential for classical biological control of weeds to result in emerging or increased environmental risks (Harris and Zwölfer 1968, Wapshere 1974, Howarth 1991, Louda et al. 2003, Sheppard et al. 2003). However, current regulatory attitudes and fiscal shortfalls (Briese 2004) are reflected in the narrow focus of agent prerelease evaluations on host specificity, at the expense of a more holistic screening and assessment process (but see Louda 1998). The lack of evaluation of agent efficacy, as well as potential ecological risks, emphasize the need for formal, well-quantified risk-benefit evaluations of insect agents introduced to manage invasive weeds.

An improved set of measurable indicators of biological control impact on weed densities would aid decision-makers in objective evaluation of tangible benefits versus potential risks when deciding whether to release nonindigenous organisms. Therefore, a quantitative evaluation of benefits should be an important part of the overall risk assessment for agents. The lack of demonstrable benefits from the release of biological control agents can have substantial consequences (Thomas and Willis 1998). Specifically, if a nonindigenous organism does not deleteriously affect the targeted weed population, the economic costs or environmental risks associated with its release and establishment may always be greater than its benefits. Therefore, it is crucial that agents approved for release will actually reduce target weed populations and not simply proliferate on them.

Although several strategies exist for determining the potential efficacy of weed biological control, using methods that characterize changes in weed growth and fitness can be very costly and time-consuming. We believe that the characterization of plant physiological response to herbivory provides a tenable alternative approach as a valuable indicator of the ability of bi-

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ological control agents to reduce target weed populations. The delineation of physiological mechanisms underlying plant responses to insect injury has been crucial to the explanation of yield loss and to the development of general models of insect-induced plant stress response in crop plants (Boote 1981; Peterson 2001; Peterson and Higley 2001). Plant gas exchange processes such as photosynthesis, water vapor transfer, and respiration represent a subset of a plant's primary physiological processes. Understanding how insect injury influences these parameters is important because these are the primary processes determining plant growth, development, and, ultimately, fitness (Peterson and Higley 1993). Although individual leaf photosynthetic rates typically are not accurate predictors of plant yield and fitness (e.g., Irvine 1975, Elmore 1980, Baker and Ort 1992, Higley 1992), they can be used to objectively quantify physiological impairment—which may lead to reductions in fitness.

Boote (1981), Pedigo et al. (1986), and Higley et al. (1993) emphasized the use of categorizing plant biotic stressors, such as weed biological control agents, based on injury type and physiological response, rather than on the taxonomic classification of the stressors or physical appearance of injury (as conventionally had been done). Furthermore, Peterson and Higley (2001) argued that similarities of plant response to specific injury types, also known as injury guilds, are effective foci for addressing many basic and applied research questions.

The objective of this study therefore was to use physiological methods to evaluate how the invasive weed, Dalmatian toadflax, *Linaria dalmatica* L. Miller, is affected by two introduced biological control agents in different injury guilds: the stem-boring weevil, *Mecinus janthinus* Germar, and the defoliating moth, *Calophasia lunula* Hufnagel. Characterizing Dalmatian toadflax primary metabolic impairment to herbivory would provide an initial step toward determining the impact of biological control agents on the weed's fitness and population dynamics.

Materials and Methods

***Mecinus janthinus*.** All studies with *M. janthinus* were conducted under field conditions at two sites in both 2003 and 2004. The two sites were located near Boulder (elevation = 1798 m) and Melstone (elevation = 939 m), MT, in rangeland that had been extensively and intensively burned by wildfire in 2000. The Boulder site has a lower average temperature and receives slightly more precipitation per year than the Melstone site. Dalmatian toadflax was the dominant plant species at both sites. Observers with pre- and postfire knowledge of the vegetation community at these sites suggest that toadflax had increased significantly in both density and biomass postburn, supporting the inference that Dalmatian toadflax is a fire-enhanced invasive weed (Jacobs and Sheley 2003a–c, Phillips and Crisp 2000). Insects were released at each site in 2002, and follow-up observations made later in the same year confirmed that the insects had fed and

oviposited in the target weed. Our physiological studies were conducted 3–19 July 2003 and 7–14 July 2004.

Two study groups were evaluated: injured and uninjured plants. In 2003, there were 18 group replicates at the Melstone site and 24 replicates at the Boulder site. In 2004, there were 18 replicates per group at each site. Individual plants served as sample units at all sites. Injured plants were chosen based on the presence of ovipositional scars and swelling of stems, which indicated the presence of larvae in the stems. Injured plants that were chosen had similar amounts of injury within each location, but plants at the Boulder site were not as visually injured as those at the Melstone site. Uninjured plants, those absent of oviposition scars and stem swelling, were chosen near infested plants. All plants at both sites were at approximately the same developmental stage: early to mid flower.

The primary limitation in our field research with *M. janthinus* was that it did not involve experimental manipulation to create treatments. We chose injured plants based on the presence of oviposition scars and stem swelling, rather than caging a group of plants and assigning treatments for two main reasons: (1) Dalmatian toadflax plants are deleteriously affected (alterations in stem and leaf thickness and whole plant architecture) by caging whole plants (N. J. Irish, personal communication), and (2) the field sites were inaccessible when the adults first emerged and females began ovipositing.

***Calophasia lunula*.** We conducted a total of five greenhouse experiments in 2003 and 2004 (Table 1). The experimental design for all greenhouse experiments was a randomized complete block, with the area under individual metal halide lamps serving as the blocking factor. Four of the experiments incorporated repeated measures into the experimental design. Treatments consisted of *C. lunula* larval injury, artificial defoliation injury, and uninjured control. We incorporated an artificial defoliation treatment to determine if manual defoliation could be used as a surrogate for *C. lunula* feeding. Treatments were replicated five or more times, and an individual pot containing one plant grown from seed served as the experimental unit. In two greenhouse experiments, we were not able to include the actual insect injury treatment because of a lack of larvae. In one greenhouse study, we did not include the artificial defoliation injury treatment because of a lack of sufficient plants.

We also conducted three field experiments: one in 2003 (Bozeman, MT) and two in 2004 (Missoula, MT). The experimental design for all studies was completely random (Table 1). Treatments for two experiments consisted of *C. lunula* larval injury, artificial defoliation injury, and uninjured control. In a third experiment, only two treatments, *C. lunula* injury and control, were used (Table 1). Treatments were replicated six times in 2003. In 2004, treatments were replicated six times in one experiment and eight times in the other. Individual plants served as experimental units. For seven of the eight experiments, a leaf at the bottom one-third of each stem also was measured for physiological parameters to determine if there were inter-

Table 1. Experimental details and statistical summaries for each *C. lunula* herbivory experiment

Year	Type	Treatments	Experimental design	Treatment P^d	Treatment $P > F$	Treatment $\times$ leaf position F	Treatment $\times$ leaf position $P > F$	Mean percentage defoliation by <i>C.</i> <i>lunula</i> $\pm$ SEM
2003	Field	3 ^a	CRD, factorial, repeated measures	2.23	0.13	0.32	0.73	38 $\pm$ 6.16
2004	Field	3 ^a	CRD, factorial	1.97	0.15	0.66	0.52	45 $\pm$ 5.98
2004	Field	2 ^b	CRD, factorial	1.94	0.19	0.001	0.96	75 $\pm$ 15.44
2003	Greenhouse	3 ^a	RCBD, factorial, repeated measures	0.24	0.79	3.31	0.07	52 $\pm$ 6.37
2003	Greenhouse	2 ^c	RCBD, factorial, repeated measures	0.57	0.47	1.91	0.2	NA
2003	Greenhouse	2 ^c	RCBD, factorial, repeated measures	0.47	0.51	0.91	0.37	NA
2003	Greenhouse	3 ^a	RCBD, factorial, repeated measures	2.73	0.11	0.25	0.78	43 $\pm$ 7.4
2004	Greenhouse	2 ^b	RCBD	1.67	0.25	NA	NA	13 $\pm$ 2.8

^a Treatments = *C. lunula* larval injury, artificial defoliation injury, and control.

^b Treatments = *C. lunula* larval injury and control.

^c Treatments = artificial defoliation injury and control.

^d Treatment effect.

CRD, completely random design; RCBD, randomized complete block design; NA, not applicable.

actions between defoliated (or undefoliated control) upper leaves and undefoliated lower leaves.

For most greenhouse and field experiments, pre-starved, *C. lunula* fourth instars were securely contained within leaf cages made from stiff, fine-mesh nylon netting (tuile) and allowed to feed for at least 12 h. The leaf cages were ≈ 11 by 11 cm and covered the top portion of the stem, usually enclosing from four to six large leaves. The open end of each cage was closed tightly around the stem with a drawstring. Leaf cages could not enclose single leaves because the Dalmatian toadflax leaves are small and lack a petiole. The leaf-cage fabric intercepted $<5\%$ of photosynthetically active radiation (Peterson et al. 1998). The artificial defoliation injury treatment was imposed by clipping leaf tissue with scissors from the plant in a spatial and temporal pattern consistent with *C. lunula* feeding; however, because of variability in *C. lunula* feeding (Table 1), we standardized artificial defoliation injury at 50% of leaf area within each cage. Leaves of both the control and artificially defoliated plants also were enclosed in leaf cages, ensuring appropriately comparable treatments. Larvae were removed, and gas exchange variables were recorded on injured leaves at 1 and 48 h after the termination of injury.

Plant Primary Metabolism. Repeated gas exchange measurements were made from the same leaf per plant at approximately the apical one-third of the stem. When it was not possible to take readings from the same leaf per plant because of breakage, the nearest leaf was measured. For all *C. lunula* experiments, measurements were recorded from injured (partially defoliated) leaves. If defoliated leaves had too little leaf area remaining to measure, the nearest undefoliated leaf was used.

All gas exchange measurements were made within 3 h of solar noon using a portable photosynthesis system (model LI-6400; LI-COR, Lincoln, NE). Variables measured and analyzed included: net CO_2 exchange rate (photosynthetic rate), stomatal conduc-

tance rate, and transpiration rate. The leaves were illuminated with a light intensity of $1400 \mu\text{mol photons/m}^2/\text{s}$ from a light source inside the 2-cm^2 leaf chamber. The leaf chamber reference CO_2 concentration was $400 \mu\text{mol CO}_2/\text{mol}$, generated from a 12-g CO_2 cylinder connected to the LI-6400.

Statistical Analysis. Because gas exchange variables usually were observed on the same leaf over time, most analyses were conducted using a repeated measures multivariate analysis of variance (MANOVA; $\alpha = 0.05$; SAS 9.0, Cary, NC).

Results

***Mecinus janthinus*.** Although establishment of *M. janthinus* was relatively recent at both sites, injury was more widespread at Melstone than at Boulder. Evidence of *M. janthinus* injury to plants was comparatively intermittent at Boulder. Results from both field sites over the 2-yr study period revealed that the primary physiology of Dalmatian toadflax was significantly affected by *M. janthinus* larval injury (Table 2). In particular, over both years and locations, photosynthetic rates for plants with injury from larvae were significantly lower than rates for uninjured plants (Table 2; Fig. 1). At the Melstone site, the mean reduction in photosynthetic rates associated with larval injury was 29.5% in 2003 and 27.3% in 2004. At the Boulder site, the mean photosynthetic rate reduction associated with larval injury was 38.6% in 2003 and 17% in 2004. There was a significant time effect between measurement dates in both years at the Boulder site, but only a significant time effect in 2003 at the Melstone site.

At the Melstone site, transpiration rates and stomatal conductances were significantly lower for injured plants in 2004, but not in 2003 (Table 2). These trends were reversed at the Boulder site, where we found that transpiration rates and stomatal conductances were significantly lower for injured plants in 2003, but not

Table 2. Mean gas exchange responses $\pm$ SEM of Dalmatian toadflax to *M. janthinus* larval injury

Location	2003 treatment	2004			
		3 Jul	7 Jul	7 Jul	12 Jul
Melstone Site					
Stomatal conductance (mol H ₂ O/m ² /s)	Uninjured	0.51 ± 0.025	0.23 ± 0.014	0.19 ± 0.032	0.16 ± 0.017
	Injured	0.48 ± 0.035	0.17 ± 0.031	0.12 ± 0.019	0.11 ± 0.014
	<i>F</i>		1.29		6.34
	<i>P</i> > <i>F</i>		0.27		0.017
	df		1,22		1,34
Transpiration rate (mmol H ₂ O/m ² /s)	Uninjured	17.3 ± 0.68	4.5 ± 0.66	6.5 ± 0.76	5.3 ± 0.41
	Injured	16.2 ± 0.74	4.1 ± 0.69	4.4 ± 0.48	3.6 ± 0.36
	<i>F</i>		1.12		12.97
	<i>P</i> > <i>F</i>		0.3		0.001
	df		1,28		1,31
Boulder Site					
Stomatal conductance (mol H ₂ O/m ² /s)		14-Jul	19-Jul	8-Jul	14-Jul
	Uninjured	0.17 ± 0.01	0.17 ± 0.01	0.25 ± 0.01	0.22 ± 0.02
	Injured	0.12 ± 0.02	0.13 ± 0.01	0.22 ± 0.02	0.22 ± 0.02
	<i>F</i>		9.9		0.2
	<i>P</i> > <i>F</i>		0.003		0.65
Transpiration rate (mmol H ₂ O/m ² /s)					
	Uninjured	3.5 ± 0.26	6.4 ± 0.42	4.5 ± 0.18	6.5 ± 0.62
	Injured	3.3 ± 0.52	4.8 ± 0.34	3.8 ± 0.23	6.6 ± 0.39
	<i>F</i>		9.28		0.41
	<i>P</i> > <i>F</i>		0.004		0.53
df		1,37		1,34	

2004. The inconsistency in these responses suggests that stem-boring injury by *M. janthinus* larvae may not be severely impairing the ability of the leaves to uptake CO₂ and transpire H₂O (stomatal limitations). Disruption of xylem tissues because of larval feeding within the stem apparently was insufficient to consistently close stomata and reduce transpiration. Therefore, larval injury did not seem to physiologically mimic drought stress. Reductions in stomatal conductances and transpiration, when they occurred, seemed to follow, rather than cause, photosynthetic reductions. This type of primary metabolic response also was observed with injury from *Epilachna varivestis* Mulsant on *Phaseolus vulgaris* L. and *Glycine max* L. Merrill (Peterson et al. 1998). Jeanneret and Schroeder (1991) suggested that photosynthetic mechanisms

may be affected if stem injury by *M. janthinus* larvae prevents translocation of carbohydrates to the roots. Our results support the contention that *M. janthinus* injury affects photosynthesis of Dalmatian toadflax. More detailed physiological measurements will be required to determine the mechanisms underlying reductions in photosynthetic rates of Dalmatian toadflax. Measurements of light-response curves, assimilation-intercellular CO₂ curves, and chlorophyll fluorescence can be used to determine precisely where biochemical limitations to photosynthesis are occurring and therefore can provide mechanistic explanations of physiological impairment. Stem-boring injury is so poorly understood that, to date, the injury guild is characterized by the physical appearance of injury rather than on physiological effects of the injury (Wel-

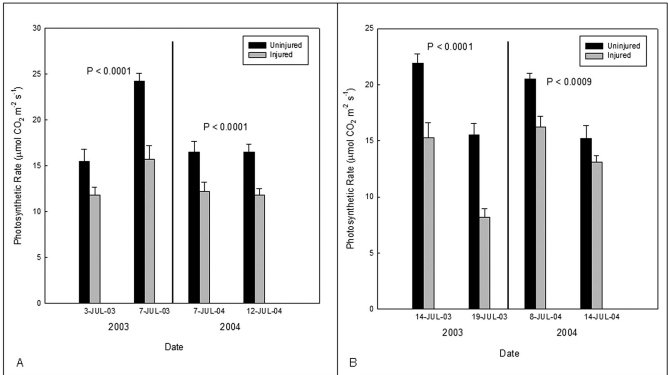


Fig. 1. Photosynthetic responses of *L. dalmatica* to injury by *M. janthinus* larvae (A, Melstone, MT; B, Boulder, MT). Note statistically significant differences in all tests between injured/uninjured plants.

ter 1989; Peterson and Higley 1993). Additional studies within this system therefore will assist in characterizing plant physiological responses to this injury guild.

Because we did not experimentally impose the treatments, we cannot discount the possibility that the plants containing *M. janthinus* larvae were previously stressed. Adult females may have selected specific plants for oviposition because those plants were already stressed. Therefore, the gas exchange responses observed in this study may not have been caused by larval injury, but by other factors. However, that explanation is unlikely for the following reasons: (1) the uninjured and injured plants were often <20 cm apart, indicating that abiotic or other biotic stress factors likely were not different between treatments, and (2) the injured plants were chosen based only on the presence of ovipositional scars and stem swelling; no other visual differences between plants were evident. Future experimental research will need to determine conclusively if the responses we observed are solely the result of larval injury.

***Calophasia lunula*.** Defoliation by *C. lunula* larvae varied considerably among plants within and among experiments (Table 1). There were significant time effects in all five of the experiments in which repeated measures statistical analysis was used. In all eight experiments, there were no significant differences among treatments for any of the gas exchange variables measured (Table 1). Therefore, our results suggest that defoliation injury did not affect Dalmatian toadflax primary metabolism (i.e., photosynthesis, stomatal conductance, transpiration) over the observation period. Larval defoliation reduced leaf area, but did not affect the photosynthetic apparatus of remaining tissue. There was no statistically significant interaction between injury of upper leaves and photosynthesis of lower, uninjured leaves on the same plant (Table 1). This indicates that there was no photosynthetic response by lower leaves linked to defoliation injury on upper leaves.

Many studies have reported that there are no changes in photosynthetic rate in the remaining leaf tissue of injured leaves in response to insects in the defoliator injury guild (e.g., Davidson and Milthorpe 1966, Poston et al. 1976, Syvertsen and McCoy 1985, Welter 1989, 1991, Higley 1992, Peterson et al. 1992, Peterson et al. 1996, Peterson and Higley 1996, Burkness et al. 1999, Peterson et al. 2004). The similarity in response across these studies suggests that the photosynthetic processes of many plant species is not affected directly by defoliation. The principal effect of this injury type seems to be linked more to the reduction in photosynthesizing leaf area than to a reduction or enhancement of photosynthetic capacity of remaining tissue of injured leaves (Peterson and Higley 1996, Peterson 2001, Peterson et al. 2004).

Measurement of the potential impact from the defoliation injury in these experiments was limited to relatively few leaves on the distal end of one stem of a plant. Therefore, the results may not translate to whole plant performance. Indeed, substantial defoli-

ation of whole plants is known to alter the pattern of normal progressive leaf senescence of some plant species (Higley 1992, Peterson et al. 1992). Consequently, Dalmatian toadflax may respond differently to defoliation injury at different levels of biological organization, as has been observed for other plant-insect systems (Peterson and Higley 1993, Peterson and Higley 2001).

Because there were no statistically significant physiological differences between leaves from *C. lunula* injured and artificial defoliation injured plants (Table 1), it is possible to simulate *C. lunula* defoliation. This is important because simulating insect defoliation often allows for better experimental control and a more rapid quantification of injury than using actual insects (Pedigo et al. 1986, Peterson et al. 2004). However, if the objective of a study is to characterize whole plant and fitness responses of Dalmatian toadflax to *C. lunula* injury, the artificial defoliation techniques must adequately reproduce the spatial and temporal pattern of injury on the plant (Pedigo et al. 1986).

Discussion

Injury Guilds. The injury guild concept has evolved from grouping herbivores by taxonomic category to grouping them by categories of plant physiological impact (Peterson 2001). Our results indicate that insect herbivores in two distinct injury guilds differentially impact Dalmatian toadflax physiology. Based on the primary physiological parameters evaluated in this study, the stem borer, *M. janthinus*, seemed to affect Dalmatian toadflax more than the defoliator, *C. lunula*.

The differences we observed in host-plant physiological response to the two herbivore species, representing two different injury guilds, may help explain their relative effectiveness in controlling the target weed. Weed ecological studies indicate that crude tissue removal or consumption, such as is produced by *C. lunula* larvae, seldom significantly affects trenchant weed infestations (Cousens and Mortimer 1995; Myers and Bazely 2003); this also holds true for Dalmatian toadflax (Robocker et al. 1972; Vujnovic and Wein 1997). Furthermore, weed species with both vegetative and seed reproduction, such as Dalmatian toadflax, are particularly resilient to simple biomass reduction (Burdon et al. 1980, Burdon and Marshall 1981, Lajeunesse et al. 1993).

Jeanneret and Schroeder (1992) reported that larval mining in high density or outbreak populations of *M. janthinus* caused premature stem wilting and suppression of flower formation, and consequently, reduction in seed production (Jeanneret and Schroeder 1991). Larval feeding also was correlated with reduced stem biomass and with an increased incidence of vegetative stem mortality during prerelease tests (Saner et al. 1994). According to Robocker (1970), the vegetative stems of Dalmatian toadflax contribute significantly to both the root carbohydrate reserves and vigor of each individual toadflax plant.

Conclusions. Successful biological control of invasive weeds with herbivorous insects is predicated on

the assumption that insect-induced injury will stress the weed sufficiently to cause reductions in individual fitness and, eventually, weed population density. These reductions in fitness can be caused through direct and/or indirect consequences of herbivory (Crawley 1989, Sheppard 1996). Herbivores may cause physiological impairment to the weed host, which may lead directly to reductions in fitness through feeding activity. Alternatively, herbivores may impair the weed host's ability to compete effectively with other plants. Regardless of the mechanism underlying fitness loss, because plant gas exchange directly affects growth, development, and fitness, we argue that characterizing how agent injury impacts these primary physiological processes may be a key indicator of the agent's potential efficacy. However, plant physiological impacts by the agent do not necessarily predict agent efficacy. Indeed, the type of injury does not solely determine yield or fitness loss. In addition to injury type (impact per unit injury), the magnitude and duration of injury (intensity of injury) also is an important determinant (Pedigo et al. 1986, Peterson and Higley 2001). The intensity of injury depends heavily on the density of herbivores on the host. For example, although defoliation injury may not alter photosynthetic rates of remaining leaves, high densities of herbivores, and therefore high rates of defoliation, may lead to reduced fitness. Therefore, to predict efficacy, it also is necessary to understand the potential intensity of injury as well as the physiological impact per unit of injury.

With such information, improved risk-benefit and cost-effective decisions can be made about whether to release exotic biological control agents. For example, if both biological control agent species were being evaluated for release, gas exchange measures would indicate that *M. janthinus* would have a higher probability of being efficacious. Consequently, further studies on *C. lumula* would be needed to evaluate whether the intensity of injury (population density potential) would be sufficient to reduce invasive Dalmatian toadflax populations.

To our knowledge, this study is the first to show primary metabolic impacts by insects introduced as biological control agents against an introduced weed species. Furthermore, these results contribute to and support current knowledge of plant gas exchange responses to insect injury types (Boote 1981, Welter 1989, Peterson and Higley 1993, Peterson 2001). Additional research within the system we studied will need to address directly the links between host-plant physiological impairment and changes in the host's population.

Understanding plant physiological responses to herbivory and tracking the impact of those responses on the weed's population and community dynamics can provide a quantitative and systematic method for evaluating agent efficacy. If physiological impairment from herbivores can be related to fitness effects, the metabolic measurements could be used as a diagnostic tool during the exploration phase of classical biological control. These physiological methods, which often are

less costly than measurements of biomass, yield, and fitness reduction, potentially improve the process of agent selection from among numerous available candidate species.

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