The lesser of two weevils: physiological responses of spotted knapweed (Centaurea stoebe) to above- and belowground herbivory by Larinus minutus and Cyphocleonus achates

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RESEARCH ARTICLE

The lesser of two weevils: physiological responses of spotted knapweed (*Centaurea stoebe*) to above- and belowground herbivory by *Larinus minutus* and *Cyphocleonus achates*

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Abstract

The physiological responses of plants to variable levels of root and shoot herbivory in the field may yield valuable insights regarding potential compensation or tolerance responses for herbivory. In an infestation of *Centaurea stoebe* (spotted knapweed) located in the Colorado foothills, we measured physiology, biomass, and flower production of individual plants undergoing isolated or simultaneous herbivory by the above- and belowground biological control insects, *Larinus minutus* and *Cyphocleonus achates*. Over the growing season, net C assimilation rate, transpiration, stomatal conductance, and Ci all decreased, while water use efficiency increased. The reductions in plant physiology were found to be correlated to late-season plant performance measures of aboveground biomass and flower production. The decrease in these physiological traits was due to an increase in the intensity of *Larinus* damage over time; effects of *C. achates* root damage to plant physiology, including transpiration were only marginally significant. The effects of these two species on plant physiology were not interactive, and *Larinus minutus* was found to exert larger negative effects on this invasive plant in terms of plant physiology and potential reproductive output than *C. achates*. While previous studies have shown *C. achates* to have significant negative effects on population densities of spotted knapweed, the addition of *Larinus minutus* to the suite of insects used in biological control of spotted knapweed should facilitate continued or enhanced reduction in densities of this noxious weed.

**Keywords**: Plant physiology; Biological control; *Centaurea stoebe* L. *ssp. micranthos*; Invasive plants; *Larinus minutus*; *Cyphocleonus achates*
1. Introduction

Among the many dynamic interactions between plants, herbivores, and their external abiotic conditions, plants employ multiple mechanisms to deal with herbivore damage, including altered photosynthetic activity (Welter, 1989; Delaney, 2008), compensatory growth (Paige and Whitham, 1987; Trumble et al., 1993), phenological escape (Coley and Barone, 1996; Saltz and Ward, 2000), or resistance through chemical defense (Baldwin and Ohnmeiss, 1994; Stamp, 2003). These mechanisms of response to herbivory deserve further attention (Tiffin, 2000). The direction and magnitude of responses to herbivory, and how those responses may influence plant fitness, are also highly complex and site specific (Maschinski and Whitham, 1989; Trumble et al., 1993; Stamp, 2003). The generalized plant responses to herbivory elucidated from short-term experiments sometimes involve artificially imposed damage, or low to moderate levels of herbivore damage inflicted in a single bout; however, in natural systems plants may undergo chronic damage over a broader range of intensities to both root and foliar tissues by multiple herbivores. In particular, introduced biological control insects released in an attempt to control invasive plants may have large impacts since these species may lack predators and reach high densities (e.g. Hunt-Joshi et al., 2004). Such is the case with spotted knapweed (Centaurea stoebe L. subsp. micranthos [Gugler] Hayek [Asteraceae]), also identified as C. maculosa and C. biebersteinii (see Ochsmann 2001, Hufbauer and Sforza 2008), a plant that infests over two million ha of range and pasture lands in North America, and causes a wide-range of economic and ecological problems (Lacey, 1989; DiTomaso, 2000; Smith, 2008). The species is a short-lived perennial that produces multiple flowering stems from June through the early fall, and that is capable of producing tens of thousands of seeds per square meter at high densities and in the absence of biological control insects (Sheley et al. 1998).
Extensive research has been attempted to understand the biotic and abiotic factors related to *C. stoebe*’s ability to invade, compete with, and attain dominance over other herbaceous vegetation (e.g., Pokorny, 2005; Hill et al., 2006; Maron and Marler, 2008). A substantial number of reports exist that suggest that biological control insects may directly or indirectly contribute to this dominance (e.g., Pearson and Callaway, 2008), or contrarily, to its control and demise as a dominant (Story et al., 2008; Knochel and Seastedt, 2010). Biological control studies provide evidence that through tissue damage and reduction of reproductive potential, the root feeder, *Cyphocleonus achates* Fahr. (Coleoptera: Curculionidae), flower head weevil *Larinus minutus* Gyllenhal (Coleoptera: Curculionidae), and the gall fly species *Urophora affinis* Fairfield (Diptera: Tephrididae), together have the ability to reduce populations of *C. stoebe* (Story et al., 2006; 2008). Some evidence indicates that drought may also interact with root and foliage herbivory to impact *C. stoebe* (Corn et al., 2006; Pearson and Fletcher, 2008), and that damage by the root-feeding weevil, *C. achates* may contribute to population control by reducing adult plant longevity and in some cases causing mortality (Cortilet and Northrop, 2006; Jacobs et al., 2006; Michels et al., 2009). *Cyphocleonus achates*, a univoltine root weevil introduced to North America in the late 1980’s, feeds on *C. stoebe* aboveground tissues from June-Sept as an adult and females oviposit during the late summer and fall near the taproot crown. There, larvae typically overwinter at first instar and continue development and the consumption of taproot tissue the following spring, with emergence of adults in June and July (Corn et al., 2009).

The univoltine flower head weevil, *Larinus minutus*, overwinters as an adult in the soil and emerges in late May-June to feed on stems, rosettes and cauline foliage. Females oviposit into...
open flowers, and larvae develop within the maturing flower head, where they consume reproductive tissues and thereby greatly reduce seed production. *Larinus minutus* in the larval stage are the primary impact to flowers—they feed within the maturing flower heads on achenes. *Larinus minutus* effects on seed production were not considered in this experiment but are reported elsewhere (Knochel and Seastedt 2010), because our focus here was to determine the relatively undocumented effects *L. minutus* in the adult life stage on other plant tissues. Adults feed primarily on stem and foliar tissues, and although they do consume a small portion of inner flower petals when preparing a location for oviposition within the top of the flower head, this damage does not eliminate flower heads. In addition to these effects of larval seed predation (Story et al., 2008; Knochel et al., 2010a), this species has recently been shown to reduce flowering and aboveground biomass of *C. stoebe* through tissue damage, at least in Colorado (Seastedt et al., 2007; Knochel et al., 2010b; Knochel and Seastedt, 2010). However, little is known about how damage by *L. minutus* and *C. achates* to non-reproductive root and aboveground tissues influences plant physiology and performance in the field.

This is important for several reasons. First, as noted above, studies have shown both positive and negative effects of biological control agents on indices of plant fitness. Examining how a range of damage intensity by *L. minutus* and *C. achates* affects *C. stoebe* physiology could improve our understanding of plant compensation responses to introduced herbivores. Second, knowledge of the effects and interactions of both insect species on individual plant physiology and performance could improve our understanding of their potential role in controlling the plant’s population-level dynamics. Third, measuring the physiological traits of *C. stoebe* plants in the field experiencing a range of damage from multiple herbivores provides a first assessment of the
Finally, this study may provide a mechanistic link between herbivory and plant performance. We directly measured how isolated and simultaneous above- and belowground herbivory by *Larinus minutus* and *Cyphocleonus achates*, respectively, influences spotted knapweed physiology and performance in a field infestation on the Colorado Front Range. We asked three questions: 1) What is the relationship between spotted knapweed physiology and plant performance outcomes in terms of biomass and potential reproductive output? 2) How does a gradient in the intensity of herbivory by *L. minutus* and *C. achates* affect plant physiology and performance? 3) Are the effects of *C. achates* root herbivory measurably superior to aboveground foliar herbivory by *Larinus minutus* in terms of its effect on growth and potential reproduction and is there an interaction between the two species?

2. Methods

2.1. Field site description

Field research occurred during the summer of 2009 approximately 15 km northwest of the city of Boulder, CO (40°07’13.86”N 105°19’26.34”W), between 1865 and 2070 m elevation, in a meadow surrounded by rugged 15 to 60 percent slopes and dominated by ponderosa pine (*Pinus ponderosa* Douglas Ex. Lawson). Soils at the site are part of the Fern Cliff-Allens Park-Rock outcrop complex, and are composed of mixed loamy alluvium parent material, with a stony sandy clay loam profile at 0 to 100 cm depth (NRCS, 2008). Mean annual air temperature is 6 to 8 degrees °C, with a frost-free period of 80 to 120 days. Precipitation during the course of this study measured 104% of the 30 year average for June and July (NOAA, 2009).
has sustained moderate cattle grazing since the mid 1900’s, however cattle were not present on
the property during this study. *Centaurea stoebe* was accidentally introduced to this area in the
late 1980’s, and since then has spread to over 40 ha of meadows, riparian areas, and forests. Also
present are other non-native species including dalmatian toadflax, *Linaria dalmatica* (L.) Mill.,
mixed grass prairie species also common in the area include sand dropseed, *Sporobolus
cryptandrus* (Torr.) A. Gray, blue grama, *Bouteloua gracilis* (HBK) Lag. ex Steud., and wild
bergamot, *Monarda fistulosa* L. Since 2001 we have observed and monitored specialist
biological control insects at the site (Knochel and Seastedt 2009). These include two gall flies of
the *Urophora* genus, and the seed head weevil *Larinus minutus*, which likely colonized the site
from nearby populations of *Centaurea diffusa*. These insects were supplemented during 2001-
2005 with releases of three thousand *Larinus minutus* weevils, and two thousand *C. achates* root
weevils.

2.2. Experimental design

The experimental meadow was at the core of a spotted knapweed infestation, located near the
stream channel of a steep gulch. Spotted knapweed stem densities in this area reach 50 stems/
\( \text{m}^2 \). Four 50 m long transects were placed across this meadow and oriented east-west and parallel
to an ephemeral stream that ceased running in mid-July, and 48 spotted knapweed plants were
selected and tagged along the transects at 1m intervals. Target individuals were chosen that were
identifiable as a single plant with one taproot, and having dead stems from the previous year
(generally > 2 years old), that had initiated upright stem growth (bolted) from the rosette stage in
the spring of 2009. Thus, most plants were likely between 2-4 years of age.
2.3. Insect census and damage estimates

All insect censuses and physiological measurements were taken for each target plant on June 26, July 7 and July 20, 2009. On each date, the number of *Larinus minutus* adults present on each plant was recorded. Weevil damage to stem and foliage tissues was visually estimated independently by three people, with each person assigning a number describing the intensity of defoliation between 0 (no damage) and 5 (removal of entire leaves and with the majority of each stem length having tissues scraped-away). The average of the three independent estimates was used as the damage value testing the relationship between damage and physiological traits. The presence of *C. achates* and damage to the taproot was estimated when whole plants were harvested upon termination of the experiment on July 20, 2009. At this time, the taproots were carefully sectioned to count the number of larvae, pupae, or adult *C. achates* root weevils present. Damage by *C. achates* was scored by the presence and number of individuals per root. The total mass of *C. achates* was also measured, and was entered into the preliminary stepwise regressions but not found to be a powerful explanatory variable. In previously published research at this field site, we have found that seasonal insect attack by these two species is highly variable, with plants sustaining variable seed head attack by *L. minutus*, as well as about 20% of plants on average not undergoing attack by *C. achates* root weevils. Thus, although we did not cage or manipulate insect attack or insect densities from naturally occurring levels at this field site, our experimental design inherently allowed comparison of a wide range of attack by both species: from very low *L. minutus* damage to heavier levels of damage, and of plants whose roots had no *C. achates* weevil attack to roots with high densities.
2.4. Photosynthetic gas exchange

Gas exchange measurements on spotted knapweed were taken using a portable closed-flow gas exchange system (LI-6400 XTi, LI-COR, Inc., Lincoln, NE) with CO\textsubscript{2} controller set at 380 ppm fitted with a standard 6-cm\textsuperscript{2} leaf chamber and red-blue light source set at 1500 µmol·m\textsuperscript{-2}·sec\textsuperscript{-1}.

All measurements were taken between 1000 and 1400 hours under clear skies. Leaves were chosen for measurement that best represented the majority of leaves on the plant or when few leaves were present, the most suitable leaf for measurement was used. As such, on highly damaged plants leaves with herbivore damage were measured because no leaves were present without damage. However, every effort was made to select intact leaves for measurements.

Because knapweed leaf margins dissected and will not occupy completely the area of the chamber, after taking each measurement, we calculated total leaf area by covering a leaf with a clear acetate sheet with a 0.25cm\textsuperscript{2} grid. The number of complete squares contained within the area of the leaf that had been enclosed in the leaf chamber was recorded, with partial squares considered 0.125cm\textsuperscript{2}. All the physiological variables dependent on leaf area were recalculated using the leaf area determined with the grid. Carbon assimilation rate (A), transpiration rate (E), stomatal conductance (g\textsubscript{s}), and intercellular leaf [CO\textsubscript{2}] (C\textsubscript{i}) were determined. Water use efficiency (WUE) was calculated as A/E (µmol CO\textsubscript{2}/mmol H\textsubscript{2}O). By the end of the experiment, four plants had either senesced, lost too much leaf tissue to herbivores for adequate physiological measurement, or died and so were excluded from the statistical analyses.

2.5. Plant trait measurements

*Centaurea stoebe* plant trait measurements were taken on July 20, 2009 at the end of the experiment. The number of flowers produced by each plant was counted, and plant stems were
clipped at ground level and collected. Both coarse and fine roots were excavated, harvested and transported to the lab. Some loss of fine roots occurred due to the difficulty of separating these tissues from the soil, however, root harvest was equivalent across all experimental plants. Plant tissues were dried at 60°C to a constant weight and weighed. Specific leaf area (SLA) was determined by measuring the fresh leaf area \(\text{cm}^2\) divided by the dry leaf mass (mg). In most cases, leaves used to calculate SLA were the same leaves used previously for photosynthesis measurements. To determine C:N content, the leaf samples were ground in a Wiley mill (40 mesh screen), and 3-5 mg per plant were analyzed by combustion on a Carlo Erba Flash EA1110 CN analyzer (CE Elantech, Lakewood, New Jersey, USA) calibrated with an Atropine standard.

2.6. Statistical analysis

We used two different procedures in the data analysis. First, we broadly analyzed the data by using an automated stepwise regression procedure that included all response and predictor variables and combined both forward addition and backward elimination. We then used a manual method with a more narrow set of variables based on the significance of initial results and the factors that we considered most important for our biological system. Using this process, the stepwise regression gave us a broad indication of the relationships between the various plant physiological measurements or growth traits (e.g. biomass, flowers) and measured herbivore traits (e.g., damage estimates and abundance of \(L.\ minutus\) per plant, number or mass of \(C.\ achates\) per plant root). The effects of the two species of insects were considered together, and then separately. When \(L.\ minutus\) and \(C.\ achates\) were considered together, the effects of \(L.\ minutus\) often outweighed those of the root weevil, \(C.\ achates\). For \(L.\ minutus\), variables other than the estimates of their damage intensity, or the density of \(C.\ achates\), were not included.
When the effects of *C. achates* were separately analyzed, the dry mass of root tissue was included in the model to test effects of this insect while holding root mass constant (the insect induces some enlargement of the root tissue when forming galls in the taproot, and, the insect tends to be in higher abundance in larger plants), and to see whether the presence of *C. achates* significantly reduces additional unexplained error. The best model was the one with the smallest AIC (Aikake’s information criterion) which indicates a better fit of the model. Next, using a subset of response variables that would minimize multicollinearity (Zar, 1999) in the regression model and most effectively explain overall relationships between plant physiology and insect attack, we created several models manually by entering predictor variables in the order we thought they affected the response variables and also in the order that would control for the variance used by the previously included variable. Once the models were determined, we report the results of individual regressions and provide R$^2$, P and F-values. When graphing the results, curvilinear lines were presented when these models provided a significantly better fit to the data points. All statistical analyses were performed using SAS (SAS Inc, Cary NC). For comparisons of physiology across time, we used repeated measures ANOVA, with date, as the main effect and plant (the replicate) included as a random term, thereby providing the appropriate degrees of freedom for the main effect of date (Gotelli and Ellison, 2004). All plant trait (biomass, flower production) response data were collected only at the termination of the experiment, thus we did not analyze *L. minutus* or *C. achates* effects for previous dates. For plant physiology, taken on three separate dates, we first analyzed *Larinus minutus* damage across the three dates. As we report, the effects of *L. minutus* on physiology intensified—the R$^2$ value increased as the season progressed, reflecting the increase in cumulative damage sustained by a majority of the individual target plants. Our primary results then report the relationship between *L. minutus*
damage and plant physiology measurements from the last date. This statistical method was used because it allowed us to account for both *L. minutus* and *C. achates* on the last date, and we did not have multiple measurements of *C. achates* over the season, but rather just one measurement when upon excavation of the roots.

3. Results

3.1. Physiological responses to herbivory

On the last sampling date in July, estimates of cumulative damage to stems and foliage by *Larinus minutus* ranged from 0.5 (10 plants had very low damage with scores of 1.5 or less) to 5 (scores of 4 or 5 indicated large amounts of tissue removed), with an average damage estimate of 2.8 ± 0.2. *Cyphocleonus achates* densities ranged from zero to nine individuals per taproot, with an average of 2.6 ± 0.4 (SE), and with 15 plants that were undamaged and contained no root weevils. The majority of plants were damaged by at least one of the two species; however 7 of the plants undamaged by *C. achates* also had very low cumulative damage by *L. minutus.*

Photosynthesis, transpiration, stomatal conductance, and *C*$_i$ all decreased 45-80% over time, while WUE increased nearly 40% over the same time period (Table 1; Fig. 1). The decrease in the same physiological traits was associated with leaf and stem herbivory by *L. minutus* (Fig. 2). Carbon assimilation, stomatal conductance and transpiration all increased at intermediate levels of *L. minutus* herbivory, then decreased as herbivore damage increased (Fig. 2). Transpiration decreased in response to aboveground damage, but surprisingly, *C. achates* damage to roots (estimated from *C. achates* census data) did not influence transpiration (Table 1), even as precipitation decreased and temperatures increased.
3.2. Plant performance measures and herbivore damage

At the time of plant harvest in July, before the emergence of *C. achates* weevils from the roots, *C. stoebae* was in full flower but flower heads had not set seed. Stem and leaf damage increased by 40% over time (Fig. 1), even though the total number of *Larinus* minutus abundance on individual plants did not increase during the experiment (P = 0.13). Aboveground biomass decreased as *Larinus* damage increased (Fig. 3). For each unit increase in estimated *Larinus* canopy damage, flower production decreased by 8.3 flowers. Finally, *Larinus* damage increased as the foliar C:N ratio increased.

Flower production and aboveground biomass were negatively influenced by *C. achates* weevil density, controlling for root biomass as an additional parameter in the model (Table 1; Fig. 3). Root biomass was positively correlated with *C. achates* root weevil numbers (Table 1), and was negatively correlated with aboveground damage by *Larinus* (Table 1), suggesting that aboveground foliar feeding decreased allocation of resources to roots (Fig. 3). However, root:shoot ratios did not change in response to *Larinus* damage ($F_{1,42} = 2.14; P = 0.15$), but did increase as more *C. achates* adults were present ($F_{1,41} = 9.92; P = 0.003$) in the roots. The SLA decreased as the total mass of *C. achates* per root increased ($F_{1,42} = 4.55 \ P = 0.039, R^2=0.10$), but was not correlated with *C. achates* density. The SLA decreased as *Larinus* damage increased ($p = 0.058$), though not as a result of the interaction between both weevils ($p = 0.55$). Indeed, we found no interaction between *Larinus* weevil damage and *C. achates* root weevil density on any physiological or plant trait measurement (Table 1).

4. Discussion

The results from a number of studies suggest that *Larinus* and/or the *Urophora* gall flies can
facilitate control of *C. stoebe* populations through direct reductions in seed production (Seastedt et al., 2007; Story et al., 2008). Here, we report that in addition to its known effects as a seed predator, *Larinus* may also substantially influence knapweed populations through the effects of foliar herbivory that reduce aboveground biomass and flower production (Fig. 3). Damage from *Larinus* was negatively correlated with the size of aboveground plant tissues (i.e. smaller plants had more damage); however, the numbers of *Larinus* weevils observed per plant was not reflective of plant size. Thus, it seems that for a similar density of weevils, smaller plants either sustained more tissue damage than larger ones, or the damage itself reduced the plant size, or both. Heavily damaged plants had a considerable amount of stem and foliar tissues removed, thus decreasing their aboveground biomass, and these plants also appeared to senesce earlier, likely leading to reductions in overall reproductive output.

*Larinus minutus* fed chronically on individual plants over the experimental period, and thus our observations of herbivory escalating over time reflect cumulative damage rather than an increase in feeding (Fig. 1). For the physiological parameters we measured, increased damage by *Larinus* was associated with decreases in plant photosynthetic rate, stomatal conductance, and transpiration (Fig. 2), but not root:shoot ratio. The trends in the plant response to aboveground herbivory suggest that at low to moderate aboveground herbivory, spotted knapweed plants actually compensate (Welter, 1989; Meyer, 1998) for damage by increasing rates of physiological activity. Higher than average precipitation in our study and better water relations might also have driven the early apparent compensation for herbivory. In *Amaranthus hybridus*, photosynthesis increased in response to herbivory only when water was highly available (Gassman, 2004). In our system, photosynthesis increased in response to herbivory at the same time water was more available. As water availability declined over the season and herbivory
increased, spotted knapweed’s ability to compensate declined and physiological responses were
depressed, including photosynthetic rates. Hill and Germino (2005) also showed that *C. stoebe*
photosynthetic rates decreased as water stress increased, and found that stem epidermal tissues
were critical for persistence of photosynthesis into the late summer, after the senescence of
rosette and cauline foliage. We found similar results, with all knapweed physiological response
depending over the course of the season, suggesting that as water availability was reduced, plant
physiological activity also declined. Water-stress induced reductions in photosynthetic rates and
other physiological traits could explain why *L. minutus* damage to stem tissues resulted in the
substantial reductions of growth and flower production reported here. The declines in all plant
physiological activity over time (Fig 1) all indicate some kind of stress leading to a reduction in
growth and potentially in the ability of plants to compensate for herbivory. These results support
the idea of a compensatory continuum or optimization model of herbivory (McNaughton, 1979;
Maschinski and Whitham, 1989) that may interact with climatic factors. In other words, the
plant’s ability to tolerate herbivory declined in response to both increased damage from multiple
herbivore species as well as climatic conditions that may have led to greater water stress (Hill et
al., 2006).

Cumulative damage over the experimental period by *Larinus* was most severe on plants with a
higher foliar C:N ratio (Fig. 3). Thus, to meet its N needs *Larinus* appears to have increased
tissue consumption when tissue N was low. This increased herbivory may have occurred instead
of (or perhaps in addition to) *Larinus* also preferentially feeding on spotted knapweed plants
possessing tissues of higher nutritional quality (White, 1988). These results are similar to the
feeding patterns of another biological control root weevil reported by Van Hezewijk et al.
(2008), and suggest that in areas where soil resources are high, *Larinus* damage may be
somewhat reduced. Not only would *L. minutus* damage be reduced in areas where soil resources are higher, but also knapweed tolerance to herbivory would likely be higher (Cronin et al., 2010). For smaller knapweed plants, *L. minutus* damage appeared to shorten their growing season (they senesced earlier) thereby reducing their reproductive output. In contrast, for larger plants that may better tolerate adult *L. minutus* foliar damage, the overall impact of *L. minutus* to plant fitness might be greater in terms of larval consumption of seeds.

Damage by the root weevil *C. achates* has also contributed to some of the reported population declines of *C. stoebe* in North America via effects that reduce aboveground biomass and flower production (Knochel and Seastedt, 2010). In contrast to the substantial impacts we found for *Larinus* on plant physiology and performance, the impacts of *C. achates* on physiology were largely insignificant. However, the root weevil did have some negative effects on flower numbers and plant aboveground biomass (Fig. 3). *Cyphocleonus achates* presence decreased flower production and aboveground biomass when controlling for the positive correlation of weevils with root biomass, an effect primarily due to the accumulation of additional tissue that forms around the damaged areas of the taproots (Fig. 3). In addition, plants typically endure damage to the taproot over multiple years, thus the root weevil may also reduce the longevity of plants and thereby influence lifetime reproductive output, an outcome we did not measure here. *Cyphocleonus achates* has been shown to reduce *C. stoebe* dominance and has been reported to significantly contribute to reductions in densities and abundance (e.g. Jacobs et al., 2006; Corn et al., 2006). Thus, we hypothesized that heavy damage by this species to the plants' taproot would have negative impacts on physiology, in particular transpiration and water use efficiency (WUE) and provide a mechanistic explanation for some of its observed impacts the plants dominance or
population densities. However, we detected no effects of belowground C. achates damage on C. 
stoebe physiology, including its water use efficiency, a physiological parameter that Hill et al. 
(2006) showed was advantageous to this invasive weed compared with native vegetation, 
especially under dryer conditions.

Thus, we speculate that C. achates damage to the root reduces spotted knapweed population 
densities during dry years, but the plant exhibits some capabilities of increased persistence in wet 
years. In other words, knapweed is tolerant of C. achates root damage when water is more 
available. Our comparisons of spotted knapweed seed production in dry upland sites versus 
wetter sites indicate that the plant appears to be able to avoid some of the negative effects of 
biological control insects in areas where soil moisture is higher (unpublished results).

Interestingly, the majority of roots dissected for the presence of C. achates also contained a 
fungus that had colonized the damaged taproot. The direct effects of this root fungus on plant 
performance or its interactions with the root weevil, if any, remain unknown.

In some systems, simultaneous herbivory by multiple species on the root and shoot tissues of the 
same plants results in interactions (Masters and Brown, 1992; James et al., 1992; Maron, 1998; 
Masters et al., 2001; Bezemer et al., 2003; Soler et al., 2007), but not in others (Hunt-Joshi et al., 
2004; for a review see Denno et al.,1995; Blossey and Hunt-Joshi, 2003). Here, we found that 
the abundance and damage inflicted by the aboveground feeder L. minutus were independent of 
the infestation levels and damage to roots of the same plants by C. achates, and vice-versa.

Furthermore, the effects of C. achates and L. minutus on plant physiological response and plant 
performance were not interactive. Although root feeding is known to influence aboveground 
tissue quality (Kaplan et al., 2008), and potentially the feeding rates or amount of tissue
consumed by foliar insects, we found no relationship between *C. achates* densities and foliar C:N. In some plants, leaf N increases in response to herbivory (Gassman, 2004), but in our study C:N ratios actually increased with *Larinus* herbivory, again suggesting that knapweed plants were not able to tolerate or compensate for herbivory, especially as the season became progressively drier. Our results here indicate a potentially larger and more important role of *L. minutus* in the control of spotted knapweed than previously reported (Story et al. 2008; Knochel and Seastedt, 2010b). This is perhaps due to inter-annual variability in *Larinus* densities, or even due to the decline in absolute knapweed abundance at the study site. Regardless, this species needs to be more thoroughly tested throughout spotted knapweed's range in North America.

5. Conclusions

Overall, damage to roots by *C. achates* had measurably smaller effects on spotted knapweed than did the quite extensive effects on physiology and performance related to canopy damage by *L. minutus*. Our results partially explain why *L. minutus* was dubbed a ‘silver bullet’ biological control agent for diffuse knapweed (Myers, 2004; Myers et al., 2009). We provide a physiological mechanism to explain the effects of *Larinus* on spotted knapweed, and we contend that this insect species, when combined with plant competition and the negative effects of *C. achates* reported here and elsewhere, may together provide a collective ‘silver bullet’ for reductions of spotted knapweed populations in North America.

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Figure legends

Figure 1 (a) Photosynthetic rate, (b) transpiration, (c) stomatal conductance, (d) $C_i$, (e) water use efficiency and (f) *L. minutus* canopy damage measured from individual spotted knapweed plants on three dates in Boulder County, CO. Points depict the mean ± 1 SE, different letters denote significant differences between dates at P<0.05.

Figure 2 Negative polynomial regressions of (a) photosynthetic rate (A), (b) stomatal conductance (log), and (c) transpiration (E) as a function of cumulative *Larinus minutus* damage (data shown is from last measurement date only) to stem and foliar tissues of individual spotted knapweed plants over a two month period.

Figure 3 Linear and polynomial regressions of insect and *C. stoebel* plant traits showing (a) foliar C:N (b) flower production and (c) aboveground biomass (log) as a function of cumulative *Larinus minutus* damage to stem and foliar tissues; and (d) root biomass as a function of *C. achates* density. Panels (e) flower production and (f) aboveground biomass are partial regression plots that depict the effects of *C. achates* root weevil density (log), holding root biomass constant.
Table 1 Repeated measures ANOVA across three sampling dates for (a) *C. stoebe* physiology (date), and regression results for insect effects on (a) physiology and (b) plant traits. Effects of foliar damage by *Larinus minutus*, and root damage by *Cyphocleonus achates*, or their interaction, are presented for measurements taken on the last sampling date. Significant effects are in bold type.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Date</th>
<th>Larinus minutus</th>
<th>Cyphocleonus achates †</th>
<th>Larinus x Cyphocleonus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>df</td>
<td>F</td>
<td>P</td>
<td>df</td>
</tr>
<tr>
<td>(a) Physiology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Photosynthesis</td>
<td>2, 44</td>
<td>15.4</td>
<td>&lt;0.001</td>
<td>1, 43</td>
</tr>
<tr>
<td>Transpiration</td>
<td>2, 46</td>
<td>49.1</td>
<td>&lt;0.001</td>
<td>1, 43</td>
</tr>
<tr>
<td>Conductance</td>
<td>2, 46</td>
<td>43.3</td>
<td>&lt;0.001</td>
<td>1, 43</td>
</tr>
<tr>
<td>Ci</td>
<td>2, 42</td>
<td>36.5</td>
<td>&lt;0.001</td>
<td>1, 38</td>
</tr>
<tr>
<td>WUE</td>
<td>2, 46</td>
<td>18.1</td>
<td>&lt;0.001</td>
<td>1, 47</td>
</tr>
<tr>
<td><em>Larinus</em> damage</td>
<td>2, 47</td>
<td>35.0</td>
<td>&lt;0.001</td>
<td>-</td>
</tr>
<tr>
<td>(b) Plant traits</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Foliar C:N</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1, 43</td>
</tr>
<tr>
<td>Flowers / plant †</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1, 46</td>
</tr>
<tr>
<td>Aboveground biomass †</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1, 38</td>
</tr>
<tr>
<td>Root biomass</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1, 38</td>
</tr>
</tbody>
</table>

† For *C. achates* effects on flowers and aboveground biomass, log-transformed root biomass was included in the model, thus df = 2.
Figure 1
Larinus minutus damage

\[ R^2 = 0.17 \]
\[ P = 0.014 \]

a) Photosynthesis

\[ A_{net} (\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}) \]

b) Conductance

\[ g_s (\text{mol} \text{H}_2\text{O} \text{m}^{-2} \text{s}^{-1}) \text{ (log)} \]

\[ R^2 = 0.30 \]
\[ P = 0.010 \]

c) Transpiration

\[ (E) \text{ mmol} \text{H}_2\text{O} \text{m}^{-2} \text{s}^{-1} \]

\[ R^2 = 0.31 \]
\[ P < 0.001 \]
R2 = 0.1689

Larinus minutus damage

R2 = 0.17
P = 0.006

Foliar % C:N

R2 = 0.49
P < 0.001

Flowers per plant (log)

R2 = 0.3195
P = 0.002

Aboveground biomass g (log)

R2 = 0.32
P = <0.001

Cyphocleonus achates root -1

R2 = 0.28
P < 0.001

Root biomass g (log)

R2 = 0.34
P = <0.001