

# Interactions and effects of multiple biological control insects on diffuse and spotted knapweed in the Front Range of Colorado

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## Abstract

Abundances and interactions among biological control insects and their effects on target invasive plants were monitored within the flower heads and roots of diffuse knapweed, *Centaurea diffusa*, and in spotted knapweed, *Centaurea stoebe*, along the Colorado Front Range. Flower weevils, (*Larinus* species) and root-feeders (*Cyphocleonus achates* and *Sphenoptera jugoslavica*) were released on knapweed that already supported biological control gall flies (*Urophora* species). At a single monitoring site, seed production by *C. diffusa* declined from 4400 seeds m<sup>-2</sup> in 1997 to zero seeds m<sup>-2</sup> on the monitoring sites in 2006, while the flowering stem density of *C. diffusa* declined from a peak of almost 30 stems m<sup>-2</sup> in 2000 to zero stems m<sup>-2</sup> in 2006. The average abundance of *Urophora* and *Larinus* in flower heads fluctuated independently during the 2001–2006 interval, while the relative abundance of *C. achates* and *S. jugoslavica* in roots exhibited a weak inverse relationship that appeared driven by climate effects. The relative abundance of insects on a population of *C. stoebe* was monitored for five years as *Larinus* species and *C. achates* became established on spotted knapweed that already supported *Urophora* species. Spotted knapweed seed production on our monitoring site declined from 4600 seeds m<sup>-2</sup> in 2003 to zero seeds m<sup>-2</sup> in 2006. Unlike *C. diffusa*, substantial numbers of rosettes of *C. stoebe* remained present. *Larinus* consumed almost all *Urophora* encountered in *C. diffusa*, and consumed about 40% of the *Urophora* in co-infested flower heads of *C. stoebe* (ca. 10–15% of the total *Urophora* population). No negative correlations between the relative densities of flower head and root-feeding insects were observed. The effects of these insects on target plants have produced results consistent with the ‘cumulative stress hypothesis’ for biological control of *Centaurea* species.

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## 1. Introduction

Large reductions in the abundances of non-native plant species have resulted from the release of non-native insects and pathogens (McFadyen, 1998). However, as reviewed by Myers and Bazely (2003), many of these releases fail to significantly affect the densities of their target species. Recent studies have challenged the dogma that escape from native herbivores is the common mechanism that is responsible for the high densities observed in non-indigenous plant species (e.g., Callaway and Ridenour, 2004; Colautti et al., 2004). Further, the effects of introduced herbivores

on introduced plants in their new environments may be markedly different from that in their native ecosystems (Keane and Crawley, 2002). If we assume a long co-evolutionary relationship between plants and their herbivores, we should expect various outcomes of plant–herbivore interactions. The outcomes for plants range from negative or neutral responses to positive (over-compensation) responses that may increase plant fitness by enhancing competitive interactions with other plants (Callaway et al., 1999; Ridenour and Callaway, 2003; Thelen et al., 2005).

The releases of different biological control agents, often done sequentially after unsuccessful results from initial releases, have been termed the “lottery approach” (i.e., the probability of controlling the target plant would likely

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increase with the number of agents released; Myers, 1985), and this approach has been criticized as one that could elevate the indirect non-target effects of biocontrol releases. Specifically, the large number of insect releases against the knapweeds (*Centaurea* species) has been used as a model system demonstrating the problematic issue of a failure to control the target species and the unintended result of non-target effects on predators in the food web that consume biological control agents (Pearson and Callaway, 2005, 2006).

Introduced forbs of the Eurasian genus *Centaurea* have collectively colonized more area of the western United States than any other non-indigenous dicotyledous plant genus (LeJeune and Seastedt, 2001), and this group currently occupies about 9 million hectares (Duncan et al., 2004). Accordingly, several species of *Centaurea* were the focus of early biological control release efforts, and over a 21-year interval 12 species of insects were released against two *Centaurea* species (Story and Piper, 2001). Many of these species have failed to establish in specific areas of the western US, but in Colorado for example, six species can be found on *C. diffusa*: two gall flies, two flower head weevils, and two root-feeders.

The present study examined the consequences of the release of biological control insects on several populations of diffuse knapweed (*C. diffusa* Lamarck) and one population of spotted knapweed (*C. stoebe*, L. subsp. *Micranthos*, also called *C. maculosa*; see Ochsmann, 2001 for synonyms), which already supported two species of *Urophora* gall flies. We documented the response of the target plants to these cumulative releases, and the net outcome of multiple releases on the abundances of the individual insect species. In particular, interactions between the two genera of flower head insects, *Urophora* and *Larinus*, were examined by comparing abundances in flower heads within and among sites and years. Correlations between the two root-feeders and among all the insects were evaluated by comparing annual relative abundances in roots or flower heads. We then evaluated the contention that these combined releases contribute to the “cumulative stress approach” (Muller-Scharer and Schroeder, 1993) envisioned to be necessary for the successful control of these knapweed species, insofar as possible, by monitoring plant seed production, rosette, and flowering stem densities of the *Centaurea* species.

## 2. Methods

### 2.1. Diffuse knapweed study sites

The introduced forb, diffuse knapweed, became abundant in grasslands along the Colorado Front Range in the 1980s. The longest and most complete data set on diffuse knapweed and insect abundance was from a single, 30 hectare mixture of restored and native mixed grass prairie found at 1700 m elevation (39°55′38.40″N 105°10′47.25″W), monitored over 10 years. Details of the dates, species, and

numbers of biological control insects released as well as the plant and soil conditions at this site are described in Seastedt et al. (2003, 2005) and LeJeune et al. (2005, 2006). In addition to the long-term monitoring site, results reported here included results from 3 to 16 additional sites sampled per year along a 200 km section of the Colorado Front Range centered near Denver, Colorado. Sites varied in composition from upland meadows at 2000 m in elevation to prairie and roadside sites at the base of the mountains at elevations of approximately 1700 m. Vegetation at all sites was a mixture of native and non-native grasses and forbs. Common native grasses included western wheat grass, *Agropyron smithii* Rydberg., blue grama, *Bouteloua gracilis* (HBK) Lag. ex Griffiths, buffalograss *Buchloe dactyloides* (Nutt.) Columbus, and junegrass, *Koeleria macrantha* (Ledebour) Schultes (taxonomy follows USDA Plant database). Common non-native grasses included two species of annual bromes, *Bromus arvensis* L., *Bromus tectorum* L., and Kentucky bluegrass *Poa pratensis* L. Common native forbs included fringed sage, *Artemisia frigida* Willdenow, fleabane, *Erigeron flagellaris* Gray, spreading daisy, *E. colomexicanus* A. Nels, and snakeweed, *Gutierrezia sarothrae* (Pursh) Britton & Rusby. Common non-native forbs in addition to diffuse knapweed included *Alyssum simplex* Rudolphi, and bindweed, *Convolvulus arvensis* L.

### 2.2. Diffuse knapweed sites: insects

The introduction of two gall fly species of the genus *Urophora*, *U. affinis* Frfld and *U. quadrifasciata* Meigen (Diptera: Tephrididae) in 1970 represented the first attempts to use biological control agents to reduce the abundance of *C. stoebe* and *C. diffusa* in North America (Harris, 1980; Muller-Scharer and Schroeder, 1993). The two species of gall flies were released by Colorado Department of Agriculture personnel along the Colorado Front Range in the late 1980s (Seastedt et al., 2003). These species, alone or in combination, were abundant on knapweed by the mid 1990s if not earlier.

The flower head weevil, *Larinus minutus* Gyll. (Coleoptera: Curculionidae), was first released in this region in 1996. The weevil lays an egg in the flower of diffuse or spotted knapweed, and the larva consumes developing seeds, pupates, and emerges as an adult from the flower head of these plants in late summer or autumn. The adults then over-winter in the soil (Lang et al., 2000). A second flower head weevil, *Larinus obtusus* (Gyll.), was released in the Boulder, Colorado area by 2001, but its abundance remains unknown. Whereas we suspect that our results are based largely on the activities of *L. minutus*, the potential presence of both species on one or more study sites must be acknowledged (Story and Coombs, 2004).

A root-feeding beetle, *Sphenoptera jugoslavica* Obenb. (Coleoptera: Buprestidae) had been released in the Colorado region in the early 1990s, and 400 of these insects were released at our monitoring site in 1997. By 1999, an addi-

tional 1100 insects had been released. A second root-feeder, *Cyphocleonus achates* Fahr. (Coleoptera: Curculionidae) was first released on Colorado grasslands in 1996, and 50 of these insects were also released at the monitoring site in 1997. Additional releases of fewer than 200 individuals of *C. achates* were made in subsequent years. While *C. achates* and *S. jugoslavica* were also released at a subset of other sites used to assess *Larinus* and *Urophora* abundance, these root-feeding insects were not systematically monitored away from the core site.

### 2.3. Spotted knapweed study site

Spotted knapweed was accidentally introduced into the Front Range region in the 1980s, but has remained relatively uncommon. The study site used here was located approximately 20 km northwest of the city of Boulder in Left Hand Canyon (40°07'13.86"N 105°19'26.34"W) in meadows and on slopes dominated by ponderosa pine (*Pinus ponderosa* Douglas Ex. Lawson) between 1910 and 2070 m elevation. A history of moderate cattle grazing within and around this study area occurred since the mid 1900s.

This population of spotted knapweed occupied over 40 hectares of meadows, riparian areas, and forest that had been burned in the 1980s. This fire apparently facilitated the invasion of other non-native species including dalmatian toadflax, *Linaria dalmatiana* (L.) Mill., sulfur cinquefoil, *Potentilla recta* L., and musk thistle, *Carduus nutans* L. Meadows contain large amounts of the invasive cheatgrass, *Bromus tectorum* although some relics of the former mixed grass prairie exist including blue grama, *Bouteloua gracilis* (HBK) Lag. ex Steud., needle grass *Hesperostipa comata* (Trin. & Rupr.) Barkworth, and sideoats grama *Bouteloua curtipendula* (Michx.) Torr.

### 2.4. Spotted knapweed site: insects

The spotted knapweed site contained both species of *Urophora* when first inspected in 2001. A few *Larinus* weevils were observed on the vegetation that year, and those insects were supplemented over the next two years with releases of approximately three thousand weevils. The weevils may have also colonized this site from other populations of *C. diffusa* in the region. In addition, approximately 100 *S. jugoslavica* and two thousand *C. achates* were released at this site during the 2001–2005 interval.

### 2.5. Diffuse knapweed: sampling procedures

#### 2.5.1. Insects

The relative abundance of *Urophora* and *Larinus* were obtained by dissecting flower heads of knapweed. For *C. diffusa* before 2005, six flower heads were sampled per plant, and 30 plants were sampled per site. In 2005 and 2006, only three flower heads were sampled on each of 30 plants, but collections were repeated several times during the July–September interval. Collections at each site were

made haphazardly among plants and among flowering stems and were assumed to be random. Additional details for the *C. diffusa* collections are reported in Seastedt et al. (2003, 2005). Harvests were made between early August and early October during the 1997–2004 interval, with collections timed to collect seeds from mature plants with intact flower heads. Flower heads were kept at 4°C until dissected, usually within a few days of collection. For each flower head the number of mature seeds, the number of *Urophora* larvae, and the presence or absence of *Larinus* were recorded. Smith and Mayer (2005) and Crowe and Bouchier (2006) indicated that *Larinus* inflicted substantial mortality on *Urophora*, and that the presence of *Urophora* might inhibit flower head selection and egg-laying by *Larinus*. In 2005 and 2006 we therefore initiated collections in July and continued these into September to understand the phenology of flower head use by the insects, and to assess the extent to which our earlier counts may have underestimated *Urophora* abundance in flower heads.

The impact of *Larinus* and *Urophora* on seed abundance and upon each other in individual flower heads was assessed using Pearson's Correlation analysis. We also attempted to detect how the presence of a second species might affect flower head selection by *Larinus* and *Urophora*. If both species of insects selected flower heads with similar characteristics, co-infestation could be enhanced. If *Larinus* avoided flower heads with *Urophora* or if it consumed *Urophora*, co-infestation of flower heads would be reduced. We attempted to measure this in *C. diffusa* and *C. stoebe* by assuming random selection of flower heads by each insect. If *Urophora* and *Larinus* presence in flower heads was independent, then one would expect the number of flower heads containing both species to be the product of their individual abundance. For example, if 50% of the flower heads contained *Urophora* and 40% contained *Larinus*, then the expected number of flower heads with both insects is  $0.5 \cdot 0.4 = 0.2$ , or 20% of flower heads. This procedure is biased because the observed *Urophora* numbers can be reduced by *Larinus*-induced mortality (Smith and Mayer, 2005). Given this mortality and disappearance of larvae, final *Urophora* numbers and therefore the number used to estimate co-infestation are lower than the original number of larvae in flower heads. However, because this procedure produces a lower estimate of co-infestation, the comparison of expected minus observed co-infestation provides a conservative assessment of *Larinus* effects on *Urophora*. While this model cannot distinguish consumption effects from avoidance effects, our procedures used in 2005 and 2006 to directly assess *Urophora* mortality by *Larinus* allowed us to distinguish between these two interpretations.

The relative abundance of *S. jugoslavica* and *C. achates* was measured by excavating roots of *C. diffusa* in May to mid June of each year at the core monitoring site. Roots of bolting plants or large rosettes were sampled at random. Each root was sectioned and the numbers and type of larvae, pupae or adults within the roots were recorded.

Sample size varied among years, but annual estimates are composed of an average of at least 50 plants. By 2006 the lack of plants at the core monitoring site required that samples be obtained from diffuse knapweed in adjacent grasslands.

### 2.5.2. Plants

Numbers of diffuse knapweed rosettes, flower heads per stem and numbers of stems per  $m^2$  were obtained at the long-term monitoring site using procedures detailed in Seastedt et al. (2003). Cover estimates for *C. diffusa* and other common species and vegetation types for the 1997–2005 interval are reported in Bush et al. (2007). Initially, collections were made within two separate areas of the monitoring site (termed the release and reference areas; Seastedt et al., 2003). As insects eventually colonized the reference plots, we have pooled all results into a single site estimate. An average of 60 plants were sampled to obtain an average of flower heads per plant, and  $1 m^2$  quadrats were used to estimate stem densities from 60 samples taken from random locations within the two areas of the monitoring site. Seed production (number of seeds produced per  $m^2$ ) was estimated at the monitoring site by the calculation:  $(\text{stems} \cdot m^{-2}) \times (\text{flower heads} \cdot \text{stem}^{-1}) \times (\text{seeds} \cdot \text{flower head}^{-1})$ . Only a single value (i.e., no error estimate) was obtained per year. Procedures for monitoring *C. diffusa* at these sites consisted of either total counts of knapweed along a fixed,  $60 m^2$  transect at each site or censused using point-intercept and presence and absence counts along a 50 m transect (Seastedt et al., 2003).

## 2.6. Spotted knapweed: sampling procedures

### 2.6.1. Insects

Procedures for sampling insects in flower heads of spotted knapweed were similar to those of diffuse knapweed. Three flower heads per plant on 30 plants were counted, and in 2005 we switched to selecting individual flower heads at the tops, middle and bottoms of randomly selected plants. Only mature flower heads were sampled. Interactions among insects and seed production was again tested using Pearson's Correlation procedure, and the same model to look at co-occupancy by insects in diffuse knapweed flower heads was also used for spotted knapweed.

The relative abundance of *S. jugoslavica* and *C. achates* was measured by excavating roots of *C. stoebe* in May to early July. Root-feeding insects were not systematically sampled at the *C. stoebe* site until 2006. Roots of bolting plants or large rosettes were sampled at random. Each root was sectioned and the numbers and type of larvae, pupae or adults within the roots were recorded. The estimate was obtained from a sample of 50 plants.

### 2.6.2. Plants

Numbers of knapweed rosettes, flower heads per stem and numbers of stems per  $m^2$  were obtained at the single spotted knapweed site. Counts of flower heads on 30 stems

were obtained for flower heads per plant, and  $0.5 m^2$  quadrats were used to estimate stem densities from 30 quadrats taken from a fixed transect at the *C. stoebe* site. Seed production (number of seeds produced per  $m^2$ ) was estimated at this site by the calculation:  $(\text{stems} \cdot m^{-2}) \times (\text{flower heads} \cdot \text{stem}^{-1}) \times (\text{seeds} \cdot \text{flower head}^{-1})$ . Only a single value (i.e., no error estimate) was obtained at this site per year.

## 3. Results

Temperatures and precipitation for the study region during the study interval was typical of a continental climate, i.e., highly variable. The thirty-year (1971–2000) average precipitation for the Boulder area is 51.4 cm (NOAA, 2006). We used a water-year (October–September) period to evaluate the effect of annual precipitation on *Centaurea* and insect abundances. The seven-year interval in which we have the most data on insects (October 1999–September 2006) produced annual precipitation that was 82, 94, 61, 115, 122, 91, and 76% of the annual average, respectively. The 2002 season was particularly dry (61% of average), with the 2004 season particularly wet (122% of average).

### 3.1. Diffuse knapweed populations

The *C. diffusa* at our long-term monitoring site was locally extirpated during the 10-year interval (Fig. 1). No rosettes or flowering stems were observed on our permanent collection areas in 2006 (or in 2007, data not shown), although a few rosettes and flowering stems could be found nearby. The short-lived recovery in knapweed densities following the drought year of 2002 was more evident from the size of plants rather than the stem counts shown here. Average flower heads per plant increased from a pre-drought high of about 50 per plant to 160 per plant in 2004 (data not shown). Even so, seed production of *C. diffusa*, estimated at over 4000 seeds per  $m^2$  in 1997, never exceeded 300 per  $m^2$  after 2000 (Table 1). Extensive defoliation of these plants accompanied by low seed production per flower head, characterized the declining population of *C. diffusa*.

The decline shown in Fig. 1 was repeated at several other sites where the same insects had been released before

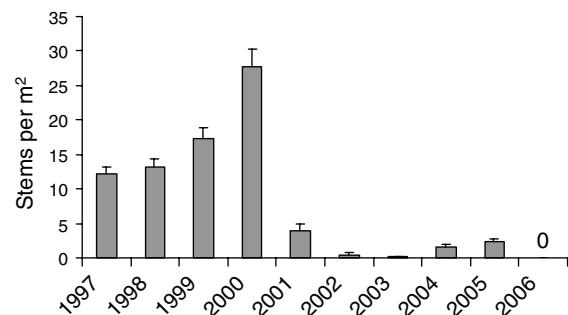


Fig. 1. Stem densities of diffuse knapweed from the long-term monitoring site near Boulder, Colorado. Values are means and standard errors from  $60 m^2$  counts obtained randomly over a 10 ha area.

Table 1

Insect abundances on *C. diffusa* and seed production at core release site, 1997–2006. Values are the average number of insects found in flower heads (*Urophora*, *Larinus*) or in roots (*Cyphocleonus*, *Sphenoptera*)<sup>a</sup>

Year	Relative abundance				Seed production (number m <sup>-2</sup> )
	<i>Urophora</i>	<i>Larinus</i>	<i>Cyphocleonus</i>	<i>Sphenoptera</i>	
1997	0.23	nd	nd	nd	4400
1998	0.48	nd	nd	nd	2535
1999	0.38	nd	nd	nd	2883
2000	0.28	0.54	nd	nd	1881
2001	0.30	0.42	0.33	0.22	61
2002	0.08	0.32	0.19	0.21	4
2003	0.26	0.69	0.02	0.46	0.1
2004	0.13	0.51	0.05	0.22	230
2005	0.51	0.41	0.40	0.08	10
2006	0.77	0.54	0.22	0.28	0.0

<sup>a</sup> nd, no data. Means based upon examination of at least 180 flower heads or 60 roots. 2006 data obtained from knapweed adjacent to original release site.

2001. At one site, densities of plants declined to zero in 2004, but then reappeared in modest densities in 2006, implying that the seed bank or reintroduction from off-site sources could re-establish the plant. The absence of any plants precluded the retention of insects, but adjacent off-site sources of *C. diffusa* allowed for all species to recolonize the knapweed. A third site has remained weed free since 2004, while five additional monitoring sites were weed free in 2006. While declines of *C. diffusa* were widespread, the plant remained common in the Front Range of Colorado, particularly on disturbed sites.

### 3.2. Insect abundance on diffuse knapweed

Estimates of the relative abundance of all biological control insects monitored at the long-term release site are shown in Table 1. Only *Urophora* species were routinely counted before 2000. No significant correlations between precipitation and relative abundance of *Urophora* were noted, and no significant correlations between the flower head densities of *Urophora* and the abundances of other insects after 2000 were observed. *Larinus* became locally common at the main monitoring site by 2000, but was not found at all sites along the entire 200 km gradient until 2004 (Seastedt et al., 2005).

Root-feeders became locally common at the release site by 2001. Survivorship of *C. achates* appeared to have been negatively affected by the 2002 drought, based upon censuses the following year (Table 1). In contrast, *S. jugoslavica* densities in *C. diffusa* declined following the relatively cool, wet year of 2004, and a comparison of densities of the two root-feeding insects suggested a marginally significant negative relationship (Pearson's correlation coefficient for annual mean values,  $R = -0.754$ ,  $P = 0.083$ ,  $n = 6$ ). The abundance of *S. jugoslavica* in roots was positively correlated with that of *Larinus* in flower heads (Pearson's correlation coefficient,  $R = 0.830$ ,  $P = 0.048$ ,  $n = 6$ ).

A correlation analysis for seeds, *Urophora* and *Larinus* obtained in the 2001–2006 interval from sites across the Front Range indicated that occupancy of flower heads by

*Larinus* had a stronger negative effect on seed number found in individual flower heads (Pearson's  $R = -0.38$ ,  $P < 0.0001$ ) than that of *Urophora* (Pearson's  $R = -0.09$ ,  $P < 0.0001$ ). *Larinus* was also negatively correlated with *Urophora* ( $R = -0.29$ ,  $P < 0.0001$ ). When average seed number per flower head is graphed as a function of insect presence, the effects of both insects, alone or in combination are evident for *C. diffusa* (Fig. 2).

More than one *Urophora* larva or pupa is often found in a single diffuse knapweed flower head. On rare occasions, more than one immature larva of *Larinus* was found in a flower head, but no more than one pupa or adult of *Larinus* was ever found in a single flower head of *C. diffusa*. Based on the assumption of a random selection model, the expected percentage of flower heads with both insects is shown in Table 2. The difference between observed numbers and the expected co-infestation obtained from all sites in the Front Range for the 2001–2005 interval indicated that the presence of *Larinus* in a flower head appeared largely to exclude its use by *Urophora* (Table 2). The 2006 results are more influenced by midsummer samples that contain developing larvae of *Larinus* yet to consume either seeds or *Urophora*.

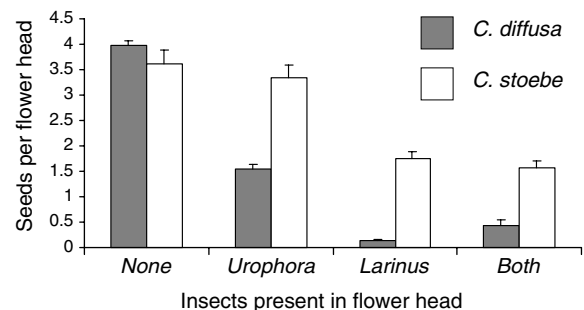


Fig. 2. Seed numbers in flower heads of diffuse and spotted knapweed containing no insects, *Urophora* only, *Larinus* only, or both insects. Results (means + 1 SE) shown for *C. diffusa* (shaded bars) are from 6242 flower heads examined from 2002 to 2006. Results for *C. stoebe* (clear bars) are from a count of 1862 flower heads obtained from 2002–2006.

Table 2  
Occupation of knapweed flower heads by *Urophora* and *Larinus*<sup>a</sup>

Year	Number of flower heads examined	Percent of flower heads with			
		<i>Urophora</i>	<i>Larinus</i>	Both	
				Observed	Expected <sup>b</sup>
<i>C. diffusa</i>					
2002	1257	15.8	45.7	1.0	7.2
2003	813	13.1	59.0	0.1	7.7
2004	1998	13.2	33.8	0.6	4.5
2005	909	39.6	42.1	2.6	16.7
2006	995	36.9	40.6	12.7 <sup>c</sup>	15.0
<i>C. stoebe</i>					
2002	296	20.2	84.1	11.8	17.0
2003	311	24.4	55.0	2.9	13.4
2004	429	50.1	9.8	6.2	4.9
2005	540	76.9 (65.7)	69.2	52.4 (30.7)	53.2
2006	288	31.2 (27.8)	54.2	12.8 (10.8)	16.9

<sup>a</sup> Estimates of flower head occupancy by live *Urophora* for 2005 and 2006 shown in parentheses. Data from previous years include only live *Urophora*.

<sup>b</sup> Assumes random selection by each insect; see text.

<sup>c</sup> Reflects relatively large numbers of flower heads counted in July, before consumption of *Urophora* by *Larinus* larvae. Prior to 2006, data were obtained later in growing season.

While a strong negative correlation occurs between *Larinus* and *Urophora* within individual flower heads, annual results obtained at the long-term monitoring site (Table 1) does not explain much of the variance in *Urophora* densities in flower heads among years. Using site means to test for differences in relative densities of *Urophora*, *Larinus* and seeds per flower head for the 2000–2006 interval, no significant temporal patterns were observed for seed production (Fig. 3). A significant reduction in *Urophora* densities after 2000 was observed. In contrast, *Larinus* had the lowest densities in 2000. Thereafter, however, densities show no correlations between the two insects. *Urophora* densities were lowest in flower heads in the relatively wet years of 2003 and 2004, whereas relative densities of *Larinus* were lower in 2000 compared to those observed in 2003 and 2006. No other yearly averages for *Larinus* were significantly different from one another (Fig. 3).

### 3.3. Spotted Knapweed population

The population of *C. stoebe* exhibited a seed production pattern similar to *C. diffusa*, with 4600 seeds per m<sup>2</sup> in 2003 declining to zero seeds per m<sup>2</sup> in 2006 (Table 3). The first seed production census occurred in 2003, which was a recovery year following the 2002 drought. Plants appeared vigorous, producing an average of about 120 stems per m<sup>2</sup> at our monitoring site (data not shown). In spite of adequate precipitation in 2004, seed production appeared to diminish, and by 2006 extensive defoliation by *Larinus* in conjunction with cattle grazing and reduced precipitation completely eliminated seed production at our monitoring site. Only 0.4 flowering stems m<sup>-2</sup> ( $\pm 0.29$  SE,  $n = 30$ ) were counted at the site. However, areas adjacent to the monitoring area exhibited flowering and seed production, and rosettes remained common, with 24.9 m<sup>-2</sup> ( $\pm 3.0$ , SE,  $n = 30$ ) rosettes found at the monitoring site in 2006.

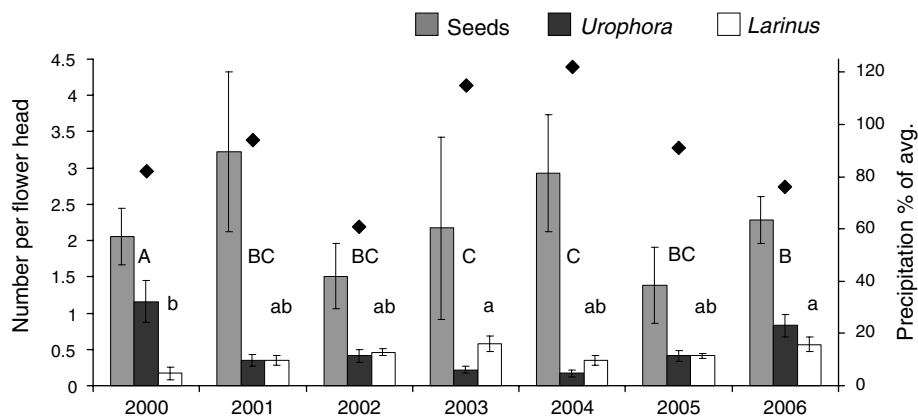


Fig. 3. Seeds, *Urophora* flies and *Larinus* weevils found in flower heads from diffuse knapweed sites, 2000–2006. Results (means + 1 SE) are for the average numbers observed at 4, 9, 17, 7, 11, 12, and 14 sites sampled in the 2000–2006 interval, respectively. Annual precipitation values (black points) are the percent of average water-year, measured from October–September. Different uppercase letters for *Urophora* and lowercase letters for *Larinus* indicate significant differences in relative densities of each species among years at  $P < 0.05$  (SNK test). Seeds per flower head did not show any temporal patterns.

Table 3  
Seeds, *Urophora*, and *Larinus* found in flower heads, and estimates of seed production for *C. stoebe* in Colorado

Year	Sample size	Seeds	Number per flower head <sup>a</sup>		Seed production (number m <sup>-2</sup> )
			<i>Urophora</i>	<i>Larinus</i>	
2002	294	1.46 (0.180)	0.30 (0.041)	0.89 (0.025)	No data
2003	311	4.26 (0.335)	0.43 (0.053)	0.63 (0.036)	4600
2004	429	3.06 (0.250)	1.15 (0.073)	0.10 (0.014)	1030
2005	540	2.15 (0.151)	1.81 <sup>b</sup> (0.065)	0.74 (0.018)	260
2006	288	1.80 (0.215)	0.57 <sup>b</sup> (0.064)	0.57 (0.032)	0

<sup>a</sup> Values are means and SE from flower heads from a single population.

<sup>b</sup> *Urophora* value includes 0.27 and 0.06 insects per flower head in 2005 and 2006, respectively, that were consumed by *Larinus*. Results obtained before 2005 based on live *Urophora* or pupae fragments.

The effect of insects on total seed abundance in individual flower heads of spotted knapweed is also not as strong as that observed in diffuse knapweed (Fig. 2). This five-year summary suggested that *Urophora* have very little direct effect on the seed production of spotted knapweed within the flower heads occupied by larvae, while the overall effect of *Larinus* and of both insects on a flower head scale was less than that observed for *C. diffusa*.

#### 3.4. Insect abundance on spotted knapweed

The relative abundance of *Urophora* and *Larinus* in flower heads of *C. stoebe* for the 2002–2006 interval indicated patterns similar but potentially more variable than those found in *C. diffusa* (Table 2 and Fig. 4). *Urophora* abundance ranged from a low of about 0.2 per flower head in 2002 to over 1.8 larvae per flower head in 2005, while *Larinus* numbers varied from about 0.1 larvae per flower head in the wet year of 2004 to almost 0.9 larvae per flower head in the dry year of 2002. Unlike diffuse knapweed, some of these flower heads contained two or more *Larinus*.

The negative relationships observed between seed numbers in flower heads and insect presence were not as strong as those observed in diffuse knapweed. An analysis of all flower heads examined over the 2002–2006 interval indicated a Pearson's correlation coefficient of  $-0.19$  between

seeds and *Larinus* ( $P < 0.0001$ ), a non-significant value of only  $-0.02$  between seeds and *Urophora*, and a negative relationship ( $R = -0.19$ ;  $P < 0.0001$ ) between *Larinus* and *Urophora*.

While a negative relationship between *Urophora* and *Larinus* is detectable at the flower head scale, an annual or within-year comparisons of average flower head occupancy of the two genera indicated that their relative abundances were not correlated. The Pearson's correlation coefficient for a comparison of average *Urophora* and *Larinus* abundance in flower heads from 15 collections made in 2005 and 2006 at this single site was  $0.07$  ( $P = 0.79$ ), while the value for annual means from five years of collection was  $0.48$  ( $P = 0.41$ ). While clearly non-significant, the unexpected result was that these correlations were positive.

The abundance of the root-feeders, *C. achates*, was quantified in 2006 for *C. stoebe*. An examination of roots of individual plants indicated that 34% ( $\pm 6.7\%$ , SE,  $n = 50$ ) contained at least one *C. achates* insect.

## 4. Discussion

### 4.1. Diffuse knapweed

Reports of large declines in the abundance of *C. diffusa* from Montana (Smith, 2004), Oregon and Washington

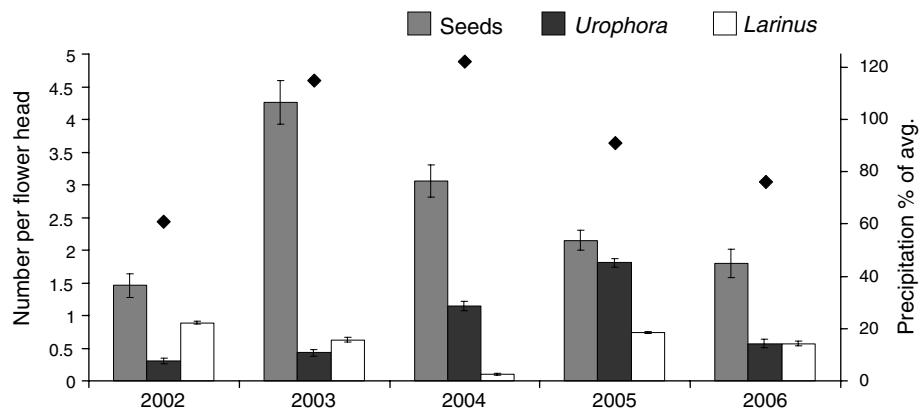


Fig. 4. Seeds, *Urophora*, and *Larinus* found in flower heads, from *C. stoebe* site in Boulder County, Colorado, 2002–2006. Results (means + 1 SE) are from a single population of spotted knapweed. Annual precipitation values (black points) are the percent of average water-year, measured from October–September. *Urophora* values include 0.27 and 0.06 insects per flower head in 2005 and 2006, respectively, that were consumed by *Larinus*. Results before 2005 include only live *Urophora*.

(Story and Coombs, 2004) and British Columbia (Myers, 2004) as well as reports from weed managers in Colorado suggest widespread control of this plant by the suite of biocontrol insects found on these sites. The common denominator would appear to be *Larinus* (presumably *L. minutus*), which is viewed as the keystone or ‘silver bullet’ control agent (Myers, 2004). Seedling success of diffuse knapweed at sites containing other, competing plant species in our region appears to require substantial seed inputs (Seastedt and Suding, 2007), and the seed production at our monitoring site, as influenced by plant survivorship under the insect regime reported here, was apparently not sufficient to maintain the population.

The contribution of root-feeders to the decline of *C. diffusa* remains unknown. At our monitoring site, dead and dying bolting *C. diffusa* were observed throughout the 2001–2006 study period, and roots of these plants almost always contained *C. achates*. If plant mortality preceded our root censuses, then we underestimated the relative abundance of these insects. We suspect that *C. achates*, in particular, has strong negative effects during drought years such as 2002 and again in 2006 when compensatory growth of plants was severely constrained by water limitation. The decline in abundance of this insect species following the 2002 drought may suggest high mortality of plants containing *C. achates*, or it may reflect low abundance of adequate-sized rosettes for egg deposition in late summer and fall of 2002.

LeJeune et al. (2005) demonstrated that *S. jugoslavica* and *C. achates* prefer to attack either different-sized *Centaurea* rosettes or rosettes found in nutrient-rich versus nutrient-poor habitats. Here, there was a marginal suggestion that the annual relative densities of the two species are negatively correlated, suggesting different climate responses. Selection of rosettes in different habitats and differential success under differing climates is consistent with the contention that the two species complement each other and collectively have a greater effect than either species alone, at least over multi-year intervals. We did find a positive correlation between *S. jugoslavica* and *Larinus* abundance, a finding attributed to both insects becoming relatively more abundant under similar climatic conditions.

Our results, in conjunction with those reported elsewhere, indicate that the biological control program that evolved over the last 40 years for *C. diffusa* has resulted in a successful outcome in terms of substantially reducing the economic and ecological concerns posed by this weed, and one that appears widespread if not universal. While few would disagree that the numbers of individual species releases for the invasive *Centaurea* species have been large and are probably in excess of the number of insect species needed to reduce densities of *C. diffusa*, the decline in abundance of *C. diffusa* results in little residual non-native plant or insect biomass to alter native food webs. Hence, for *C. diffusa*, the concerns about direct and indirect non-target effects of the biological control effort appear minimal, and its application appears warranted throughout North America.

#### 4.2. Spotted knapweed

Clearly, *Larinus* and *Urophora* appear able to better coexist in the larger flower heads of *C. stoebe* than in *C. diffusa*. However, when *Larinus* was abundant, numbers of *Urophora* observed in flower heads containing both species were reduced relative to the expected number based on a random selection model (Table 2). Using the flower head results for *C. stoebe* reported in Fig. 2, *Urophora* densities averaged 1.27 insects ( $\pm 0.05$  SE,  $n = 901$ ) in flower heads lacking *Larinus*, versus an average of 0.76 insects ( $\pm 0.04$  SE,  $n = 961$ ) found in flower heads containing *Larinus*. If this difference of 0.51 insects reflects a consumption value, then *Larinus* consumed about 40% of the *Urophora* they encountered. We estimated that this represents 15% of the total *Urophora* population in 2005, and 11% of the population in 2006. At our site, therefore, some of the indirect negative effects on food webs caused by *Urophora* abundance (Pearson and Callaway, 2006) are being reduced by the presence and activities of *Larinus*. However, since *Larinus* has a large over-wintering biomass (albeit in the soil), the species may be causing other alterations in food webs.

While *Larinus* appears to be a key component of biological control for *C. diffusa*, this species has yet to be reported as significant on *C. stoebe* in Montana (Smith, 2004; Story et al., 2006; Sturdevant et al., 2006), but does seem capable of high flower head attack rates on *C. stoebe* in British Columbia (Crowe and Bouchier, 2006). At this time there are no studies to indicate why *Larinus* has not assumed the dominance observed here at the Montana sites.

The question as to whether biocontrol insects released in North America can effectively reduce densities of *C. stoebe* across multiple ecosystem types remains unresolved. This species remains abundant throughout most of its introduced range. However, in the presence of the same biocontrols reported here, densities of spotted knapweed have been reported to decline in Minnesota (Cortile and Northrop, 2006). In Montana *C. achates* (Jacobs et al., 2006) or *Urophora* and *C. achates* (Corn et al., 2006; Story et al., 2006) have been reported to reduce densities of this plant. Unlike *Larinus*, the root-feeder *C. achates* does not directly compete with or consume *Urophora*, and the cumulative stress effects of the combined biocontrol agents may be additive or synergistic (e.g., McEvoy and Coombs, 1999). While we attribute the lack of flower heads and seeds at our *C. stoebe* site in 2006 to low precipitation and foliage herbivory by insects and cattle, effects of *C. achates* on this plant were suggested by the presence of stunted plants incapable of regrowth. Further, the decline in seed production of *C. stoebe* in 2004 and 2005, with 1030 and 260 seeds per  $m^2$ , supports the notion that the long-term reproductive output of the plant has been affected by the addition of *Larinus* and *C. achates*. These observations are consistent with the findings for *C. stoebe* populations obtained in Montana, which lacked large densities of *Larinus*. These results suggest that there may be more than one combination of two or more biological

control agents capable of reducing *C. stoebe* seed production and perhaps plant densities as well.

The negative relationship observed between *Larinus* and *Urophora* in diffuse knapweed is not as strong in *C. stoebe*. Crowe and Bouchier (2006) reported an antagonistic relationship between these species in *C. stoebe*, and that *Larinus* attempted to avoid flower heads containing *Urophora*. Those authors demonstrated that *Larinus* densities in flower heads of spotted knapweed were reduced in the presence of *Urophora*. Their results suggested that the inferior biological control agent, *Urophora*, could interfere with the efficacy of the more effective agent. Our results do not provide further insights into this competitive relationship, but argue that it may not be of strong ecological significance for two reasons. First, relative abundances of the two insects do not show correlations when averaged across collection dates or across years implying that other factors are driving relative densities. Second, the Montana findings suggest that *Larinus* may not be essential to *C. stoebe* declines provided *C. achates* is abundant (Story et al., 2006). In spite of relatively high rates of consumption of *Urophora* by *Larinus*, both insects appear to maintain substantial densities in flower heads at our site. The declining numbers of flower heads per unit area of both *Centaurea* species may be the best explanation for this pattern, i.e., the insects have become resource-limited. This resource limitation is the result of climatic constraints on the plants, the influence of the current and past populations of insects (i.e., the reduction in seed production and consumption of tissues), or, most likely, both of these factors.

## 5. Conclusions

The current procedures used to develop biological control programs include stochastic components (Myers, 1985; McEvoy and Coombs, 2000), and the problems with this approach are many (Tenner, 1996). Denoth et al. (2002, p. 29) concluded, “Taking into account... that each introduced control agent adds to the complexity of a natural system and may entail negative effects, the risks from introductions should be carefully balanced against the potential benefits.” Louda et al. (2005) noted that there are multiple outcomes with the release of biological control agents, and only a fraction of those outcomes are likely to be positive or neutral with respect to native species diversity. The *Centaurea*—biological control insect story in particular has been used as the model system in this discussion (Pearson and Callaway, 2005, 2006). Clearly, and as stated by many authors, the solution to problems such as the one addressed here are to find biological agents with strong host specificity and with large negative effects on the host species (Pearson and Callaway, 2005). Getting this right with the first release is important but may not always be possible because more than one type of herbivory may be required to produce sufficient negative effects on target plants; i.e., not all species in all environments are vulnera-

ble to single “silver bullet” species obtained from their native lands.

Clearly, the presence of *Urophora*, alone, has been insufficient to reduce densities of *Centaurea* species to acceptable levels (e.g., Myers and Bazely, 2003; Story et al., 2006). In contrast, *Larinus*, alone, may be able to control *C. diffusa* (Myers, 2004), but probably not *C. stoebe* (Smith, 2004; Story et al., 2006). With widespread reports of a successful outcome for *C. diffusa* and what may be an emerging success story for *C. stoebe*, the multiple releases of biological control agents against these two *Centaurea* species may represent a less-than-perfect but successful biological control effort. *Urophora* species were released before other agents and have dispersed throughout North America. Because these species persist in declining populations of the host plant, field tests of other combinations of biological control agents that exclude *Urophora* species, while of interest, are unrealistic from a management perspective. The combination of flower head insects and root-feeders appear to provide results consistent with a ‘cumulative stress’ effect on target species (Muller-Scharer and Schroeder, 1993), and the comparison of results reported here with the Montana findings by Story et al. (2006) suggest that this effect can be generated with different combinations of insects. All studies to date indicate that releases of multiple species of biological control agents do not lead to the ‘overcompensation responses’ observed when only the effects of a single biological control agent are measured (e.g., Thelen et al., 2005). Our study indicated that while some antagonistic interactions do occur among the multiple releases of biological control agents for *Centaurea* species, these are not sufficient to diminish what appeared to be a strong negative effect on the target plant species.

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