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Field cage assessment of interference among insects attacking seed heads of spotted and diffuse knapweed

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Abstract
Field studies were conducted to determine the competitive interactions between introduced biological control agents that attack the seed heads of spotted knapweed (*Centaurea stoebe* ssp. *micranthos*) and diffuse knapweed (*Centaurea diffusa*). Two weevils, *Bangasternus fausti* and *Larinus minutus* (Coleoptera: Curculionidae), were each paired with the previously established fly, *Urophora affinis* (Diptera: Tephritidae). Each species was released either alone or in pair-wise combinations inside screen cages placed over existing knapweed plants at six field sites in Montana and one in Oregon. *Larinus minutus* produced almost three times as many progeny on diffuse knapweed as on spotted knapweed. *Larinus minutus* reproduction was not affected by competition with *U. affinis*, but *U. affinis* reproduction was reduced by the presence of *L. minutus* (by 71% on spotted and 77% on diffuse knapweed). *Bangasternus fausti* reproduction generally was not affected by competition with *U. affinis*, nor was *U. affinis* affected by *B. fausti* on either host plant. There were extremely few cases of successful production of both weevil and fly in the same capitulum, which was probably because weevil larvae consume the developing flies. Both weevils increased the total proportion of seed heads infested on diffuse knapweed, and *B. fausti* increased it on spotted knapweed. However, the release of either weevil did not significantly further reduce seed production on either plant. The results and experimental design are discussed in light of the subsequent establishment and impact of these agents.

Keywords: *Biological control, herbivore, interspecific competition, weed,* Centaurea stoebe *ssp.* micranthos, Centaurea maculosa, Centaurea diffusa

Introduction

There is an increasing need to improve the efficiency of developing biological control agents that are safe and effective (Louda et al. 2003; Sheppard et al. 2003). In some cases, a single agent proves sufficiently effective to provide satisfactory control (Denoth et al. 2002), but when an established agent is not providing effective control, what should be done? Not only has the weed problem not been solved, but the ineffective agent may cause undesirable ecological effects merely by contributing to the food web (Pearson & Callaway 2003). The only reasonable remedy to such a situation is to find new agents that can result in effective control, regardless of whether they complement the first agent or outcompete it. This perspective adds new emphasis

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to the old question of whether releasing an additional agent will increase the level of control achieved by a previously established agent (Ehler 1990). However, measuring the interspecific interactions among agents attacking the same resource in the wild can be difficult because of differences in life history, seasonality, microhabitat requirements, overwintering strategies, etc. (Denno et al. 1995; Reitz & Trumble 2002). For biological control of weed projects, such studies are often difficult or impossible to do in the land of origin, where the target weed populations are usually low. The alternative is to conduct experiments under containment conditions; however, designing such an experiment may not be as straightforward as it first appears. In this paper we analyze the results of an unpublished field experiment conducted by the late Sara Rosenthal to evaluate the prospects of two new agents that were thought likely to compete with an established agent. In light of the increasing need to predict efficacy of prospective agents and to minimize the number of species introduced (Balciunas 2004), it is important to learn how such experiments may be better designed.

Most critical to designing an effective experiment is understanding the ecology, life history and behavior of the target plant and the natural enemies. Spotted knapweed (*Centaurea stoebe* L. ssp. *micranthos* (Gugler) Hayek [Asteraceae], but often reported as *Centaurea maculosa* Lamarck in North America [Ochsmann 2001]) and diffuse knapweed (*Centaurea diffusa* Lamarck) are important rangeland weeds in the northwestern third of the continental United States (Roché & Roché 1999; Sheley et al. 1999; Story 2002). Both plants are invasive aliens that were accidentally introduced from Europe and have been targeted for classical biological control (Maddox 1982; Rees et al. 1996). The plants have similar biologies, but spotted knapweed produces larger capitula (flower heads; 1.2 cm diameter) that produce more seed (about 26 per capitulum) than diffuse knapweed (0.8 cm diameter; 12 seeds) (Watson & Renney, 1974). Spotted knapweed usually has fewer capitula per plant (about 16) than diffuse (about 74) (Watson & Renney 1974).

Two tephritid flies that attack capitula, *Urophora affinis* (Frauenfeld) and *U. quadrifasciata* (Meigen) (Diptera: Tephritidae), became established in Montana in 1973 and 1980, respectively (Story 1995a). Both the *Urophora* files established well in Canada and reduced seed production by up to 95% (Harris 1980), but this was generally considered insufficient for effective management of diffuse knapweed (Harris 1980; Myers et al. 1990; Powell 1990). Two weevils that attack capitula, *Bangasternus fausti* (Reitter) and *Larinus minutus Gyllenhal* (Coleoptera: Curculionidae), were approved for introduction in 1990 and 1991, respectively (Rees et al. 1996). Although there was good reason to expect that increasing the number of insect species would increase overall impact on the plant (Harris 1990), there was a risk that a subsequently released species might reduce the impact of the previously established gall flies (Denoth et al. 2002).

Files

Urophora affinis is multivoltine and overwinters as mature larvae in knapweed capitula (Berube 1980; Rees et al. 1996). Adults appear in June and oviposit on immature capitula. Each larva transforms a flower ovary into a hard gall, and many galls can occur inside one capitulum (each capitulum contains many individual florets, each of which can produce one seed). Ovipositor damage can cause some florets to abort

(Shorthouse 1990), and florets adjacent to developing galls may abort (Harris 1980). *Urophora quadrifasciata* has a similar life history to *U. affinis,* except that it prefers to oviposit on larger, more mature flower buds; it produces a gall with a thin, papery wall (Berube 1980). *Urophora affinis* populations tend to displace *U. quadrifasciata* because they oviposit in capitula first and infestation slows capitulum development, leaving fewer capitula suitable for oviposition by *U. quadrifasciata* (Berube 1980*;* Myers & Harris 1980). Both species can produce two to three generations in Montana (Story et al. 1992*)*. Fecundity of *U. affinis* is about 120 eggs (Zwolfer 1970), and development time is about 5–6 weeks (Harris & Shorthouse 1996). Because *U. affinis* was the dominant species attacking knapweed at the research sites, the experiment used only this species.

Weevils

Bangasternus fausti is a univoltine insect that overwinters as an adult in debris on the soil or rarely in capitula (Sobhian et al. 1992; Rees et al. 1996). Adults appear on plants in early May, and eggs are deposited externally on or below immature knapweed capitula. In laboratory cages, average longevity of active females is 33–58 days, and average fecundity is 67–106 eggs (Sobhian et al. 1992). Oviposition in the field occurs from mid-May to mid-August, peaking in late June to early July. Larvae tunnel up the stem and/or into the capitulum, where they feed on developing seeds. Pupation occurs inside the capitulum, and adults usually emerge during the summer. Development time is about 4–5 weeks.

Larinus minutus is also univoltine, and adults overwinter in debris on the soil (Groppe 1990; Kashefi & Sobhian 1998; Lang et al. 2000a) and under knapweed rosettes (G. Piper, personal communication). Adults appear on knapweed plants in May and feed on leaves; when flowers open they feed inside them. Adult feeding is necessary for ovariole development. Females oviposit into open flowers from June into August. In the laboratory, females can oviposit for up to 11 weeks, and average fecundity is 85 eggs (Kashefi & Sobhian 1998). Larvae feed by chewing on florets and developing seeds, pupate inside the capitulum, and emerge later in the same summer.

The purpose of this study was to measure competitive interactions between *B. fausti* or *L. minutus* and the previously established fly, *U. affinis*, and determine their potential impact on seed production of spotted and diffuse knapweed.

Methods

Field experiments were conducted in 1992 at five spotted knapweed sites in Montana: Dupuyer, Pondera county; Grassrange, Fergus county; Hruska, Fergus county; Seilsted, Fergus county; Woods, Chouteau county; and two diffuse knapweed sites: Heppner, Morrow county, Oregon; and Ware, Fergus county, Montana. Sites with heavy infestations of knapweed were selected with the help of cooperators from state and federal land management agencies. *Urophora affinis* and *U. quadrifasciata*, were widely established in Montana (Story & Nowierski 1984; Story 1985) and were present at all the study sites.

In April, exclosure fencing was erected around several sites, where it was necessary to exclude cattle. Bottomless cubic cages (61-cm sides) made of aluminum screen (1.2-mm openings) were placed over existing knapweed plants and anchored with

rebar stakes. The cages were designed to exclude any previously established knapweed insects and to retain the experimentally released insects. Dirt was shoveled around the base of all cages to help seal them. All knapweed capitula from the previous season were removed from within each cage to prevent the emergence of overwintering *Urophora* spp. later that spring.

Treatments consisted of releasing different combinations of capitulum-attacking insects: *B. fausti* alone; *L. minutus* alone; *U. affinis* alone; *B. fausti* and *U.* affinis; *L. minutus* and *U.* affinis; and no insects were released in the check cages. These treatments were randomly assigned to the cages at each site. All treatments at a site usually had four replicates, but the treatments varied somewhat among the locations because of logistics (Table I). Both species of weevils were collected in Greece, shipped to the Montana State University Insect Quarantine facility, where they were identified and screened for pathogens before release in the field experiments. The number of adult insects released per cage was 16 *B. fausti,* 16 *L. minutus,* or 12 *U. affinis.* These numbers were based on rough estimates of fecundity, an allowance for higher mortality in the confines of a cage, and the anticipated average number of capitula per cage. The sex of the beetles was not determined, but the sex ratio was presumed to be about 1:1, which is close to that found in field observations (Sobhian et al. 1992; Kashefi & Sobhian 1998). At each site, adult *U. affinis* were collected from neighboring knapweed plants, and six females and six males were released in each appropriate cage. Releases of beetles and flies occurred 30 June through 23 July at the various research sites.

Individual capitula on knapweed plants inside each cage were bagged between 3 August and 28 August to retain seeds and any emerging insects. Bags (5-cm square) made of nylon tulle were placed over individual capitula and attached to the stem using plastic Gripper Ties™. Up to 100 capitula per cage (depending on availability) were randomly chosen from all portions of the plants to be bagged. Bagged capitula were collected in late September. Each capitulum was sealed in a 30-mL plastic cup and held in a refrigerator (5°C) for at least 3 months, after which the emerged insects were recorded, and the capitula were dissected to count unemerged insects and seeds. Seeds were classified as being mature or immature (incomplete development).

Table I. Number of cage replications for each of the treatment combinations at the different sites.

	Treatment[a]					
Site	C	B	L	U	BU	LU
Spotted knapweed						
Dupuyer	4	–	4	4	–	4
Grassrange	4	3	3	4	4	3
Hruska	8	8	3	4	–	4
Seilsted	4	3	–	4	4	–
Woods	4	4	4	4	4	4
Diffuse knapweed						
Heppner	4	4	4	4	4	4
Ware	4	4	4	4	4	4

[a]C, check (no release); B, release *Bangasternus fausti;* L, release *Larinus minutus;* U, release *Urophora affinis;* BU, release *B. fausti* and *U. affinis;* LU, release *L. minutus* and *U. affinis.*

Because the number of capitula present in each cage was not controlled, we analyzed both the number of progeny and the proportion of capitula infested. Total numbers of insects and seeds per cage were divided by the number of capitula that were collected from the cage and multiplied by 100 to standardize for minor cage-to-cage variation in sample size. Count data were transformed by square root of $(Y+0.5)$ for analysis of variance (ANOVA). Specific hypotheses were tested by using contrasts. Proportion data were analyzed by chi-square tests.

Results and discussion

Because of logistics, not all release treatments were applied at all study sites (Table I). At several sites *Urophora* spp. heavily contaminated non-*Urophora* treatment cages (Dupuyer, Hruska and Heppner). This indicates that we failed to remove all of the naturally infested capitula (probably because they broke off during winter and were hidden in leaf litter) from the cages prior to the start of the experiment. This left three useful sites for spotted knapweed (Grassrange, Seilsted and Woods) and one site for diffuse knapweed (Ware) to analyze interspecific interactions. The mean number of bagged capitula that were collected from each cage did not differ with respect to treatment at any site; however, the overall numbers were lower at the Woods site (84.4 ± 3.4 SH) than at the other sites (Grassrange 95.5 ± 1.3, Seilsted 98.2 ± 0.7, Ware 96.7 ± 1.0).

Insect establishment

Urophora affinis established at all release cages at all seven sites (treatments U, LU and BU). *Larinus minutus* established at 69% of spotted knapweed cages and 100% of diffuse knapweed cages at the seven sites (treatments L and LU; $\chi^2 = 6.21$, $df = 1$, $P < 0.025$). Some European studies reported that the insect more commonly infests spotted knapweed than diffuse knapweed (Groppe, 1990; Groppe et al. 1990; Jordan 1995) although more were reared from field-collected capitula of diffuse knapweed than spotted knapweed in Greece (Groppe 1988). *Bangasternus fausti* established at 65% of spotted knapweed cages and 81% of diffuse knapweed cages at the seven sites (treatments B and BU), but the difference was not significant. The fact that the two weevils did not establish at all cages, suggests that either they were old, in poor condition (after collection and shipment from Europe), or susceptible to predation inside the cages. *Larinus minutus* adults generally survive 5–14 weeks in the laboratory (Groppe 1990) and *B. fausti* for 4–8 weeks, but both species usually first appear about 4 weeks before the knapweed flower buds form (e.g., *B. fausti* on the first week of May in Greece) (Sobhian et al. 1992). Thus, insects released in July would be old, and therefore would probably not oviposit very much. The subsequent successful establishment of both these species (Lang et al. 2000b; E. Coombs, unpublished data) tends to support the hypothesis that the weevils used in this experiment were old or in poor condition.

Host plant suitability

Urophora affinis infested $48 \pm 5\%$ (SE) of spotted and $31 \pm 11\%$ of diffuse knapweed capitula in cages where it was the only insect released (not significantly different). *Larinus minutus* infested $11 \pm 3\%$ of spotted and $30 \pm 4\%$ of diffuse knapweed capitula

in cages where it was the only insect released (ANOVA, $F_{(1,\ 9)} = 15.0$, $P < 0.004$). *Bangasternus fausti* infested 15 ± 6% of spotted and 9 ± 2% of diffuse knapweed capitula in cages where it was the only insect released (not significantly different). Therefore, only *L. minutus* performed significantly differently with respect to host plant, both establishing at a higher proportion of cages and infesting a higher proportion of capitula on diffuse knapweed.

The number of insects produced per infested capitulum was determined from analyzing only capitula from which that insect species was recovered, that came from cages in which only that species had been released. The average number of *U. affinis* produced per infested capitulum was 22% greater on spotted knapweed than on diffuse knapweed (2.91 ± 0.07 [SE] vs. 2.39 ± 0.08; $F_{(1,\ 1069)} = 17.1$; $P = 0.0001$), which may simply be a reflection of the larger size of the capitula of spotted knapweed (uninfested capitula of spotted knapweed had 18.5 ± 0.3 SE seeds per capitulum versus 11.6 ± 0.4 for diffuse knapweed). However, the number of *B. fausti* (1.02 ± 0.02) and *L. minutus* (1.02 ± 0.01) per capitulum did not differ with respect to host plant. *L. minutus* and *B. fausti* were usually solitary insects, whereas up to 16 *U. affinis* larvae were found in a capitulum.

Overall, *L. minutus* produced almost three times as many progeny per 100 capitula on diffuse knapweed (30.9) as on spotted knapweed (11.1) (based on comparison of L treatment cages; Table II). Reproduction by *B. fausti* (9.6 vs. 21.5) and *U. affinis* (74.7 vs. 133.5) did not differ significantly with respect to the two host plants.

Insect reproduction

Reproduction by *L. minutus* was not affected by the simultaneous presence of *U. affinis* on either spotted knapweed (L vs. LU treatments; Figure 1a) or diffuse knapweed (Figure 1b). Reproduction by *U. affinis* was reduced 71% by competition

Table II. Number of insect progeny per 100 capitula that were exposed to only one insect species (mean ± SE).

	Treatment[a]		
Site	B	L	J
Spotted knapweed			
Dupuyer	–	2.2 ± 1.0[b]	160.5 ± 32.8
Grassrange-2	4.0 ± 2.1	7.0 ± 3.8	153.6 ± 14.2
Hruska	3.3 ± 1.2[b]	3.1 ± 1.0[b]	113.5 ± 57.9
Seilsted	0.4 ± 0.4[b]	–	91.9 ± 26.3
Woods	34.6 ± 5.5	14.1 ± 4.5	142.8 ± 54.1
Mean[c]	21.5 ± 6.9 a	11.1 ± 3.1 a	133.5 ± 17.1 a
Diffuse knapweed			
Heppner	3.0 ± 1.4[b]	14.6 ± 2.7[b]	83.5 ± 23.8
Ware	9.6 ± 2.4	30.9 ± 3.8	65.9 ± 30.6
Mean[c]	9.6 ± 2.4 a	30.9 ± 3.8 b	74.7 ± 18.3 a

[a]B, number of *Bangasternus Fausti* in B release cages; L, number of *Larinus minutus* in L cages; and U, number of *Urophora affinis* in U cages (see Table I).

[b]Sites where some B or L treatment cages were excluded from analysis because of contamination by *Urophora* spp.

[c]Comparison of spotted and diffuse knapweed; means with the same letter in the same column are not significantly different; one-way ANOVA, $P \leq 0.05$; contaminated cages were excluded).

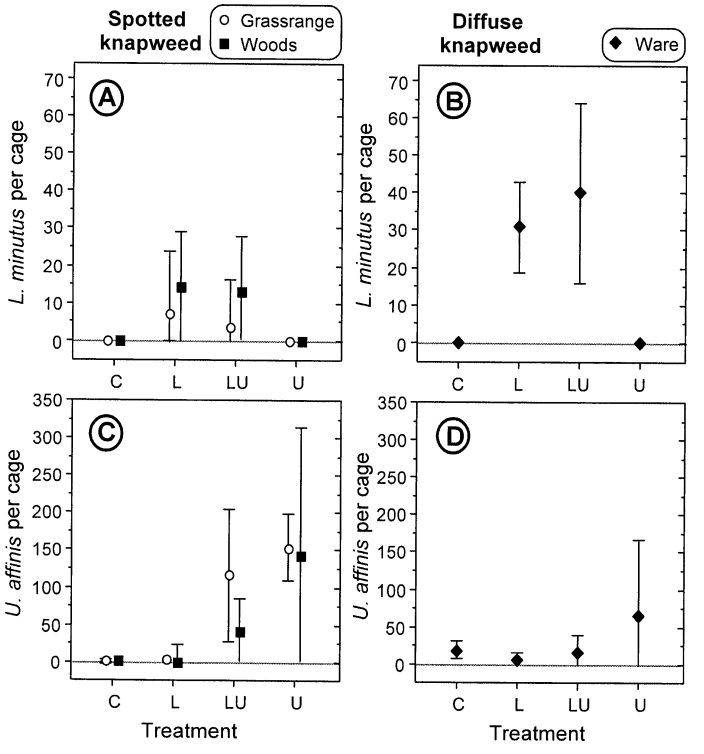

Figure 1. Reproductive interactions of *Larinus minutus* and *Urophora affinis* on spotted and diffuse knapweed (mean number of progeny per 100 capitula ± 95% CI). C, check (no release); L, release *L. minutus;* LU, release *L. minutus* and *U. affinis;* and U, release *U. affinis.*

with *L. minutus* on spotted knapweed at Woods (contrast of U vs. LU treatments; $F_{(1)} = 8.86$, $P = 0.012$; Figure 1c), where *L. minutus* reproduction was relatively high, but not at Grassrange, where *L. minutus* reproduction in the LU cages was low. Reproduction by *U. affinis* was reduced 77% by competition with *L. minutus* on diffuse knapweed ($F_{(1)} = 5.88$, $P = 0.033$; Figure 1d).

Reproduction by *B. fausti* on spotted knapweed was reduced by the presence of *U. affinis* by 85% at Grassrange (B vs. BU treatments; $P(1) = 8.2$, $P = 0.015$; Figure 2a), but not at the Woods or Seilsted sites, nor on diffuse knapweed (Figure 2b). At Grassrange and Seilsted, *B. fausti* infestation rates were extremely low, even in the absence of *U. affinis.* The biological significance of the 85% reduction at Grassrange is dubious because the infestation rates were so low (4.0 vs. 0.6 insects per 100 capitula). The strongest evidence for lack of impact of *U. affinis* on *B. fausti* reproduction is at Woods, where infestation rates of both insects were high. Reproduction by *U. affinis* was not reduced by competition with *B. fausti* on either plant at any of the sites (U vs. BU treatments; Figure 2c and d).

Proportion of capitula infested

The infestation rate of *L. minutus* was not affected by the presence of *U. affinis* on either spotted knapweed or diffuse knapweed (L vs. LU; Figure 3a, b, c). Infestation by *B. fausti* was not affected by *U. affinis* on spotted knapweed at Woods, nor on

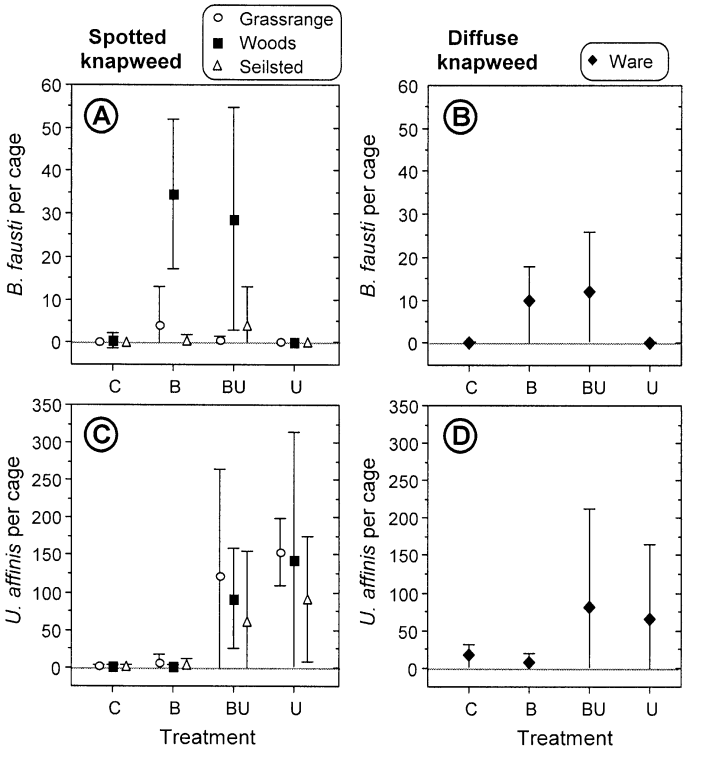

Figure 2. Reproductive interactions of *Bangasternus fausti* and *Urophora affinis* on spotted and diffuse knapweed (mean number of progeny per 100 capitula ± 95% CI). C, check (no release); B, release *B. fausti;* BU, release *B. fausti* and *U. affinis;* and U, release *U. affinis.*

diffuse knapweed at Ware (B vs. BU; Figure 3e and f). *Bangasternus fausti* decreased 85% at Grassrange and increased 975% at Seilsted (Figure 3d and g); however, at both these sites *B. fausti* numbers were very low (Figure 2), suggesting that the observed differences are not biologically significant. On spotted knapweed, infestation by *U. affinis* decreased 31% in competition with *L. minutus* at Grassrange and 68% at Woods (U vs. LU; Figure 3a and b). On diffuse knapweed, infestation by *U. affinis* decreased 71% in competition with *L. minutus* (Figure 3c). Presence of *B. fausti* decreased infestation rate of *U. affinis* on spotted knapweed 29% at Woods and 29% at Seilsted, but not at Grassrange, nor on diffuse knapweed at Ware (U vs. BU; Figure 3d, e, f, g). In general, these results showed the same patterns as the reproduction data presented above.

There were only eight cases of simultaneous infestation of capitula by *B. fausti* and *U. affinis* out of 839 infested capitula that had been exposed to the two species, and only three cases of simultaneous infestation by *L. minutus* and *U. affinis* out of 816 capitula. Thus it appears that when *L. minutus* attacks a capitulum, it usually kills any developing *U. affinis.* This is probably also the case for *B. fausti,* though adverse impact on *U. affinis* reproduction was generally limited because of the low weevil infestation rates that occurred in our study.

Total infestation rate (infestation of capitula by any insect) is another way to examine the possible interference between two species (Figure 4). Adding *L. minutus*

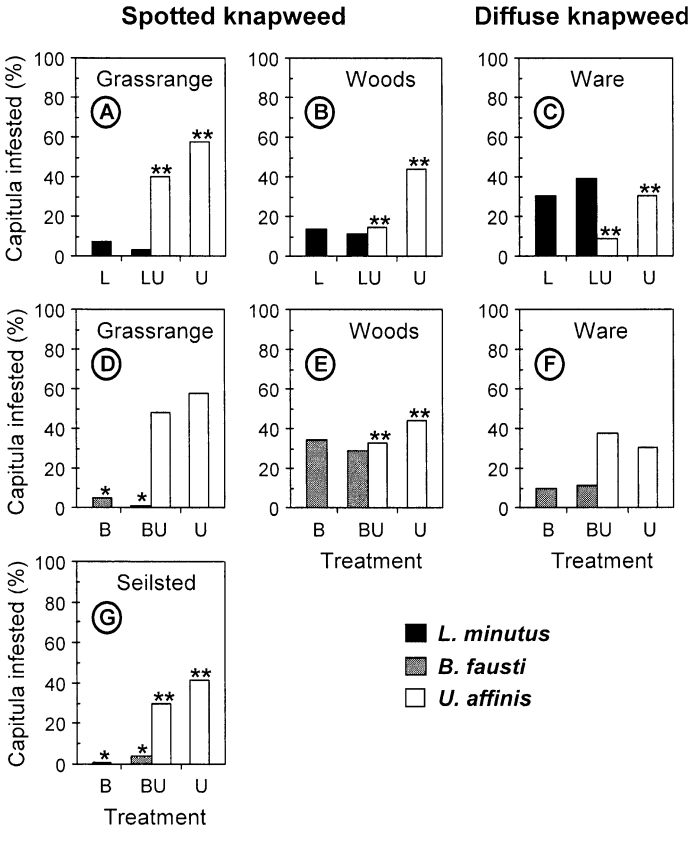

Figure 3. Effect of interspecific interactions on proportion of knapweed capitula infested by each species of insect (significance of difference between columns of the same pattern in the same group; $*p < 0.05$; $**p < 0.01$; chi-square tests).

to *U. affinis* increased total infestation rate on diffuse knapweed at Ware (U vs. LU; Figure 4c). However, on spotted knapweed, adding *L. minutus* to *U. affinis* decreased the total infestation rate at both Grassrange and Woods (U vs. LU; Figure 4a, b). This suggests that this weevil interferes with the fly and reduces its impact on spotted knapweed. Adding *B. fausti* to *U. affinis* increased total infestation rate on diffuse knapweed at Ware and on spotted knapweed at Woods, but not at Grassrange or Seilsted, where *B. fausti* attack was very low (U vs. BU, Figure 4d, e, f and g). Adding *U. affinis* to either of the weevils increased total infestation rate on either plant at all the sites (L vs. LU or B vs. BU). If the study had been conducted earlier in the season, with correspondingly younger weevils and plants, then we would expect higher attack rates by the weevils and probably stronger interspecific competition. However, there is no reason to expect this to change the general conclusions that both weevils increase total infestation on diffuse knapweed and that *B. fausti* increases it on spotted knapweed. The interference of *L. minutus* on spotted knapweed deserves further investigation because our experiments were conducted under confined conditions, which may have forced the weevils to damage capitula or otherwise interfere with *U. affinis* more than they would under unconfined conditions.

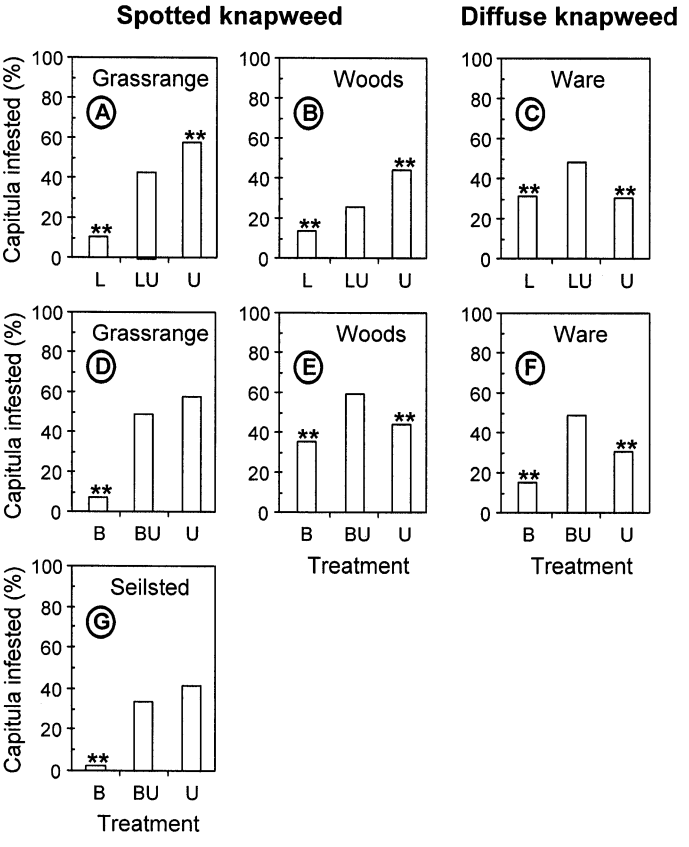

Figure 4. Effect of interspecific interactions on total infestation of knapweed capitula (significance of difference between releasing one species or both species at each site, e.g. L vs. LU or U vs. LU; $*p < 0.05$; $**p < 0.01$; chi-square tests).

Seed reduction per cage

There was no treatment effect or site-by-treatment interaction on the proportion of seeds that were mature, although there were differences among the sites (3.2 ± 0.8 [SE], 7.7 ± 3.0, 20.8 ± 4.4, and $9.4 \pm 4.4\%$ at Grassrange, Woods, Seilsted and Ware, respectively). Because only mature seeds can germinate, and are therefore important for managing the weed population, we present only those data here.

On spotted knapweed, *L. minutus* reduced production of mature seed by 95% at Grassrange (L vs. C treatments; $F_{(1,\ 15)} = 7.5$, $P = 0.015$), but the 61% decrease at Woods was not statistically significant (Figure 5a and b). *Bangasternus fausti* reduced seed production 99% at Grassrange (B vs. C; $F_{(1,\ 16)} = 8.29$, $P = 0.012$) and 99% at Seilsted ($F_{(19,\ 11)} = 9.2$, $P = 0.011$), but the 87% reduction at Woods was not statistically significant. It is surprising that *L. minutus* and *B. fausti* appeared to have such a large impact at Grassrange because they produced progeny in only 7 and 4%, respectively, of capitula (Figure 3a and d). Perhaps these insects cause sterility of flowers without producing larvae. *Larinus minutus* might cause such damage by adult feeding on flower structures and *B. fausti,* by larval tunneling followed by early larval death, but neither type of damage has been quantified in the literature.

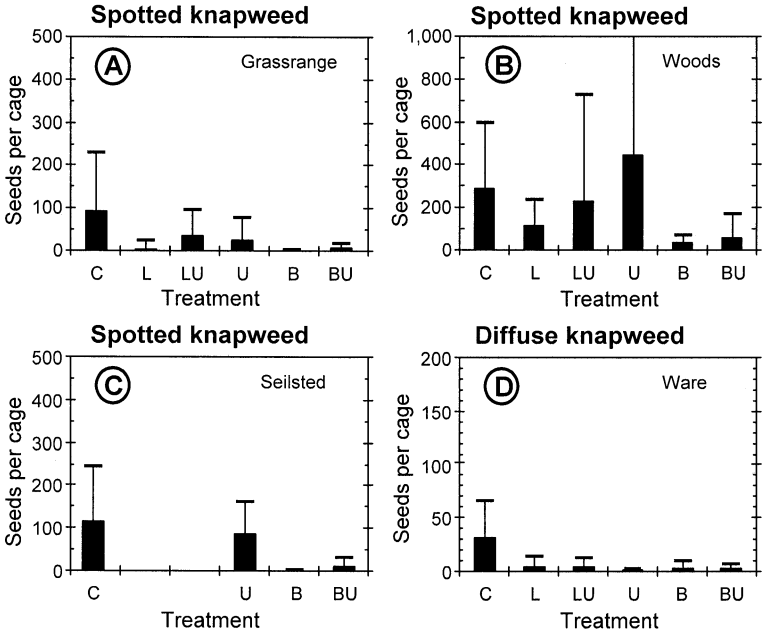

Figure 5. Effect of releasing various combinations of insects on the production of mature knapweed seeds in field cages (includes uninfested capitula).

Urophora affinis reduced seed production 77% at Grassrange (U vs. C; $F_{(1,\ 15)} = 5.00$, $P = 0.041$; Figure 5a), but not at Seilsted or Woods, where a slightly lower percentage of capitula were infested. The simultaneous release of two species of insects did not significantly further reduce seed production of spotted knapweed at any site. However, *B. fausti* plus *U. affinis* was lower than *U. affinis* alone on spotted knapweed sites when the three sites were combined and analyzed using 'site' as a block ($F_{(1,\ 34)} = 4.19$, $P = 0.048$). Percent reduction of mature seeds (from U to BU treatment) was 81% for the three sites (71, 85 and 87% at Grassrange, Seilsted and Woods, respectively).

On diffuse knapweed, *L. minutus* reduced seed production by 87% (L vs. C; $F_{(1,\ 18)} = 19.7$, $P = 0.0003$), *B. fausti* reduced it by 93% (B vs. C; $F_{(1,\ 18)} = 22.8$, $P = 0.0002$), and *U. affinis* reduced it by 98% (U vs. C; $F_{(1,\ 18)} = 25.06$, $P = 0.0001$) (Figure 5d). The simultaneous release of two species of insects did not significantly further reduce seed production of diffuse knapweed.

In this experiment, *L. minutus* and *B. fausti* each appeared to cause more reduction of spotted knapweed seed than that caused by *U. affinis* alone (U vs. L and U vs. B; Figure 5a, b, c); however, these differences were not statistically significant, except for the 99% difference between B and U treatments at Seilsted ($F_{(1,\ 11)} = 5.03$, $P = 0.046$). Infestation rates by the weevils were always low (always less than 40% of capitula and often less than 10%) (Figure 3). If either of the weevils were to cause higher rates of infestation, then they would be expected to have more impact on seed production and perhaps interfere more with *U. affinis*.

Seed production

Spotted and diffuse knapweed are outcrossing species, dependent on insect pollinators (Harrod & Taylor 1995). The number of mature seed per capitulum in the check cages was much lower (Table III) than that reported for unconfined plants; 26 for spotted knapweed and 12 for diffuse (Watson & Renney 1974). The experimental cages presumably excluded most insect pollinators, and there was no attempt to artificially pollinate the knapweed plants because of the remoteness of the sites. In a separate experiment conducted in 1993 to compare seed production of plants in cages with or without lids, the number of mature seeds increased by 2–9-fold when the cages did not have a lid (unpublished data; ANOVA on uninfested capitula in check cages; nine sites tested, each with $P = 0.0001$). This suggests that the cages reduced pollination of knapweed flowers, which may also have adversely affected the infestation rates and reproduction of the capitulum-feeding insects.

Seed reduction in infested capitula

The direct impact of insect infestation on the production of mature seeds was determined by comparing capitula that were infested by a single species to those that were not infested. Capitula that produced neither mature seed nor insects were excluded from analysis, based on the assumption that they were not pollinated. *Larinus minutus* reduced seed production of spotted knapweed by 79% at Grassrange but not at Woods, and by 86% on diffuse knapweed at Ware (Table III). *Bangasternus fausti* reduced mature seed production of spotted knapweed by 96% at Grassrange, 97% at Seilsted, 82% at Woods, and 85% on diffuse knapweed at Ware. *Urophora affinis* reduced mature seed production of spotted knapweed by 83% at Grassrange, 40% at Seilsted, 27% at Woods, and 98% on diffuse knapweed at Ware.

Spotted knapweed capitula are larger than those of diffuse knapweed, which explains why the insects leave more seeds in capitula of spotted knapweed than diffuse knapweed. A plausible explanation for the lack of seed reduction by *L. minutus* at Woods is that the insect prefers to oviposit on the largest capitula. Such capitula would contain more seeds than can be consumed by one insect, thus possibly leaving more remaining in infested capitula than are produced by smaller capitula that are not

Table III. Reduction of mature seeds in infested capitula [mean ± SE (percent reduction compared to check)].

	Number of mature seeds per capitulum[a,b]			
Site	C	B	L	U
Spotted knapweed				
Grassrange	2.23 ± 0.16	0.10 ± 0.07 (96%)**	0.47 ± 0.19 (79%)**	0.38 ± 0.12 (83%)
Seilsted	3.15 ± 0.35	0.08 ± 0.08 (97%)**	–	1.89 ± 0.32 (40%)
Woods	4.63 ± 0.29	0.84 ± 0.17 (82%)**	4.32 ± 0.50 (6%)	3.38 ± 0.42 (27%)
Diffuse knapweed				
Ware	1.02 ± 0.12	0.16 ± 0.10 (85%)**	0.14 ± 0.07 (86%)**	0.0.02 ± 0.01 (98%)

[a]Treatments: C, uninfested; B, infested by *Bangasternus fausti;* L, infested by *Larinus minutus;* U, infested by *Urophora affinis.* [b]Comparison of infested versus uninfested capitula within the same site; one-way ANOVA: $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

infested. Fertile uninfested capitula (treatment C) produced more seeds at Woods (4.6 ± 0.3 SE) than at Grassrange (2.2 ± 0.2) or Seilsted (3.2 ± 0.4), suggesting that the capitula at Woods were larger than at the other two sites ($F_{(2,\ 516)} = 19.5$, $P = 0.0001$). Thus, at a location with large capitula, our data misleadingly suggest that the insect consumes almost no seeds. Future studies should control for capitulum size when making such comparisons to avoid this misleading paradox.

Conclusions

Larinus minutus was more successful on diffuse than spotted knapweed. *Larinus minutus* reproduction was not affected by competition with *U. affinis* on either spotted or diffuse knapweed. The addition of *L. minutus* to *U. affinis* increased the total proportion of capitula infested by 17 percentage points above that of *U. affinis* alone on diffuse knapweed but reduced it on spotted knapweed. However, *L. minutus* attack rates were much lower than are now commonly observed in the field (Lang et al. 2000a, Smith 2004), so there were probably some effects caused by the cages (e.g., lack of pollination), the quality of the insects, or timing of release.

Bangasternus fausti was equally successful on the two knapweeds, as was *U. affinis*. Reproduction by *B. fausti* was not affected by *U. affinis* on either plant. The addition of *B. fausti* to *U. affinis* increased the total proportion of capitula infested by 15–18 percentage points above that of *U. affinis* alone on both spotted and diffuse knapweed.

Urophora affinis reproduction was reduced 77% by the presence of *L. minutus* on diffuse knapweed and 71% on spotted knapweed, but not by the presence of *B. fausti* on either plant.

Addition of either weevil did not significantly further reduce seed production per cage compared to that caused by *U. affinis* alone on either plant. The lack of additive impact on seed production is probably largely because of the often low weevil attack rates in our study, because of exclusion of other insect pollinators and because of high variation among replicates at some of the sites.

Based on these results, we would expect *B. fausti* to have more impact on spotted knapweed and *L. minutus* more impact on diffuse knapweed. We would hesitate to release either of the weevils because of no evident further reduction in seed production to that caused by *U. affinis*. However, in reality the outcome was quite different. *Larinus minutus* became very abundant on spotted and diffuse knapweeds and is considered to be the most important agent causing the decline in diffuse knapweed (Lang et al. 2000a; Smith 2001, 2004; Story & Piper 2001; Seastedt et al. 2003; E. Coombs, personal communication), but its success is due to two reasons not observed in this study. First, it avoids overwintering predation, which is now severely impacting *Urophora* populations (Story 1995b; Pearson et al. 2000). Second is that foliage consumption by adult weevils in the spring causes substantial damage to rosettes and young bolting plants (Piper 2004). *Bangasternus fausti* is not well established in Montana, and is limited in Oregon (Duncan 2001; E. Coombs, personal communication). The causes limiting its success are not well known.

In retrospect, there were several problems which should be avoided in future studies. Cages were used to control treatments, but it was much more difficult to exclude the *Urophora* flies than anticipated, and there was no compensation for the exclusion of pollinators. Both weevils apparently depend on pollination of the flower to produce seed, which the larvae consume, but this is not a requirement for *Urophora*

development. Thus, exclusion of pollinators probably greatly limited the reproduction of the weevils but not of the flies. The insects should have been released earlier to better represent natural phenology. *Bangasternus fausti* oviposits on capitula at the youngest developmental stage, the *Urophora* flies oviposit on capitula at later stages, and *L. minutus* oviposits on open flowers. Although individual capitula mature at different times, there is a temporal order of attack which may enable some species to preempt individual capitula. Thus, it is important that the cages are large enough to provide sufficient capitula at the appropriate stage of development for each insect. Insect overcrowding is always a concern in such experiments, but often there is little information on which to base the decision. Aspects such as other habitat requirements (e.g., for feeding, resting, overwintering), dispersal, and vulnerability to natural enemies were not addressed, but they may affect the distribution and abundance of these insects. Perhaps the best way to help us evaluate future prospective agents is to learn more from retrospective studies of previously released agents.

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