



# How do biological control and hybridization affect enemy escape?

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## Abstract

Two mechanisms often linked with plant invasions are escape from enemies and hybridization. Classical biological control aims to reverse enemy escape and impose top-down population control. However, hybridization has the potential to alter interactions with enemies and thus affect biological control. We examined how introductions of biological control agents affect enemy escape by comparing specialist enemy loads between the native and introduced ranges of two noxious weeds (spotted and diffuse knapweed; *Centaurea stoebe* L. and *C. diffusa* Lam.) that have been the targets of an extensive biological control program. Hybrids between spotted and diffuse knapweed are often found within diffuse knapweed sites in North America, so we also compared enemy loads on plants that appeared morphologically like diffuse knapweed and hybrids. Finally, we tested the preference for diffuse knapweed, hybrids, and spotted knapweed of one of the agents thought to be instrumental in control of diffuse knapweed (*Larinus minutus*; Curculionidae). In North America spotted knapweed has largely escaped its root herbivores, while seedhead herbivore loads are comparable in the introduced and native ranges. Diffuse knapweed exhibited seedhead herbivore loads five times higher in the introduced compared to native range. While this pattern of seedhead herbivory is expected with successful biological control, increased loads of specialist insect herbivores in the introduced range have rarely been reported in the literature. This finding may partially explain the better population control of diffuse vs. spotted knapweed. Within North American diffuse knapweed sites, typical diffuse knapweed and hybrid plants carried similar herbivore loads. However, in paired feedings trials, the specialist *L. minutus* demonstrated a preference for newly created artificial hybrids over North American diffuse knapweed and for European diploid spotted knapweed over North American tetraploid spotted knapweed. Overall though, hybridization does not appear to disrupt biological control in this system.

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## 1. Introduction

When an organism leaves its native range, it escapes its natural predators and parasites (Keane and Crawley, 2002), although new, mainly generalist natural enemies may be acquired (Cripps et al., 2006). Overall, introduction to a new range often results in significantly reduced enemy

damage (Maron and Vilá, 2001; Keane and Crawley, 2002; Mitchell and Power, 2003; Torchin et al., 2003). Enemy escape appears to be common for invasive plants: comparisons of the native and introduced ranges show that enemy load is often significantly lower in the introduced range (Wolfe, 2002; Vilá et al., 2005; Liu and Stiling, 2006). The enemy release hypothesis, one of the most often cited hypotheses to explain biological invasions, is based on the assumption that, due to the reduced enemy load (often measured as the number of individual enemies per host or biomass of enemies per host) in the introduced range, invasive species experience a decrease in top-down regulation

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by natural enemies, resulting in rapid increase in population growth rate and distribution (Keane and Crawley, 2002; White et al., 2008).

Classic biological control of weeds is also based on the observation of enemy escape (DeBach, 1964; Wapshere et al., 1989; Coombs et al., 2004; van Klinken and Raghu, 2006). Natural enemies of an invasive weed are imported from the native range of that pest in an attempt to increase enemy load and establish top-down population control in the introduced range. As Elton (1958) noted, many introduced agents fail to establish or are ineffective, while others 'have done splendid work in ameliorating disastrous situations.' When classical biological control is implemented, typically only one or a few specialist natural enemies from the plant's component community (sensu Root, 1973) are introduced. Thus, even with biological control, invasive weeds might still experience reduced enemy load relative to the native range due to the reduction in total specialist enemy diversity. Alternatively, introduced biological control agents (i.e. specialist enemies) might attain considerably higher densities in the introduced range than in their native range for numerous abiotic and biotic reasons (Keane and Crawley, 2002), leading to an increase in specialist enemy load despite the lower diversity of natural enemies. One mechanism in particular that might result in greater enemy densities in the introduced range is that the introduced agent escapes its own natural enemies and competitors. Thus, following biological control, it is not obvious whether invasive species will have lower enemy load than in the native range due to a drop in specialist diversity or higher enemy load than in the native range due to changes in the population dynamics of the specialist biological control agents themselves.

Evolutionary changes within populations of invasive weeds might also affect the degree of enemy escape following the introduction of biological control agents. In particular, hybridization can alter interactions with natural enemies (Whitham, 1989; Fritz et al., 1999). Hybrids may be more, less, or equally resistant to enemy attack than their parental species (Hjälten, 1998; Fritz et al., 1994, 1999, 2001), although greater resistance seems to be rare. Roley and Newman (2006) found that a native weevil had highest survival on an introduced watermilfoil, lowest survival on the native watermilfoil, and intermediate survival on the hybrid between the two species. In contrast, Whitham (1989) found that hybrid cottonwoods were more susceptible to aphid attack than either pure parent species, and these hybrid trees acted as sinks for the aphids, perhaps preventing adaptation to the more numerous parental trees; although hybrids comprised only 3% of the population, they contained 85–100% of the aphid population. Thus, hybridization may have important consequences for biological control, as hybrids may be attacked differently than one or both parent species.

This study focuses on spotted and diffuse knapweed, which were inadvertently introduced to North America from Eurasia in the late 1800s or early 1900s, and have become a

major threat to rangeland productivity and quality across western North America (Watson and Renney, 1974; Roché and Roché, 1991; Sheley et al., 1999). Additionally, diploid spotted knapweed and diploid diffuse knapweed can hybridize (Gáyer, 1909; Ochsmann, 1999), and hybrids are present within diffuse knapweed sites in North America (A.C. Blair, unpublished data; Ochsmann, 2001). As these weeds infest more than two million hectares (Sheley et al., 1998; Duncan et al., 2004), it is difficult or impossible to control them with cultivation, and herbicide application is often not economically feasible because of the low productivity of the land. Therefore, 13 specialist herbivorous insects from the native range have been introduced in an effort to impose biological control (Rosenthal et al., 1991; Sheley et al., 1999). These 13 species feed on both diffuse and spotted knapweed, and each is considered to be a biological control agent for both weeds. Overall, diffuse knapweed seems to be better controlled by these herbivores than spotted knapweed (Smith, 2004; Seastedt et al., 2007). Using a biogeographical approach (e.g. Hinz and Schwarzlaender, 2004; Hierro et al., 2005), we conducted cross-continental field surveys to quantify seedhead and root herbivore loads in the introduced and native range of each species to determine if the introduction of specialist insects has resulted in different outcomes for the two invasive plants, which may serve as a first step towards understanding the greater control of diffuse knapweed. In North America, seedhead and root herbivory is almost certainly attributed to specialist insects, as there are no known native generalist herbivores that damage these knapweeds in those specific plant parts. Similarly in Europe, most seedhead and root herbivory is likely to be due to specialists (U. Schaffner and P. Häfliger, personal observation), especially given the complex defense chemistry of the genus *Centaurea* (Djeddi et al., 2007 and references within). Thus, overall our study most likely compares herbivory between regions by specialists and not generalists.

To examine the consequences of hybridization for biological control efforts, we surveyed herbivore loads on diffuse knapweed and hybrids found within diffuse knapweed sites across western North America and experimentally tested the preference of an important biological control agent, *Larinus minutus* Gyllenhal (Coleoptera: Curculionidae) for the parent species and the hybrids.

The research presented here addressed the following questions:

- (1) How has implementation of biological control affected specialist herbivore loads in the introduced range relative to the native range of diffuse and spotted knapweed?
- (2) Within North American diffuse knapweed sites, do hybrid and diffuse knapweed plants experience different or equivalent specialist herbivore loads?
- (3) Do adults of the seedhead weevil *L. minutus* show a feeding preference for diffuse knapweed, hybrids, or spotted knapweed?

## 2. Materials and methods

### 2.1. Study species

*Centaurea stoebe* L. subspecies *micranthos* (Gugler) Hayek (= *C. maculosa* Lam.; = *C. biebersteinii* de Candolle) (spotted knapweed) and *C. diffusa* Lam. (diffuse knapweed) are problematic weeds in North America that can increase soil erosion (Lacey et al., 1989; Sheley et al., 1997), alter plant community composition (Tyser and Key, 1988), negatively impact biodiversity (Ortega et al., 2006), and are thought to have allelopathic effects on other plants (Fletcher and Renney, 1963; Callaway and Aschehoug, 2000; but see Locken and Kelsey, 1987; but see Blair et al., 2005, 2006). Both species appear to have been introduced several times, as introduced populations harbor high levels of genetic diversity (Hufbauer and Sforza, 2008; Marrs et al., 2008).

It is reported that both species have diploid ( $2n = 18$ ) and tetraploid ( $4n = 36$ ) cytotypes (Ochsmann, 2000). Both cytotypes of diffuse knapweed are referred to simply as *C. diffusa* Lam. The tetraploid seems to be rare, as it has only been reported twice in the literature from one specimen in Bulgaria (Löve, 1979) and one in the former Yugoslavia (Löve, 1978). All diffuse knapweed in this study are likely to be diploid. Diffuse knapweed is an annual to short-lived perennial. The two cytotypes of spotted knapweed both fall under the name *C. stoebe* L., which takes precedence over the commonly used *C. maculosa* (Ochsmann, 2000). The biennial diploid is designated *C. stoebe* subsp. *stoebe* L., and the polycarpic tetraploid is designated *C. stoebe* subsp. *micranthos* (Gugler) Hayek (for which *C. biebersteinii* DC. is a synonym). The spotted knapweed plants that have been surveyed in North America are tetraploids (i.e. *C. stoebe* subsp. *micranthos*) (Moore and Frankton, 1954; Ochsmann, 2000; H. Müller-Schärer, personal communication). Thus, when we refer to spotted knapweed of North American origin, it is likely to be the tetraploid *C. stoebe* subsp. *micranthos*, while spotted knapweed from Europe may be either cytotype. When ploidy level of European spotted knapweed is known, we clearly specify it. Diploid hybrids between diploid spotted and diffuse knapweed were first identified in the native range in 1909 (Gáyer, 1909; Ochsmann, 1999), and were given the name *Centaurea xpsammogena* Gáyer. They tend to occur only in a narrow zone of overlap between the two diploid parent species ranging from Romania to the Ukraine (U. Schaffner, personal communication). Through field observations and molecular techniques, we have recently found that diffuse knapweed in North America was introduced with hybrid individuals containing significant introgression from diploid spotted knapweed. Individuals of hybrid origin are found in most North American diffuse knapweed sites, but not in spotted knapweed sites, and hybridization does not appear to be ongoing (A.C. Blair, unpublished data).

Floral traits are used to diagnose species in this complicated genus (Ochsmann, 2000). Diffuse knapweed has

smaller white flowers (rarely pink), no bract pigmentation, and a pronounced terminal spine, while spotted knapweed has larger purple flowers, obvious bract pigmentation, and lacks a terminal spine (Watson and Renney, 1974; Ochsmann, 2000). Hybrids typically have intermediate morphology with purple ray flowers and white disc flowers, pigmented bracts, and terminal spines. In this paper, designation of a plant as a hybrid or typical diffuse knapweed is based on floral morphological characters. As this classification has not been confirmed at the molecular level for the specific plants studied here, we denote the plants as hybrid-like or diffuse-like to highlight that the classifications contain some uncertainty at the genome level. Molecular work has corroborated that the presence of intermediate individuals within a region correctly predicts interspecific admixture (A.C. Blair, unpublished data).

### 2.2. Biological control program

The thirteen specialist insects that have been introduced to North America from the native range can all attack both spotted and diffuse knapweed. The larvae of these insects damage the plant either in the root ( $n = 5$ ; three moth and two beetle species) or the seedhead ( $n = 8$ ; four fly, one moth, and three beetle species). The seedhead weevil *L. minutus* was first introduced to North America in 1991 from Greece (Lang et al., 1996). The decline of diffuse knapweed populations in some areas of North America has been attributed to this weevil (Seastedt et al., 2003; Smith, 2004), but it appears to play a relatively smaller role in spotted knapweed control (e.g. Smith, 2004; Story et al., 2006). Seastedt et al. (2007) reported that upon introduction of *L. minutus*, seed production of diffuse knapweed declined from 4400 seeds/m<sup>2</sup> in 1997 to 0 seeds/m<sup>2</sup> in 2006. Other reports of successful control of diffuse knapweed have come out of Montana, Oregon, Washington, and British Columbia (Smith, 2004; Story and Coombs, 2004; Myers, 2004), and *L. minutus* may have played a large role in each area (Seastedt et al., 2007). The weevil is univoltine; adults overwinter in leaf litter and emerge in late spring/early summer. While all of the biological control agents introduced against the knapweeds cause damage as larvae, adult *L. minutus* are also able to significantly defoliate knapweed plants prior to flowering (Wilson and Randall, 2003; Piper, 2004; Norton et al., 2008). At flowering, the weevils switch to feeding on knapweed flowers. Eggs are laid in open flowers, and developing larvae can destroy all of the seeds in a diffuse knapweed capitulum and 25–100% of the seeds in spotted knapweed capitulum (Lang et al., 1996). *L. minutus* is capable of developing in several *Centaurea* species including *C. stoebe* (spotted knapweed), *C. diffusa* (diffuse knapweed), *C. arenaria*, and *C. calcitrapa* (Jordan, 1995). Several studies have explored the preference and performance of *L. minutus* on spotted vs. diffuse knapweed, but found conflicting results (Table 1).

Table 1

A summary of the studies that compare the preference or performance of *Larinus minutus* for spotted (SK) vs. diffuse knapweed (DK)

Author(s)	Measure of preference or performance	Preferred species
Smith and Mayer (2005)	Establishment of <i>L. minutus</i> in release cages <i>L. minutus</i> infested capitula in release cages <i>L. minutus</i> progeny production in release cages	<b>DK NA–100% SK NA –69%</b> <b>DK NA–30 ± 4% SK NA–11 ± 3%</b> <b>DK NA–30.9/100 capitulum SK NA–11.1/100 capitulum</b>
Groppe et al. (1990)	Dispersal preference in native range Rate of attack in the field in the native range	DK EU–Insignificant preference over SK EU <b>SK EU–27.7% DK EU –12.6%</b>
Jordan (1995)	Adult feeding on leaf tissue Percentage of flowers attacked, choice test Number of eggs laid, choice test Percentage of flowers attacked, choice test Number of eggs laid, choice test Percentage of flowers attacked, choice test Number of eggs laid, choice test Survival pupa to adult, performance test	<b>SK NA, EU–Yes DK NA–Yes DK EU–No</b> <b>SK EU–66.7% DK EU–27.8%</b> <b>SK EU–19 DK EU–8</b> <b>SK EU–66.7% DK NA–16.7%</b> <b>SK EU–12 DK NA–3</b> SK EU–66.7% SK NA–55.6% SK EU–19 SK NA–20 <b>SK NA–70% SK EU–55% DK NA–55% DK EU–50%</b>

EU, plants of European origin; NA, plants of North American origin. The preferred plant type or the plant type with greater *L. minutus* performance is shown in bold.

### 2.3. Cross-continental field surveys of spotted and diffuse knapweed specialist herbivore loads

Three sets of surveys were conducted: summer 2005 North America, summer 2005 Central Europe, and summer 2006 North America. The main focus of the 2005 surveys was to compare seedhead and root herbivore loads of spotted and diffuse knapweed between the native and introduced range (Table 2). The history of releases of biological control agents was largely unknown for nearly all surveyed sites in North America 2005. The main focus of the 2006 surveys was to compare seedhead herbivore loads between diffuse-like and hybrid-like individuals within North American diffuse knapweed sites. About half of the 2006 sites were surveyed without prior knowledge of where biological control agents had been released, while the other half were <6 km from seedhead herbivore release sites (Table 2). To obtain a relative measure of herbivore loads for comparisons across sites and between plant types (i.e., diffuse-like and hybrid-like), we recorded herbivores or evidence of herbivores as present/absent data within individual seedheads and roots (described further below).

Between June and September 2005, we surveyed seven spotted knapweed sites and five diffuse knapweed sites across North America to assay specialist seedhead herbivory (Table 2). Surveys focused on regions where these plants are considered quite invasive. We found sites with spotted and/or diffuse knapweed by contacting county weed supervisors prior to the collection trip, and sites were then selected either by driving until encountering a site or from directions from a weed supervisor. At each site we haphazardly ran a 50 m transect through the population and surveyed approximately 30 plants on one side of the tape measure every 1-m (or more if plants were spaced further apart). This sampling scheme ensured the inclusion of a representative sub-sample of the site. To measure herbivore load of seedhead feeders, we surveyed from 13 to 30 plants per site (Table 2), depending on the availability of

mature seedheads, and we opened five seedheads per plant. Spring 2005 was unseasonably cool, so flowering was delayed across much of the west. Therefore, although all sites had >30 plants, not all sites had 30 plants with mature seedheads at the time of the survey. We recorded whether seedhead feeders were present or absent per seedhead. In addition to actual larvae or insects, we scored seedheads as having seedhead feeders present if we found freshly laid egg(s) or damage with frass (even in the absence of an insect). Within the diffuse knapweed sites, approximately half of the plants surveyed were diffuse-like ( $n = 61$ ), and half were hybrid-like ( $n = 63$ ). Five of the nine North America diffuse knapweed sites were assayed for ploidy, and only diploid plants were detected (A.C. Blair, unpublished data; Table 2).

During the same set of surveys across North America, we also measured herbivore load of specialist root miners in eight diffuse knapweed sites, nine spotted knapweed sites, and one diffuse + spotted knapweed site (Table 2). We evaluated whether root miners were present by excavating the root from the soil and opening it. We surveyed from 11 to 30 plants per site (Table 2). Within the diffuse knapweed sites, again approximately half of the plants sampled were diffuse-like ( $n = 114$ ), and the other half hybrid-like ( $n = 99$ ).

To quantify seedhead and root miner loads in the native range, in August 2005 we visited five spotted knapweed sites and five diffuse knapweed sites in Europe and in 2006 one diffuse knapweed site in the Ukraine (Table 2). Either local botanists identified sites for us, or we found sites while driving through the countryside. Only one of the six diffuse knapweed sites contained hybrid-like plants, and at that site those plants made up <5% of the population; therefore, we consider these sites to be relatively pure diffuse knapweed. Approximately thirty plants were assayed per site, as described above in North America 2005 (Table 2). Ploidy was assayed for three of the five spotted knapweed sites; one of these sites was diploid,

Table 2

The proportion of seedheads and roots with evidence of herbivory per surveyed diffuse and/or spotted knapweed site in the introduced (North American) and native (European) ranges in 2005 and 2006

Site	GPS location	Altitude (m)	Seedhead	Root
<i>2005 North America diffuse knapweed (all sites likely 2n)</i>				
Wolcott, CO (2n)	N39°42'10.2" W106°40'32.8"	2130	—	0.04 (24)
Denver, CO (2n)	N39°41'48.5" W105°11'32.7"	1848	0.69 (29)	0.28 (29)
Idalia, CO	N39°40'30.5" W102°33'22.3"	1260	—	0.0 (16)
Pendle, OR (2n)	N45°54'58.8" W119°33'31.8"	94	0.68 (30)	0.57 (30)
Dalles, OR (2n)	N45°36'17.1" W121°11'02.3"	27	0.58 (27)	—
Wenatchee, WA	N47°33'40.4" W120°16'11.3"	249	—	0.73 (26)
Wenatchee, WA	N47°28'14.4" W120°20'11.5"	232	—	0.32 (28)
Kaycee, WY	N43°50'13.7" W106°52'28.6"	1849	0.13 (16)	0.0 (20)
Natrona, WY (2n)	N43°23'07.9" W107°03'45.6"	1888	0.05 (22)	0.0 (25)
<i>2005 North America spotted knapweed (all sites likely 4n)</i>				
Eagle, CO	N39°39'22.3" W106°35'59.9"	2262	—	0.0 (18)
Boise, ID	N43°38'35.5" W116°15'19.7"	795	—	0.0 (12)
Big Timber, MT	N45°46'23.7" W109°47'56.8"	1216	—	0.04 (21)
Big Timber, MT	N46°04'29.3" W109°56'08.7"	1507	—	0.0 (27)
Laurel, MT	N45°41'21.8" W108°46'17.2"	1076	0.83 (21)	0.0 (23)
Westport, NY	N44°16'42" W073°31'52.6"	168	0.56 (20)	0.15 (20)
Ithaca, NY	N42°17'33.9" W076°42'49"	345	0.68 (30)	—
Buffalo, WY	N44°29'59.9" W109°12'44.3"	1664	0.08 (13)	0.0 (11)
Buffalo, WY	N44°22'38.7" W106°42'37.2"	1435	0.66 (14)	0.0 (18)
Casper, WY	N42°54'14.6" W106°27'09.7"	1628	0.64 (27)	—
Flam, Ontario	N43°20'58.5" W080°06'43.6"	252	0.23 (20)	0.0 (20)
<i>2005 North America diffuse + spotted knapweed</i>				
Fairfield, ID	N43°18'16.6" W114°48'6.4"	1528	—	0.07 (28)
<i>2005 Europe diffuse knapweed</i>				
Romania 3	N43°54'8.76" E28°34'26.1"	17	0.03 (29)	0.43 (30)
Romania 4 (2n)	N44°23'22.8" E28°31'35.9"	17	0.15 (29)	0.63 (30)
Romania 5 (2n)	N44°94'34.3" E28°91'4.9"	26	0.08 (30)	0.37 (30)
Romania 6 (2n)	N45°11'8.8" E28°47'8.3"	9	0.07 (30)	0.37 (30)
Romania 7 (2n)	N45°29'52.3" E27°54'42.5"	17	0.12 (30)	0.57 (30)
Ukraine 5	N48°48'22.8" E30°33'54.9"	174	—	0.83 (30)
<i>2005 Europe spotted knapweed</i>				
Austria 1	N47°53'3.0" E16°16'40.9"	573	0.32 (30)	0.40 (30)
Hungary 1 (2n)	N46°48'1.7" E17°12'20.0"	151	0.27 (30)	0.20 (30)
Romania 2 (4n)	N47°13'59.9" E26°30'57.4"	327	0.80 (30)	0.87 (29)
Romania 8 (4n)	N47°28'30.3" E26°16'6.0"	401	0.67 (30)	0.37 (30)
Romania 9	N46°21'33.1" E25°47'38.7"	727	0.43 (30)	0.23 (30)
<i>2006 North America diffuse knapweed</i>				
Adams 2, CO	N39°52'02.5" W104°55'30.8"	1551	0.40 (20)	—
Doug1, CO	N39°24'36.7" W104°52'12.0"	1848	0.79 (28)	—
Doug2, CO	N39°20'23.8" W104°49'53.3"	1960	0.68 (28)	—
Estes, CO	N40°22'09.5" W105°31'54.2"	2183	0.53 (29)	—
Mosier, OR <sup>a</sup>	N45°41'01.9" W121°24'08.3"	43	0.95 (14)	—
Tygh, OR <sup>a</sup>	N45°15'14.9" W121°09'05.8"	361	0.80 (31)	—
I84, OR <sup>a</sup>	N45°47'28.2" W120°01'51.8"	96	0.31 (29)	—
Hepner, OR <sup>a</sup>	N45°20'34.4" W119°32'58.1"	646	0.69 (27)	—
Condon, OR <sup>a</sup>	N45°14'35.7" W120°10'55.5"	859	0.83 (29)	—
Antelope, OR <sup>a</sup>	N44°50'34.6" W120°54'01.7"	593	0.40 (27)	—
Laramie, WY	N41°04'55.1" W105°29'06.2"	2275	0.01 (25)	—

Number in parentheses shows the number of plants surveyed within the site. Ploidy is presented when known (2n or 4n). DK = diffuse knapweed, SK = spotted knapweed.

<sup>a</sup> Sites <6 km from seedhead herbivore (*Larimus minutus* and *Bangasternus fausti*) release points.

while the other two were tetraploid (H. Müller-Schärer, unpublished data; Table 2). Three of the European diffuse knapweed sites were assayed for ploidy and only diploid plants were detected (H. Müller-Schärer, unpublished data; Table 2).

To further examine if seedhead herbivores respond to the different floral morphology between diffuse-like and hybrid-like plants, in 2006 we compared seedhead herbivore loads between diffuse-like and hybrid-like plants in North America. Between July and September 2006, we

visited an additional 11 diffuse knapweed sites throughout western North America (Table 2). We assayed seed-head feeders as described above for 14–31 plants per site ( $\approx 2/3$  hybrid-like and  $1/3$  diffuse-like). On average, we assayed  $\approx 30$  seedheads per plant, for a total of 8649 seedheads. Surveys for root herbivores were not conducted in 2006.

#### 2.4. *Larinus minutus* collection and colony conditions for leaf preference tests

*Larinus minutus* weevils were collected during the first week of June 2007 at Hughes Stadium, Fort Collins, CO (N40°33'27.88" W105°7' 59.60") from a large diffuse knapweed infestation. Insects were kept in mesh cages with one or two live plants. The plants used for feeding the weevils were from a diffuse knapweed site in North America not used in the choice study (Roosevelt, WA, USA). As plants were defoliated, new ones were added. The cage was kept under artificial lights that were set on a 14 h light, 10 h dark cycle. Collecting weevils from and maintaining them on diffuse knapweed may have introduced a bias for preference of diffuse over spotted knapweed; however, our results suggest this was not a problem (see Sections 3 and 4). Limited spotted knapweed infestations in the area prevented collection of weevils from that species.

#### 2.5. Plant material for *L. minutus* feeding preference trials

Plants from the following sites were grown for this experiment:

- two pure diffuse knapweed sites in Crimea, Ukraine
- two diffuse knapweed sites that contained both hybrid-like and diffuse-like plants in North America
- three verified diploid spotted knapweed sites in the Ukraine
- two verified tetraploid spotted knapweed sites in North America

Additionally, 20 Back Cross 1 (BC1) seeds created in the lab were planted. As the hybrid-like individuals found within diffuse knapweed sites are likely to have experienced many generations of introgression since their introduction at the turn of the century, BC1 seeds were included to better understand how individuals with a greater portion of their genome derived from spotted knapweed may influence preference. BC1 seeds were created by crossing North American diffuse knapweed with European diploid spotted knapweed. An F1 from this cross was then back-crossed with diffuse knapweed to create BC1 seed. We used micro-satellite markers to confirm the identity of the F1 and BC1 individuals (A.C. Blair, unpublished data).

Plants were grown in pots (diameter 1.5", depth 8.25") in Sunshine Mix #3 potting soil. The plants were started in a greenhouse in May 2007 and subsequently they were moved outside to a lath house. Pots were misted daily until

most seeds had germinated (approximately 2 weeks) and then watered daily. Plants were fertilized (Osmocote Classic 14-14-14 Scotts) as needed. At three weeks all plants were sprayed to control a thrips outbreak (Borer, Bagworm, Leafminer, and Tent Caterpillar Spray, Ferti•lome).

#### 2.6. *Larinus minutus* leaf preference tests

Two sets of feeding trials were conducted (July and September, 2007). Weevils were starved for 24 h prior to the feeding trials, and then were presented with pairs of fresh leaves that were similar in age, size, and shape. Harvested leaves were placed in a 150 × 15 mm polystyrene Petri dish; the cut end of the leaf was placed in a moistened paper towel to keep the leaf fresh. Twenty weevils were then added to each Petri dish and allowed to feed for 24 h.

Leaves were scanned with a flatbed scanner (Microtek, ScanMaker 6800) pre- and post-feeding. We calculated the area (mm<sup>2</sup>) of the leaf pre- and post-feeding with the software program VegMeasure (v.1.6, D.E. Johnson, Oregon State University). This program uses an algorithm to select pixels that correspond to green vegetation from color photographs. We then calculated the relative amount of damage per leaf as follows: [initial green area–final green area]/initial green area.

Twenty replicates of the following four paired feeding trials were conducted in July 2007 with the plants grown in the lath house (described above):

- (1) European diffuse knapweed vs. North American diffuse knapweed
- (2) European diffuse knapweed vs. BC1
- (3) European diploid spotted knapweed vs. North American diffuse knapweed
- (4) European diploid spotted knapweed vs. BC1

The second set of feeding trials was performed in September 2007 with newly emerged weevils collected from the same site. We performed 20 choice feeding trials for the following three pairs:

- (1) North American diffuse knapweed vs. BC1
- (2) North American tetraploid spotted knapweed vs. European diploid spotted knapweed
- (3) North American diffuse knapweed vs. North American tetraploid spotted knapweed

#### 2.7. Statistical analyses

##### 2.7.1. Field surveys

To compare specialist herbivory of diffuse knapweed between North America (2005) and Europe (2005), we combined all North American hybrid-like and diffuse-like plants into one category—North American diffuse knapweed, as we were interested in the overall differences at the continent scale instead of among morphological vari-

ants within a site. Each seedhead or root was assigned a 0 if there was no evidence of herbivory or a 1 if there was evidence of herbivory. For each plant, we then analyzed the seedhead data as  $x$  seedheads with presence of herbivory out of a total of  $y$  seedheads (i.e. events/trials = response variable). Thus, the unit of measure is the seedhead, while the plant is the unit of analysis. Data in this events/trials format are properly analyzed with a binomial distribution and a logit link function (Littell et al., 2006, pp. 542–543). For both seedhead and root infestation, we used mixed models in SAS (PROC GLIMMIX) with continent as a fixed effect and site nested within continent as a random effect. We used the same mixed model to compare seedhead and root infestation of spotted knapweed across continents. To compare seedhead (2005 and 2006) and root herbivory (2005) between North American hybrid-like and diffuse-like plants, we used a model with plant classification as a fixed effect and site as a random effect. In 2005 we collected size data on all of the plants surveyed for biological control (A.C. Blair, unpublished data). Initially, we included plant size (using the equation for the volume of a cylinder =  $[\pi * \text{plant diameter}^2 * \text{plant height}]/4$ ) as a covariate for the 2005 data analyses, but we removed this term from the models, as it did not alter patterns of significance.

### 2.7.2. Leaf preference test

We used PROC TTEST to compare the percent change of leaf tissue between the seven paired plant types. The percentage change data were normally distributed. We used the same approach to compare the total amount eaten (initial green area–final green area) between pairs; the same results were found, so we only report the findings from the analyses of percent change.

## 3. Results

### 3.1. Cross-continental field surveys of spotted and diffuse knapweed specialist herbivore loads

**Question 1.** How has implementation of biological control affected specialist herbivore loads in the introduced range relative to the native range of diffuse and spotted knapweed?

The 2005 surveys revealed less seedhead damage on European than on North American diffuse knapweed ( $F_{1,8} = 5.62$ ,  $P = 0.04$ ; Fig. 1A). Attack by root miners showed the opposite pattern; a greater proportion of diffuse knapweed plants were attacked in Europe than North America ( $F_{1,13} = 5.32$ ,  $P = 0.04$ ; Fig. 1B).

For spotted knapweed, no difference was found in the 2005 surveys for attack by seedhead feeders ( $F_{1,10} = 0.01$ ,  $P = 0.94$ ; Fig. 1C). However, European spotted knapweed was much more often attacked by root miners than North American plants ( $F_{1,17} = 21.62$ ,  $P < 0.001$ ; Fig. 1D). In fact, only a total of four spotted knapweed plants in two out of ten sites in North America had root herbivory.

**Question 2.** Within North American diffuse knapweed sites, do hybrid and diffuse knapweed plants experience different or equivalent specialist herbivore loads?

Across the sites in 2005, hybrid-like and diffuse-like plants did not differ in the amount of seedhead ( $F_{1,118} = 0.01$ ,  $P = 0.94$ ; hybrid-like = 44%, diffuse-like = 58%) or root herbivory ( $F_{1,201} = 0.41$ ,  $P = 0.52$ ; hybrid-like = 18%, diffuse-like = 34%). Within diffuse knapweed sites from the North American 2006 field surveys, the percentage of seedheads with herbivory ranged from 1% to 95% (Table 2). Similarly, across all 2006 sites hybrid-like and diffuse-like plants did not differ in the level of seedhead herbivory ( $F_{1,274} = 1.97$ ,  $P = 0.16$ ; hybrid-like = 66%, diffuse-like = 62%).

### 3.2. *Larinus minutus* leaf preference tests

**Question 3.** Do adults of the seedhead weevil *L. minutus* show a feeding preference for diffuse knapweed, hybrids, or spotted knapweed?

In the first set of paired feeding trials (July 2007), weevils consumed comparable amounts of leaf tissue between the paired plants (Fig. 2A–D). In the second set of paired feeding trials (September 2007), the amount of tissue consumed significantly differed for two of the three pairs (Fig. 2E–G). European diploid spotted knapweed was consumed more than North American tetraploid spotted knapweed, and North American diffuse knapweed was consumed more than hybrid BC1 individuals.

## 4. Discussion

In this study our goal was to examine how biological control affects enemy escape and what role hybridization might play. We found that whether or not enemy escape was observed depended on both the plant species (i.e. spotted or diffuse knapweed) and the type of herbivory by specialist insects (i.e. seedhead or root). Hybrid-like plants in the field in North America had similar herbivore loads to diffuse-like plants. The specialist insect *L. minutus* showed a preference for North American admixed diffuse knapweed over hybrids created in the lab that contained more of the diploid spotted knapweed's genome. These findings, discussed below, could have implications for successful management of these noxious weeds by biological control agents and lead to a better understanding of how biological control interacts with mechanisms put forward to explain invasion success.

### 4.1. Biological control of spotted knapweed: impacts on enemy loads and management implications

Spotted knapweed now infests more than 1.2 million hectares in North America (Sheley et al., 1998), in spite of a rigorous biological control program begun in North America more than 30 years ago (reviewed in Müller-Schärer and Schroeder, 1993). Although biological control

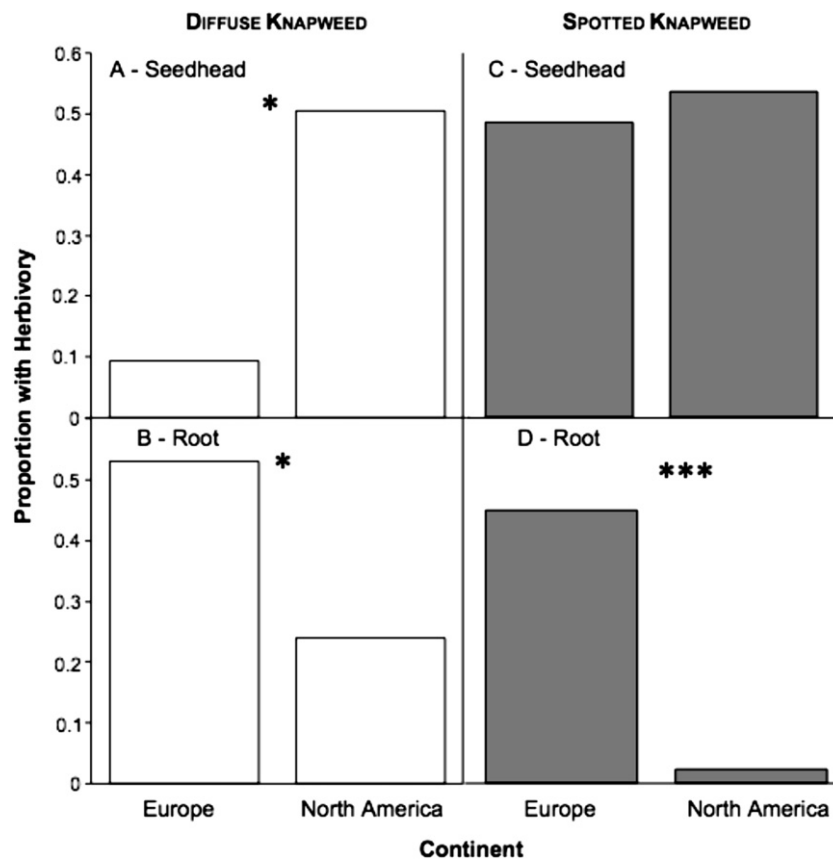


Fig. 1. The proportion of diffuse knapweed (white bars) (A) seedheads and (B) roots and spotted knapweed (gray bars) (C) seedheads and (D) roots with evidence of herbivory in Europe vs. North America. \*  $P < 0.05$ , \*\*\*  $P < 0.001$ .

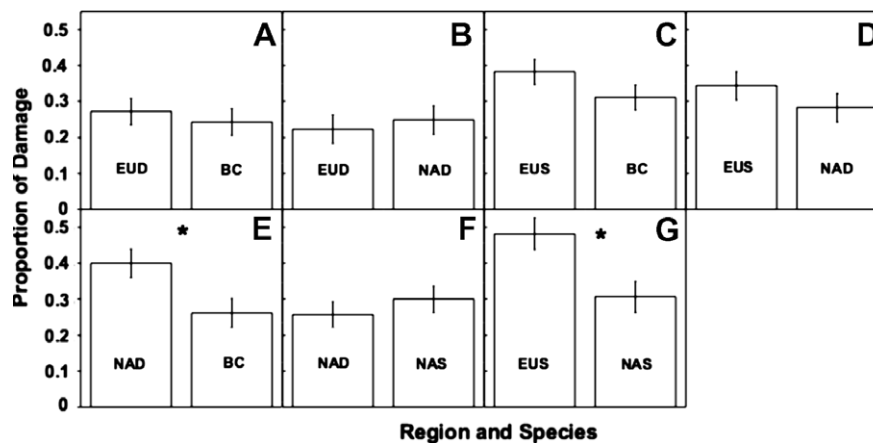


Fig. 2. The proportion of damage (i.e. leaf tissue consumption) by the biological control agent *Larinus minutus* in two sets of paired preference tests (A–D), set 1; (E–G) = set 2. Pairs of trials were analyzed with paired *t*-tests. BC, Back Cross 1 hybrid individuals; EUD = European diffuse knapweed; EUS, European spotted knapweed (verified diploid); NAD, North American diffuse knapweed; NAS, North American spotted knapweed (verified tetraploid). Values represent the mean  $\pm$  1 SE, \*  $P < 0.01$ .

introductions have largely reversed the escape from specialist seedhead herbivores, this type of herbivory may not be a strong top-down regulator of spotted knapweed. Spotted knapweed capitula are relatively large, and even when attacked, seeds are often still able to develop (Maddox, 1982; Smith and Mayer, 2005). Indeed, Story et al. (1989) concluded that the 36–41% reduction of seed production

by the seedhead fly *Urophora affinis* documented in a field study would not likely exert a strong enough impact to control spotted knapweed alone. A similar conclusion was reached when Story (1989) reported seed reduction by the seedhead flies around 50–75%. The seedhead weevil, *L. minutus*, has had a significant impact on introduced diffuse knapweed (discussed below), and it is currently not

having such a dramatic impact on spotted knapweed (Seastedt et al., 2007).

Unlike the high seedhead herbivore loads found on spotted knapweed, it appears that this species still largely escapes its specialist root herbivores in North America. Other studies have found that the root weevil *Cyphocleonus achates* has the potential to decrease infestations of spotted knapweed at some locations (Corn et al., 2006; Jacobs et al., 2006; but see Clark et al., 2001). Story et al. (2006) found that at two sites, spotted knapweed decreased by 99% and 77% as *C. achates* numbers increased. Reductions of spotted knapweed density did not take place at six additional sites where *C. achates* was absent, although six other biological control agents were present (Story et al., 2006). Recently, it was shown that *C. achates* can reduce spotted knapweed size regardless of drought conditions, and it seems unlikely that recent declines observed in western Montana have resulted just from persistent drought conditions (Corn et al., 2007). It is possible that as populations of root feeding *C. achates* grow and spread, the patterns of root miner loads we observed here may shift and reduction of spotted knapweed may occur.

*Agapeta zoegana*, a root-mining moth introduced for biological control, seems less likely to successfully control spotted knapweed, as infested plants were found to compensate for herbivory (Steinger and Müller-Schärer, 1992; Callaway et al., 1999; Newingham et al., 2007). Müller (1989) found that spotted knapweed from North America infested with *A. zoegana* increased root growth, but he also found that this insect reduced survival of immature plants. In conclusion, spotted knapweed appears to have reduced root herbivore loads in North America, as predicted by the enemy escape hypothesis, and recent evidence suggests that at least one, but perhaps not all, of the introduced root feeding biological control agents may shift that pattern and has the potential to successfully control this plant species.

#### 4.2. Biological control of diffuse knapweed: impacts on enemy loads and management implications

Diffuse knapweed infests at least 700,000 hectares in North America (Duncan et al., 2004). Compared to spotted knapweed, this species seems to be better controlled by biological control agents (Coombs et al., 2004; Seastedt et al., 2003, 2007), and this may partly be attributed to adult feeding damage by *L. minutus* that causes water stress and consequently death of plants. The smaller capitulum size of diffuse knapweed may also result in this better control, as seedhead herbivores often consume all of the seeds in an infested seedhead. Additionally, the significantly greater seedhead herbivore loads in North America relative to Europe may partly explain this phenomenon. We detected seedhead herbivory at a level of 9% across European sites. This low level of seedhead infestation in the native range is interesting because the introduced biological control agents represent only a subset of the specialists enemies found to attack seedheads in Europe (Schroeder, 1985), and generalists may

cause some damage. Within North America across two field seasons, we found that approximately 60% of the seedheads showed evidence of herbivory, with a high of 95%; this damage can be attributed to the introduced specialist insects because there are no known native seedhead herbivores of the knapweeds in North America. Similarly, Smith (2004) reported seedhead infestation of 99% and 59% within two sites in Montana, USA. As intended, biological control of diffuse knapweed has increased enemy loads, resulting in seedhead herbivory in North America exceeding that found in Europe. Plants in the native range may not be controlled by top-down seedhead feeders; one plant in North America can produce 925 seeds in one season (Watson and Renney, 1974), even if one assumes seed production is roughly half of that in the native range due to disease and herbivory, a 9% reduction would still leave approximately 420 seeds for dispersal. This scenario supports the idea that top-down regulation of a plant by a certain enemy or guild of enemies in the native range is not requisite for successful population control by such specialists in the introduced range (Müller-Schärer and Schaffner, 2008).

The elevated rates of specialist seedhead herbivory on diffuse knapweed in North America compared to Europe could be due to one or more of the following: (1) the introduced biological control agents have escaped their enemies and competitors upon introduction to North America, allowing them to attain higher population densities, (2) larger plant populations in North America have allowed the insects to attain higher densities (Root, 1973; Kareiva, 1985) (3) the abiotic conditions are more favorable for seedhead herbivores in the introduced range, (4) plants in North America may have experienced selection for a trade-off of reduced defenses for increased growth [i.e. the 'Evolution of Increased Competitive Ability' hypothesis, Blossey and Nötzold, 1995], (5) hybridization has altered enemy dynamics (Fritz et al., 1999), and/or (6) regulation in the native range by specialist seedhead feeders fluctuates through time.

Increased enemy loads by specialist herbivores in the introduced vs. native range is a phenomenon that may be commonly found when biological control is successful; however, such a pattern has rarely been reported in the literature (but see Zwölfer and Zimmerman, 2004). While Young (2003) similarly found higher levels of floral herbivory by specialists in the introduced (Nebraska, USA) vs. native range (United Kingdom) on the introduced thistle *Cirsium vulgare*, the elevated herbivory in the introduced range resulted from a shift of specialists native to North American thistles (Takahashi, 2006), and not from introduced biological control agents. Counter to our findings, Wolfe (2002) found that two specialist herbivores of the weed *Silene latifolia* were either absent or found at very low levels in the introduced range. Unlike the knapweeds though, the *S. latifolia* specialists have not been introduced as part of a biological control program. Sheppard et al. (1994) found comparable levels of an important biological control weevil of *Carduus nutans* in its native and introduced regions.

Diffuse knapweed has, to some extent, escaped its specialist root herbivores in North America compared to Europe, but the difference is less pronounced than with spotted knapweed. Compared to other reported values of root herbivore loads (i.e. 74% and 69% in MT) (Smith, 2004), we found overall lower levels of diffuse knapweed root herbivory (24%). However, two of our sites had levels nearing the previously reported values (57% and 72%), and it seems likely that these insects are regionally rare, but locally abundant when present. While *C. achates* preferentially attacks spotted knapweed because of its larger root diameter (Stinson et al., 1994), it may play a role in diffuse knapweed control, as it might be more damaging to smaller roots (Smith, 2004). The root feeding beetle *Sphenoptera jugoslavica* also has the potential to reduce some infestations of diffuse knapweed (Powell and Myers, 1988). No research has been done to examine whether diffuse knapweed compensates for root herbivory like spotted knapweed has the potential to do (Steinger and Müller-Schärer, 1992). A recent study found that instead of compensation, sub-lethal above-ground feeding of diffuse knapweed by two biological control agents reduced knapweed performance. This reduction resulted in a small, but significant, increase in the performance of two native species (Norton et al., 2008).

#### 4.3. Hybridization, enemy load, and biological control

Hybridization has the potential to alter interactions with enemies (Fritz et al., 1999). A number of invasive plant species are both of hybrid origin (Ellstrand and Schierenbeck 2000) and the target of control efforts through biocontrol introductions, e.g. over 20 agents have been targeted at and established on the hybrid weed *Lantana camara* (Zalucki et al. 2007). Thus, it is important to understand how biological control and hybridization interact. In this study, in the field, hybrid-like plants did not exhibit different levels of specialist seedhead or root herbivore loads compared to diffuse-like plants. Similarly, in laboratory preference tests *L. minutus* did not discriminate between individuals from North American admixed diffuse knapweed sites and European genetically 'pure' diffuse knapweed ( $P = 0.54$ ). Recent hybridization in the BC1 individuals did not alter the preference when compared to 'pure' European diffuse knapweed ( $P = 0.59$ ). However, BC1 individuals were consumed significantly less when paired with North American diffuse knapweed ( $P = 0.004$ ). This finding raises the possibility that hybridization between the two species may initially result in a reduction of herbivore damage on hybrid individuals. Introgression with diffuse knapweed would, however, likely erase that advantage quickly through time, especially if the hybrids are rare within the population. As the introduced diffuse knapweed is not currently undergoing hybridization with spotted knapweed, these findings do not presently affect the population dynamics of diffuse knapweed in North America. If diploid spotted knapweed were to

invade North America, the impacts of hybridization on biological control may warrant further investigation.

#### 4.4. *Larinus minutus* preference of spotted vs. diffuse knapweed

Previous studies found various patterns of preference and/or performance of *L. minutus* for spotted vs. diffuse knapweed (Table 1). We did not, however, find a strong feeding preference when we paired either diploid European or tetraploid North American spotted knapweed with North American diffuse knapweed. We predicted that *L. minutus* would favor diffuse over spotted knapweed because *L. minutus* was collected for release from diffuse knapweed, and to a lesser extent, from a closely related species *C. grisenbachii* (U. Schaffner, personal communication). Additionally, we collected and maintained weevils on diffuse knapweed, which may have resulted in a bias for diffuse knapweed. While not significant, in both feeding trials with spotted and diffuse knapweed, *L. minutus* tended to prefer spotted over diffuse knapweed. If rearing conditions influenced host preference, inclusion of weevils maintained on spotted knapweed may have revealed a significant preference for that species.

*Larinus minutus* showed a significant preference for diploid European spotted knapweed over tetraploid North American spotted knapweed. While ploidy alone may be driving this pattern, it would be interesting in further studies to include European tetraploid spotted knapweed to tease apart whether ploidy or other differences cause this preference.

In summary, many hypotheses have offered mechanistic explanations for why an organism flourishes in its introduced region. This study set out to examine how biological control programs interact with such mechanisms. We found that biological control has the potential to reverse enemy escape and even result in herbivore loads that greatly exceed those found in the native range. Additionally, while hybridization has the potential to alter interactions with enemies, we did find a strong role for hybridization influencing biological control in this system.

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