

Trait-mediated interactions and lifetime fitness of the invasive plant *Centaurea solstitialis*

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Abstract. Plants interact with numerous enemies and mutualists simultaneously and sequentially. Such multispecies interactions can give rise to trait-mediated indirect effects that are likely to be common in nature but which are also inherently difficult to predict. Understanding multispecies interactions is also important in the use of biological control agents to control invasive plants because modern approaches to biocontrol rely on releasing multiple agents for each target weed. *Centaurea solstitialis* is one of the most problematic invasive weeds in California, USA, and the weevil *Eustenopus villosus* is its dominant biological control agent. We conducted a field experiment to quantitatively assess the direct effect of the recently approved biocontrol pathogen *Puccinia jaceae* f.s. *solstitialis* on plant performance and any indirect effects that might arise by altering the plant's interactions with *Eustenopus* or its pollinators (principally the nonnative *Apis mellifera*). We documented both synergy and interference between the two biocontrol agents depending on the life stage of the weevil. *Puccinia* infection increased the impact of bud-feeding by the adult weevils but reduced the impact of seed-feeding by larval weevils. Neither infection nor *Eustenopus* attack had an effect on pollinator visitation. The net effect was that attack by both the pathogen and the weevil did not reduce plant fitness relative to plants attacked only by the weevil. Because the consequence to the plant of interacting with one species may depend on the presence or absence of other interacting species, a careful consideration of multispecies interactions may be necessary for the selection of biocontrol agents that act in a complementary manner to reduce plant fitness. Likewise, relatively tractable weed–biocontrol systems allow us to examine multispecies interactions that can be difficult to study experimentally in native systems that are composed of numerous species with well-established populations.

Key words: biological control; California, USA; *Centaurea solstitialis*; cumulative stress; *Eustenopus villosus*; indirect interactions; interference; *Puccinia jaceae* f.s. *solstitialis*; synergy; yellow starthistle.

INTRODUCTION

In simple, pairwise interactions, herbivores and pathogens are generally expected to have a direct negative effect on plant performance while pollinators are expected to directly increase it. But plants interact with numerous enemies and mutualists simultaneously and sequentially and the net effect is rarely measured and poorly understood (Strauss and Irwin 2004). When an interaction with one species modifies a trait in the plant (e.g., its biochemistry, phenology, or morphology) that is important to a subsequent interactor, the net effect will be nonadditive if it changes the frequency or intensity of those subsequent interactions. For example, pathogen infection may elicit a biochemical defense in the plant that also protects it from later attack by herbivores (Karban et al. 1987, Baldwin and Schmelz 1996, Conrath et al. 2002). Masters et al. (2001) showed that root herbivory by one insect species increased seed

predation by another and hypothesized that root herbivory increased soluble nitrogen and carbohydrates and consequently the plant's attractiveness to the seed predators. Summing the interaction coefficients from pairwise tests would overestimate the net negative effect of two enemy species in the first case (Karban et al. 1987) and underestimate it in the second case (Masters et al. 2001).

Several studies have shown that herbivory can reduce a plant's ability to attract and reward its pollinators (Euler and Baldwin 1996, Strauss et al. 1996, Lehtilä and Strauss 1997) while in other cases herbivory can alter a plant's architecture and phenology in a way that increases the frequency of plant–pollinator interactions (Strauss et al. 2001). In a meta-analysis, Morris et al. (2007) found that the mean positive effect of mutualists was smaller than the mean negative effect of enemies in pairwise tests, yet in three-way interactions, mutualists cancelled out the negative effect of enemies. Here, summing interaction coefficients from pairwise tests would underestimate the positive effect of mutualists in the presence of enemies (Morris et al. 2007). Because

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enemies and mutualists can interact with one another to produce sub- or super-additive effects, it may be necessary to measure the impact of one species in the presence of others.

The study of multispecies interactions is particularly relevant to the biological control of invasive plants. As currently practiced, classical biological control promotes the use of several enemy species to control a single weed species (Denoth et al. 2002, Myers 2007, Van Driesche et al. 2008). Two alternative rationales underlie this approach. In the “lottery ticket” model of biocontrol (sensu Myers 1985), the expectation is that by introducing multiple agents, we increase the likelihood that we fortuitously discover a single effective agent. Alternatively, the “cumulative stress” model of biological control posits that multiple enemy species attacking the same plant will together have an impact sufficiently large to reduce plant performance even when the same species acting in isolation have only minimal impacts (Myers 1985, McEvoy and Coombs 1999, Van Driesche et al. 2008). Ideally, in this latter model, multiple enemies will even have a super-additive effect on the plant (as in Masters et al. 2001). However, multiple enemies may interfere with one another either directly, via competition (Ehler and Hall 1982, Denno et al. 1995), or indirectly, via trait modification as when pathogen infection primes the plant against subsequent attack by herbivores (Karban et al. 1987, Baldwin and Schmelz 1996, Conrath et al. 2002).

We took advantage of the release of a new biological control agent (hereafter biocontrol agent) to test the effect of multispecies interactions on the lifetime fitness of the invasive thistle *Centaurea solstitialis* (yellow starthistle) in California, USA. We performed a field experiment to assess how the new agent (*Puccinia jaceae* f.s. *solstitialis*) might alter the plant’s interactions with a well-established agent (*Eustenopus villosus*). *Puccinia jaceae* f.s. *solstitialis* is a fungal pathogen that produces nonsystemic foliar infections in the plant (see Plate 1). The weevil *Eustenopus villosus* is the most abundant biocontrol agent for this plant in California and acts as both a bud herbivore and a predispersal seed predator.

We wanted to know whether plants attacked by two enemies (pathogen and herbivore/seed predator) suffered greater reductions in fitness than plants attacked by only one (herbivore/seed predator), the idea that underpins the cumulative stress model of biocontrol. We also wanted to determine whether pathogen infection and weevil attack altered the plant’s interactions with its insect pollinators. Because *Puccinia* had not yet established in California at the time of this study, we were able to experimentally manipulate its presence in a way that is difficult to do with established pathogens.

To determine the direct effect of *Puccinia* infection on plant performance we asked (1) whether *Puccinia* infection reduces plant size, the number of buds and inflorescences each plant produces, or the number of

achenes and viable seeds produced per inflorescence. To determine whether *Puccinia* infection alters the plant’s interactions with adult *Eustenopus*, we asked (2) whether infected plants lose more (or fewer) buds to *Eustenopus* bud herbivory or (3) more inflorescences on infected plants are selected as oviposition sites. To determine whether *Puccinia* infection alters the plant’s interactions with larval *Eustenopus* (predispersal seed predators) we asked (4) whether *Puccinia* infection alters *Eustenopus* larval survival or (5) the proportion of seeds consumed by *Eustenopus* larvae. And finally, to determine whether *Puccinia* infection alters the plant’s interactions with its pollinators, we asked (6) whether infected plants are visited less frequently by pollinators or produce fewer viable seeds.

METHODS

Study system

Centaurea solstitialis L. (Asteraceae) is an annual thistle native to Eurasia. It was introduced to California in the 1860s and remained largely an agricultural pest until the 1950s when it began to spread into wildlands (Maddox and Mayfield 1985). It is now one of the most common species in California grasslands, occupying ~6 million hectares and is considered to be one of the most problematic invasive species in the state (Pitcairn et al. 2006).

Our study population was located at Mount Diablo State Park (37°51' N, 121°55' W, 432 m elevation; Contra Costa County, California). A population of *C. solstitialis* has been present at this particular location within the Park since at least 1944 (Ertter and Bowerman 2002). This site was chosen because it lies within what is currently the center of the plant’s geographic range in California and has climatic conditions that are typical of inland areas throughout the state where *C. solstitialis* has been most successful.

In 1990, the USDA introduced the weevil *Eustenopus villosus* (Coleoptera: Curculionidae) as a biocontrol agent, and it has since established in the vast majority of *C. solstitialis* populations in the state (Pitcairn et al. 2002), including our study site. Adult *Eustenopus* weevils feed on the youngest flower buds at the beginning of the flowering season and this feeding kills the bud. Adult females oviposit a single egg (up to 20 in a lifetime) in the capitulum where the larvae develop and consume the seeds as they mature. *Eustenopus* is univoltine.

Bud herbivory and seed predation by *Eustenopus* has not satisfactorily reduced *C. solstitialis* density in California (DiTomaso and Healy 2007). Consequently, the USDA approved the release of *Puccinia jaceae* f.s. *solstitialis* (Uredinales: Pucciniaceae) in 2003 and field releases began the following year at three dozen sites across the state. *Puccinia* is a rust fungus that produces nonsystemic foliar infections that reduce root biomass in a laboratory setting but rarely cause mortality (Shishkoff and Bruckart 1996). Aside from our experimental

infections of individual plants for this study, *Puccinia* was not present at Mt. Diablo State Park.

The plant's interactions with *Puccinia* and *Eustenopus* are spatially and temporally separated from one another and so these two agents do not interact directly. *Puccinia* infection is nonsystemic and confined to the leaves during the cooler, wetter winter and spring months. The plant is exposed to *Eustenopus* attack only after it drops its leaves and begins to produce buds and flowers at the beginning of summer. Because *C. solstitialis* is an annual and it is attacked first by *Puccinia* and then by *Eustenopus* after which the plant dies, *Puccinia* can indirectly affect *Eustenopus* but *Eustenopus* cannot indirectly affect *Puccinia*. Both species are specialists that do not use other plants at any life stage. *Centaurea solstitialis* is self-incompatible and depends on generalist insect pollinators for reproduction (Sun and Ritland 1998).

Experimental Puccinia infections and Centaurea–Puccinia interactions

We randomly selected 200 naturally recruiting plants at the seedling stage in an established invasion; half of the seedlings were assigned to the control group (uninfected plants) and the other half were experimentally infected with *Puccinia* on 10 February 2006, five months prior to flowering. One hundred percent of our experimental infections were successful and no uninfected plants showed signs of infection. To experimentally infect plants we used a solution of 100 mg urediniospores and 100 mL of distilled water and the wetting agent Tween20 (polyoxyethylene sorbitan monolaurate; Acros Organics, Morris Plains, New Jersey, USA). Uninfected plants were sprayed with distilled water and Tween20. After each plant was sprayed, it was covered with a 20 × 20 cm plastic tent for 16 h to encourage germination of the pathogen (uninfected plants were also tented).

We checked the survival of each plant weekly and when a plant senesced in the field we measured height and counted the number of buds and inflorescences and then collected it by clipping it at ground level to measure biomass (dried at 60°C for 24 h).

Interactions with adult Eustenopus: herbivory and oviposition choice

Eustenopus is present throughout our study population. Buds that have been fed on by adult *Eustenopus* are dead and have an obvious feeding hole at the base. *Eustenopus* also leaves a diagnostic oviposition wound on the outside of the capitulum, making it possible to nondestructively identify all inflorescences used as oviposition sites. We assume a one-to-one correspondence between an oviposition wound and an egg laid because the wound is sealed with frass and it is unlikely that a weevil would seal the wound if she rejected it as an oviposition site. We counted the number of undamaged

and herbivore-damaged buds and the number of inflorescences with and without oviposition wounds for each plant. Our response variables were both the total number of damaged buds and inflorescences as well as the proportion of buds and inflorescences that had been attacked.

In other work, we have attempted to reduce *Eustenopus* bud-feeding and egg-laying on selected experimental plants by spraying them with the insecticide Ortho Systemic Insect Killer (formerly Isotox; Scotts, Marysville, Ohio, USA), without success. At higher concentrations, Isotox also appeared to have phytotoxic effects. Isotox has effectively reduced larval seed feeding by other species (e.g., Louda 1982), including the tephritid fly *Chaetorellia succinea* (Garren and Strauss 2009), which is another *Centaurea solstitialis* biocontrol agent. *Chaetorellia succinea* was rare at our study site and appears to be more susceptible to the insecticide's effects than *Eustenopus* (Garren and Strauss [2009] found a similar differential response between the two agents to the insecticide). A full factorial design in which we reduced *Eustenopus* abundance would have been ideal but we found no effective way to do this so we did not attempt to manipulate its presence in this study.

Interactions with larval Eustenopus: pre-dispersal seed predation

Puccinia infection might affect *Eustenopus* larvae in two ways. It might alter larval survival or it might change the number of seeds they consume as they develop in the capitulum. While it is possible to nondestructively identify inflorescences in which a female *Eustenopus* has laid an egg, it is necessary to destructively sample inflorescences to determine the fate of each larva. To do this, we removed mature inflorescences immediately prior to seed dispersal by clipping them from the plant, storing them in separate coin envelopes, and carefully dissecting them in the laboratory. It is very unlikely that removing inflorescences in this manner caused a compensatory response in the plant because we removed inflorescences immediately prior to seed dispersal, only once the pedicle had senesced and the petal cap had loosened but not fallen from the capitulum.

Signs of larval mortality and survival are easy to distinguish from one another, and this allowed us to quantify larval survival. When the larvae died, we found only a very small amount of damaged plant tissue inside the capitulum directly behind the oviposition wound; when the larvae survived, we found a well-developed pupal chamber with frass and partially eaten seeds (and frequently an adult weevil had emerged in the coin envelope).

We quantified the impact of larval feeding on seed production in the following manner. On each weekly visit to the site, we checked every plant for two inflorescences at the same phenological stage (immedi-

ately prior to seed dispersal), one with an oviposition wound and one without. By matching inflorescences in this way we accounted for plant-to-plant variation in seed production as well as temporal variation due to changes in resource availability and/or the pollinator community. We estimated the proportion of seeds consumed as

$$\frac{(\text{no. viable seeds UF} - \text{no. viable seeds } E_S)}{\text{no. viable seeds UF}}$$

where UF refers to unattacked inflorescences (those without an oviposition wound) and E_S refers to inflorescences in which a *Eustenopus* larva has survived to maturity. Viable seeds are easy to distinguish from nonviable achenes with a dissecting scope based on size, shape, and color.

We were unable to get such a pairing for each plant. Ultimately, we were able to calculate seed consumption from 81 pairings for 48 uninfected plants and 75 pairings for 44 infected plants. Occasionally, seeds began to disperse between visits to the field site. In these cases we were unable to count the number of seeds or to calculate larval seed feeding. We were, however, still able to determine whether the larva had survived.

Interactions with pollinators

To assess whether *Puccinia* infection and/or *Eustenopus* attack altered the rate at which pollinators visited *Centaurea solstitialis* plants, we conducted a single 30-min pollinator observation for each plant. (Previous work [Barthell et al. 2001; S. M. Swope, unpublished data] found that 30-min observation periods were sufficient to characterize pollinator visitation for *C. solstitialis* at several sites in California.) Each plant was observed individually and in random order when it reached peak flowering. Observations took place between 07:30 and 09:00, peak activity for pollinators at this site (S. M. Swope, unpublished data), and were suspended if conditions were unsuitable (typically because of wind) and were conducted as soon as conditions permitted. We recorded the number of pollinator visits and the pollinator species. A visit was defined as a pollinator landing on an inflorescence for ≥ 2 s.

It can be difficult to relate pollinator visits to effective pollination. In particular, we counted multiple visits to the same plant equally, even though a visit to a second or third inflorescence may be less effective than the first visit. We know that sequential visits of *Apis mellifera* can carry enough outcross pollen to effectively pollinate multiple flowers in other species (Cresswell et al. 1995, Michaelson-Yeates et al. 1997), but pollen carryover rates have not been estimated for *C. solstitialis*. With multiple visits counted per pollinator, there is the potential in our data for inflating the significance of differences in visitation between treatment groups. However, we found no such differences (see *Results*).

To assess effective pollination beyond visitation rates, we also compared the percentage of achenes that produced viable seeds for inflorescences from infected and uninfected plants. This may be considered the more important metric from the plant's perspective as it reflects the outcome of pollinator visitation. For this analysis, we restricted the comparison to unattacked inflorescences to avoid the obvious complications of losses of achenes (viable and nonviable) to seed-feeding larval weevils. This approach can reveal if *Puccinia* infection leads to a change in the proportion of seeds set. Such a change would suggest a change in pollination as the mechanism, although we cannot exclude the possibility that resource limitation may also contribute.

Statistical analyses

All analyses were conducted using Systat version 10.2 (SPSS, Chicago, Illinois, USA). To determine whether *Puccinia* infection had a direct effect on *C. solstitialis* performance, we used a two-tailed independent-samples *t* test. The response variables were *C. solstitialis* height, $\ln(\text{biomass})$, $\ln(\text{number of buds})$, $\ln(\text{number of inflorescences} + 1)$, and both the number and percentage of viable seeds per unattacked inflorescence. We used a *G* test to determine if *Puccinia* infection reduced seedling survival to flowering.

We compared the number of damaged buds and the number of inflorescences with *Eustenopus* oviposition wounds on *Puccinia*-infected vs. -uninfected plants (both $\ln + 1$ -transformed) using *t* tests. We also compared the proportion of buds killed by herbivory and the proportion of inflorescences with oviposition wounds. We tested for an effect of *Puccinia* on the proportion of larvae that survived and on the proportion of seeds consumed by the larvae on a per capitulum basis.

To determine whether *Puccinia* infection and/or *Eustenopus* attack affected pollinators' choice of which plants to visit, we used ANCOVA with infection status as the independent variable and the proportion of inflorescences with oviposition wounds as a covariate, and the response variable was the total number of pollinator visits to each plant.

To assess how all direct and indirect effects combined to alter lifetime fitness of the plant, we compared total seed production on a whole-plant basis. Due to the logistical challenges of collecting every seed produced by plants with several dozen inflorescences, we were only able to obtain total seed counts for a subset of uninfected ($n = 19$) and infected ($n = 21$) plants. For the remaining plants, we estimated whole-plant seed production by counting the number of inflorescences in each attack category (unattacked, with an oviposition wound but within which the larva died, and with an oviposition wound but within which the larva survived) and multiplying by the mean number of seeds produced by inflorescences in each of these categories. Mean seeds per inflorescence was calculated separately for each plant to account for differences in per inflorescence seed

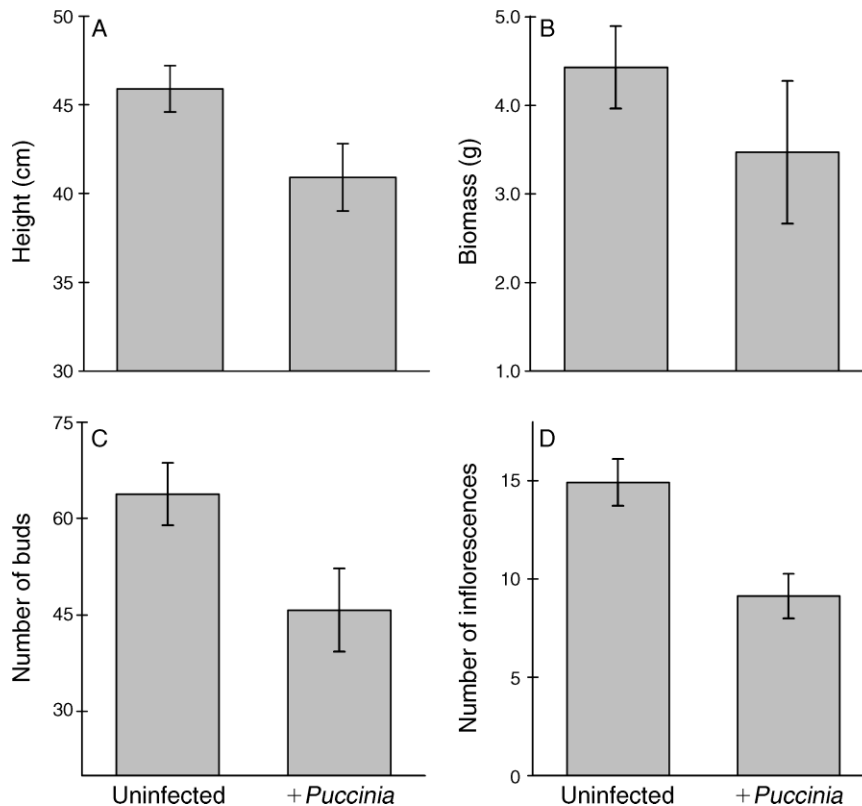


FIG. 1. Direct effects (mean \pm SE) of *Puccinia jaceae* f.s. *solstitialis* infection on *Centaurea solstitialis* performance: (A) plant height ($t_{120} = 2.10$, $P = 0.04$); (B) biomass at senescence ($t_{120} = 1.88$, $P = 0.06$); (C) total number of buds produced ($t_{115} = 2.04$, $P = 0.04$); (D) total number of buds that survived to flowering ($t_{91.8} = 3.53$, $P = 0.001$). Variables were transformed for analysis as described in *Methods*; untransformed data are shown in the figure. When Bonferroni-corrected to control for family-wise error rate, the critical P value would be 0.0125. Our study population was located at Mount Diablo State Park, California, USA.

production among plants. Data were ln transformed for analysis.

RESULTS

Direct effect of Puccinia infection on individual plant performance

Pathogen infection did not reduce the likelihood that *C. solstitialis* plants survived to flowering ($G^2 = 0.523$, $P = 0.23$). Sixty-three of the 100 uninfected plants and 58 of the 100 *Puccinia* plants survived to flowering. But *Puccinia* infection did have a direct negative effect on other aspects of plant performance. Infected plants were shorter than uninfected plants (Fig. 1A) and biomass was lower (Fig. 1B). Infected plants produced fewer buds (Fig. 1C) and inflorescences (Fig. 1D) than uninfected plants. However, there was no difference among infected and uninfected plants in terms of the number of achenes (maximum potential seed production) or viable seeds produced by unattacked inflorescences. On average, infected plants produced 34.3 ± 2.8 achenes per capitulum (mean \pm SE) and uninfected plants produced 36.0 ± 1.9 achenes per capitulum ($t_{241} = 1.16$, $P = 0.25$). Infected plants produced 28.6 ± 1.19 viable seeds per capitulum and uninfected plants

produced 30.0 ± 2.1 viable seeds per capitulum ($t_{241} = 1.36$, $P = 0.57$).

Interactions between Puccinia and adult Eustenopus

There was no difference in the number of herbivore-damaged buds between infected and uninfected plants (damaged buds on infected plants, 15.2 ± 2.3 ; uninfected plants, 15.9 ± 1.5 ; $t_{120} = 0.28$, $P = 0.78$). But because infected plants produced fewer total buds, they suffered greater attack than uninfected plants when measured on a proportional basis (Fig. 2A). Likewise, there was no difference between infected and uninfected plants in the number of inflorescences selected by adult *Eustenopus* as oviposition sites (damaged inflorescences per infected plant, 13.6 ± 2.7 ; uninfected plant, 15.6 ± 1.6 ; $t_{120} = 1.22$, $P = 0.23$). But because infected plants produced fewer total inflorescences, they suffered proportionally higher attack than uninfected plants (Fig. 2B).

Interactions between Puccinia and larval Eustenopus

Puccinia did not alter *Eustenopus* larval survival. On average, $50\% \pm 6.5\%$ larvae survived to emergence when they developed in the inflorescences of infected plants as compared to $55\% \pm 5.8\%$ developing on uninfected

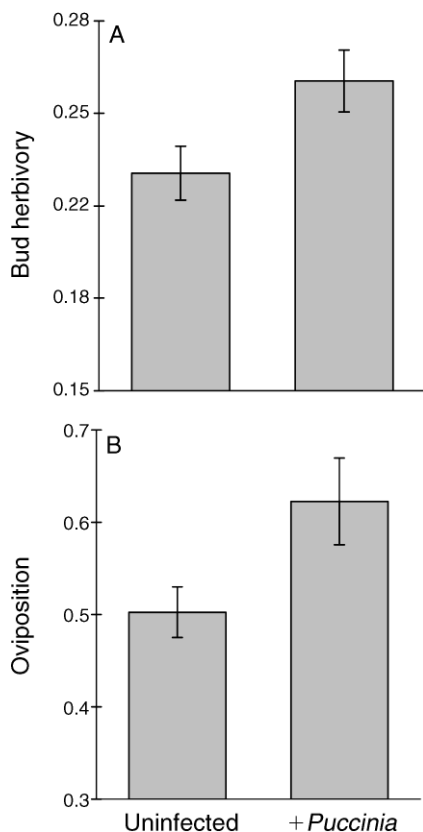


FIG. 2. Indirect effect (mean \pm SE) of *Puccinia* infection on *Centaurea solstitialis*' interactions with adult *Eustenopus villosus* weevils: (A) the proportion of *C. solstitialis* buds consumed by adult *Eustenopus* in the feeding stage ($t_{84,8} = 2.30$, $P = 0.025$); (B) the proportion of *C. solstitialis* inflorescences selected as oviposition sites by adult *Eustenopus* in the egg-laying stage ($t_{114} = 2.29$, $P = 0.02$). Variables were transformed for analysis as described in *Methods*; untransformed data are shown in figure. When Bonferroni-corrected to control for family-wise error rate, the critical P value would be 0.025.

plants ($t_{120} = 0.15$, $P = 0.60$). But when *Eustenopus* larvae survived, they consumed proportionally fewer seeds within the capitulum when they were developing on infected plants than when they were developing on uninfected plants (Fig. 3). Because there was no difference in the number of viable seeds produced by unattacked inflorescences on infected plants as compared to uninfected plants (see *Direct effect of Puccinia infection...*), this difference cannot be explained by fewer seeds (per inflorescence) on infected plants.

Interactions between Puccinia, Eustenopus, and pollinators

Peak flowering for all plants occurred over a 17-d period, and our data do not suggest a change in the composition or abundance of pollinators over that time. Pollinator visitation was frequent, about one every 3 min (8.9 ± 2.6 pollinator visits per plant [mean \pm SD]) and was dominated by *Apis mellifera*, which represented

97% of the total pollinator visits (and 91–100% of the visits on each day of observation). Together, one native bee (*Bombus vosnesenskii*) and an unidentified bee fly (Bombyliidae) represented 3% of the total pollinator visits we observed. *Centaurea solstitialis*' pollinators were unaffected by *Puccinia* infection and *Eustenopus* attack ($F_{1,120} = 0.12$, $P = 0.76$).

There was also no difference in the proportion of achenes that produced viable seeds among inflorescences from infected and uninfected plants ($t_{145} = 0.58$, $P = 0.56$). On average, $73.5\% \pm 6.3\%$ [mean \pm SE] of achenes from inflorescences on infected plants produced viable seed and $65.9\% \pm 10.5\%$ of the achenes from inflorescences on uninfected plants produced viable seed.

Whole-plant seed production

We wanted to determine how these direct and indirect effects together affected the total number of seeds *C. solstitialis* plants produced and specifically whether plants attacked by both *Puccinia* and *Eustenopus* produced more or fewer seeds than those attacked only by *Eustenopus*. When comparing plants for which we had whole-plant seed counts, we found no significant difference in the number of seeds produced by plants attacked by both agents and those attacked only by *Eustenopus* (Fig. 4). When comparing estimates of whole-plant seed counts, we also found no difference between infected and uninfected plants (infected plants, 241 ± 51.0 seeds; uninfected plants, 262 ± 27.6 seeds; $t_{112} = 1.43$, $P = 0.17$).

DISCUSSION

All plants interact with multiple species in their lifetimes, some of which may enhance their performance (e.g., pollinators) while others may reduce it (e.g., herbivores and seed predators). Multispecies interac-

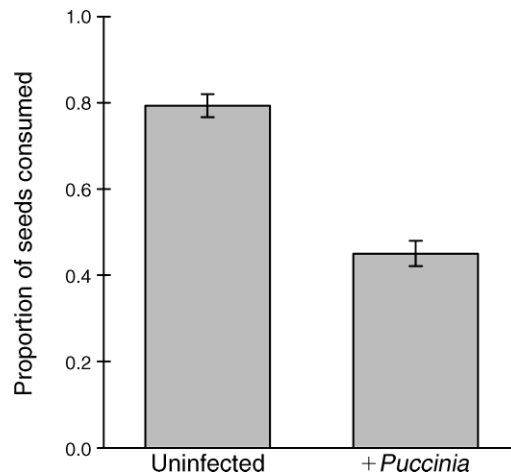


FIG. 3. Proportion (mean \pm SE) of seeds consumed by *Eustenopus* larvae developing in the inflorescences of *Centaurea solstitialis* plants uninfected and infected by *Puccinia* ($t_{154} = 8.63$, $P = 0.001$).

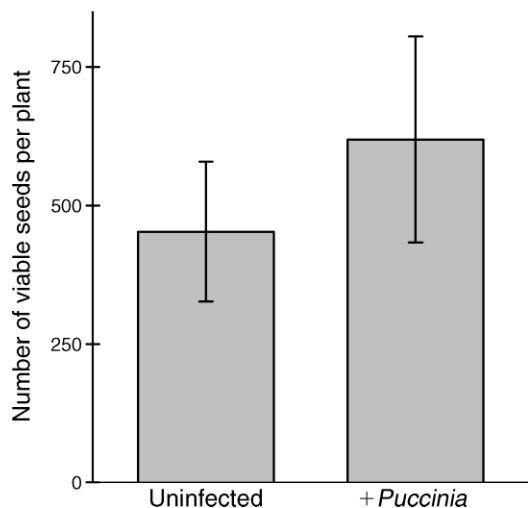


FIG. 4. Net effect (mean \pm SE) of *Eustenopus* herbivory and seed predation and pollinator visitation on the total number of seeds produced by *Centaurea solstitialis* plants uninfected and infected by *Puccinia* ($t_{38} = 0.477$, $P = 0.55$). Data were transformed for analysis as described in *Methods*; untransformed data are shown in figure.

tions can give rise to trait-mediated indirect effects that appear to be common in natural systems (reviewed by Strauss and Irwin 2004, Stout et al. 2006, Kaplan and Denno 2007, Morris et al. 2007). Our goal was to examine how interactions among two specialist enemies and generalist pollinators affected growth and seed production in an annual invasive plant that is the target of biological control.

In our study, plants were visited almost exclusively by *Apis mellifera*, which is known for being a highly effective pollinator. Visitation rates were high and pollinators did not discriminate against infected plants nor did they respond to the proportion of inflorescences attacked by *Eustenopus*. This is in contrast to other systems in which enemy attack has been shown to reduce pollinator attraction, leading to lower seed set (Karban and Strauss 1993, Strauss et al. 1996, Lehtilä and Strauss 1997, Mothershead and Marquis 2000). While it is possible that reproduction is limited by pollinator visitation in this self-incompatible annual plant, our observations in multiple populations and multiple years suggest that high visitation rates are typical (Barthell et al. 2001; S. M. Swope, unpublished data). Therefore, our results suggest that the effectiveness of these two biocontrol agents will not be enhanced (or diminished) by their incidental effects on pollinators.

We expected that sequential attack by *Puccinia* and *Eustenopus* could have one of two opposing effects on *C. solstitialis* seed production. The net effect of being attacked by two enemies ought to be greater than the effect of being attacked by one (additive or super-additive) if *Puccinia* infection reduced overall plant performance and/or increased susceptibility to *Eustenopus* and if *Eustenopus* did not discriminate against

infected plants. Alternatively, plants attacked by both enemies could have higher fitness if the enemy with the weaker direct effect (*Puccinia*) reduced the frequency or intensity of attack by the enemy with the greater direct effect (*Eustenopus*). This might reasonably occur if *Eustenopus* actively avoided infected plants, if infected plants were smaller and therefore had a greater probability of avoiding discovery by *Eustenopus*, or if *Eustenopus* consumed fewer seeds on infected plants.

We found evidence for both synergistic interactions and interference between *Puccinia* and *Eustenopus*, depending on the life stage of *Eustenopus*. *Puccinia* infection reduced the number of buds and inflorescences plants produced, and because *Eustenopus* showed no avoidance of smaller infected plants, infected plants suffered greater proportional attack by both bud-feeding and egg-laying *Eustenopus*. But we also found that the species interfered with one another when *Eustenopus* was at the larval stage. Larval seed predators consumed fewer seeds per inflorescence when they matured on infected plants. The net effect was that there was no difference in the number of seeds produced by plants attacked by both enemies vs. those attacked only by one.

One possible mechanism underlying the reduced seed predation of larval *Eustenopus* on infected plants is a biochemical response on the part of the plant to infection that is also effective against seed predators. Attack by biotrophic pathogens can induce systemic acquired resistance (SAR) in plants. While *Puccinia* infection is itself nonsystemic, numerous studies have shown that induced defenses such as SAR tend to be systemic even when damage is localized; SAR can be induced by both pathogens and insects and can be broadly effective against both groups of enemies (e.g., Karban et al. 1987, Baldwin and Schmelz 1999, Conrath et al. 2002, Rojo et al. 2003).

Another possible mechanism is that infection changes the quality of the plant as a food source for *Eustenopus*. Other work has shown that root herbivory can induce drought stress in plants, which, by reducing tissue water content, effectively increases the concentration of soluble nitrogen and carbon, making the plant a higher quality food source for the larvae (Masters and Brown 1992). *Puccinia* infection reduces taproot growth and biomass in the greenhouse (Shishkoff and Bruckart 1996) and *C. solstitialis* is dependent on its taproot to access deep soil moisture during the hot, dry summer months when it flowers. It is plausible that infection also induces drought stress, and, because drought-stressed plants may increase allocation of resources to inflorescences (Chapin 1980), *Puccinia* infection may increase the quality of the seeds in particular as a food source. Seed predators are highly responsive to small changes in the nutritional status of the host plant (Tamura and Hiara 1998) and so may be able to complete metamorphosis while consuming fewer seeds.



PLATE 1. *Puccinia jaceae* fs. *solstitialis* infection on a leaf of *Centaurea solstitialis*. Photo credit: Dale Woods.

We might expect larval survival to be lower on infected plants if SAR is the mechanism and expect survival to be the same or higher if infection leads to higher nutrient concentrations. We found that larval survival was not different on infected and uninfected plants, consistent with the latter hypothesis. We are currently testing for the possible role of SAR and/or plant tissue nitrogen and carbon in the interference between *Puccinia* and larval *Eustenopus*.

Implications for biological control

Classic niche-based competition theory predicts that interference is most likely to arise between species that attack the same part of the plant, either directly via interference competition or indirectly via exploitation competition (Schoener 1974, Connell 1980), and this idea has long guided the selection of biocontrol agents. But our understanding of how plants respond to both herbivory and pathogen infection is evolving and it appears that plant-mediated interactions occur frequently between species that never encounter one another (reviewed by Strauss and Irwin 2004, Stout et al. 2006, Kaplan and Denno 2007, Morris et al. 2007). In fact, in their recent meta-analysis, Kaplan and Denno (2007) found that the strength of the competitive interaction between phytophagous insects was not correlated with the degree of spatiotemporal overlap. This has important implications for biocontrol because it means that selecting agents that attack different parts of the plant at different times may not reduce the likelihood that they will interfere with one another. Further, even modest herbivore and/or pathogen damage, including cases in which there was no detectable effect on plant growth or

fitness, can reduce the impact of subsequent interactors (Stout et al. 2006, Denno and Kaplan 2007, Kaplan and Denno 2007; S. M. Swope and I. M. Parker, *unpublished data*). In other words, attack by an ineffective agent can still have important indirect effects. But just as interference may readily occur among species that are spatiotemporally separated from one another, indirect synergistic interactions are also possible (Rieske and Raffa 1998, Kluth et al. 2001, Masters et al. 2001, Wallin and Raffa 2001). This is the ideal outcome in a biocontrol scenario, and an explicit consideration of indirect interactions may help practitioners realize the goal of the cumulative stress model of biocontrol, namely that multiple agents will act in a complementary manner to reduce plant performance. Whether interactions are synergistic or antagonistic, it is clear that they need not be direct to be important.

Whether the nature of these interactions is synergistic or antagonistic (or neutral) may depend on the particular species involved (Van Zandt and Agrawal 2004). For example, Kluth et al. (2001) measured the response of several oligophagous insects to *Puccinia punctiformis* infection of their host plant *Cirsium arvense* and found that different species of insects responded in different ways. The majority of the insects showed neither a preference for nor an avoidance of infected plants, but stem-boring *Apion* weevils were more abundant on infected plants while another stem-borer (*Melanagromyza aeneoventris*) showed strong avoidance of infected hosts. *Cirsium arvense* is invasive in North America, and this work, which was conducted in the native range, suggests that *Puccinia*–*Melanagromyza* would not be an effective pair of biocontrol agents but

that *Puccinia*–*Apion* spp. may be. The idiosyncratic nature of these interactions means that we cannot assume that because one agent interferes with another that it would not function synergistically with a third. In practical terms this means that every proposed biocontrol agent needs to be tested independently for its interactions with all other agents.

We have pointed to numerous examples from the literature that demonstrate that the outcome of many interactions is species-specific. Our study shows that the outcome may even depend on the life stage of the species involved: *Puccinia* infection enhanced the impact of adult *Eustenopus* but reduced the impact of their larvae. The effectiveness of biocontrol agents is frequently assessed primarily or exclusively using attack rates (e.g., Crowe and Bouchier 2006). In the case of *Centaurea solstitialis* biocontrol, measuring only interactions between the pathogen and adult weevils (bud herbivory and oviposition choice) would have revealed only super-additive effects on the plant and led to the conclusion that these agents successfully complement one another. Larval seed consumption is more rarely considered in evaluating the effectiveness of biocontrol but in *C. solstitialis*, it was essential to measure larval seed consumption in order to reveal the full range of indirect interactions between *Puccinia* and *Eustenopus*.

Biocontrol remains a potentially powerful, and perhaps our only, tool for controlling widespread invasive plants, but it is not without inherent risks. The principle risk of biocontrol is nontarget attack, i.e., when an agent attacks native and/or agricultural species, and much attention has deservedly been paid to this topic (e.g., Louda and Potvin 1995, Simberloff and Stiling 1996, Strong and Pemberton 2001). Many have argued for releasing as few agents as necessary to minimize the risk of nontarget attack (e.g., Waage 2001, Crowe and Bouchier 2006). We suggest that the possibility of indirect interference between agents may be an underappreciated risk of the multi-agent approach to biological control of invasive plants. It is tempting to release an ineffective agent after investing time and money to demonstrate that it is host specific. However, because even an agent that has no apparent direct effect on plant performance may still have indirect effects on other interacting species, ineffective agents should not be released because they may interfere with established agents or even with prospective agents that have yet to be identified.

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