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Does dimethyl sulfoxide increase protein immunomarking efficiency for dispersal and predation studies?

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Abstract

Marking biological control agents facilitates studies of dispersal and predation. This study examines the effect of a biological solvent, dimethyl sulfoxide (DMSO), on retention of immunoglobulin G (IgG) protein solutions applied to *Diorhabda carinulata* (Desbrochers) (Coleoptera: Chrysomelidae), an important biological control agent of saltcedar, either internally by feeding them protein-labeled foliage or externally by immersing them in a protein solution. In addition, we determined whether internally or externally marked DMSO-IgG labels could be transferred via feeding from marked *D. carinulata* to its predator, *Perillus bioculatus* (Fabricius) (Heteroptera: Pentatomidae). The presence of rabbit and chicken IgG proteins was detected by IgG-specific enzyme-linked immunosorbent assays (ELISA). DMSO-IgG treatments showed greater label retention than IgG treatments alone, and this effect was stronger for rabbit IgG than for chicken IgG. Fourteen days after marking, beetles immersed in rabbit IgG showed 100% internal retention of label, whereas beetles immersed in chicken IgG showed 65% internal retention. Immersion led to greater initial (time 0) label values, and longer label retention, than feeding beetles labeled foliage. The DMSO-IgG label was readily transferred to *P. bioculatus* after feeding on a single marked prey insect. This investigation shows that addition of DMSO enhances retention of IgG labels, and demonstrates that protein marking technology has potential for use in dispersal and predator–prey studies with *D. carinulata*. Moreover, our observation of *P. bioculatus* feeding on *D. carinulata* is, to our knowledge, a new predator–prey association for the stink bug.

Introduction

Successful establishment and population growth of weed biological control agents are fundamentals to their success. Natural enemies of weed biological control agents can impact establishment and population growth, as well as population dynamics, and thus can be a detriment to weed suppression (Goeden & Louda, 1976). The role that top-down forces play in weed suppression appears to vary with the system in question, but their occurence is not uncommon (Hunt-Joshi et al., 2005; Dávalos & Blossey, 2010; Paynter et al., 2010). Nearly half of the failures of exotic herbivores to establish in weed biological control projects worldwide have been attributed to natural enemies (Julien & Griffiths, 1998). At times when agent densities are relatively low, such as after their initial release or after periods of intense herbivory when suitable host plants are not available, natural enemies can act as a drain on the agent's local population and thus limit its chance for establishment. A better understanding of predator–prey interactions of weed biological control agents would lead to strategies for improved success of biological control programs (van Driesche & Bellows, 1996).

Studies of arthropod predation in the field have adopted several approaches, including direct visual observation of

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predation events, predator exclusion or inclusion cage experiments, experimental measurement of predator–prey dynamics, molecular gut content analysis, and transference of a labeled prey to a predator (Southwood, 1978; van Driesche & Bellows, 1996). Hagler et al. (1992) described an immunological approach to mark insects for dispersal studies using protein markers. A subsequent study showed that immunomarking methodology can also be a powerful method to study arthropod predation (Hagler & Durand, 1994). Specifically, protein-marked prey can be detected in the guts of predators that consumed them using protein-specific enzyme-linked immunosorbent assays (ELISA) (Hagler, 2006, 2011; Buczkowski et al., 2007; Mansfield et al., 2008; Kelly et al., 2012).

Dimethyl sulfoxide (DMSO) is an organosulfur compound with important uses in molecular biology (i.e., to minimize interfering reactions in PCR studies), and in cell cryoprotection research (Santos et al., 2003; Rowley, 2009). The solvent properties of DMSO also make it a useful vehicle for medication (Mathes et al., 2010). In insect pheromone research, DMSO facilitates the uptake of compounds applied topically to pheromone glands (Choi et al., 2005). We know of no studies exploring the use of DMSO to enhance protein label uptake and retention in studies of arthropod dispersal or predation.

Saltcedar [*Tamarix* spp. (Tamaricaceae)] was intentionally introduced from Eurasia to the southwestern USA in the 1800s to reduce soil erosion and provide windbreaks (DiTomaso, 1998). This perennial shrub-tree spread rapidly, invading riparian habitats and other wetlands in many western states (DiTomaso, 1998; Friedman et al., 2005). More than 800 000 ha of highly valuable watersheds in North America are currently infested with saltcedar, and there is potential for further spread (Morisette et al., 2006). Saltcedar infestations impact water reserves, soil salinity, flooding and fire frequency, and health of native species (DiTomaso, 1998; Lovich & de Gouvenain, 1998; Shafroth et al., 2005). Economic impact of saltcedar-caused losses is estimated at 16 billion USD to consumers, producers, and the natural environment in the USA (Zavaleta, 2000).

In 2001 a complex of four *Diorhabda* species (Coleoptera: Chrysomelidae) was released in the USA for the classical biological control of saltcedar (Tracy & Robbins, 2009). One of these, *Diorhabda carinulata* (Desbrochers), has established in Nevada, Utah, Wyoming, and Colorado, where it is killing saltcedar in some areas (DeLoach et al., 2008). However, observational evidence suggests that predation by ants and other arthropods may adversely affect population dynamics of *Diorhabda* species (Moran et al., 2009).

The two-spotted stink bug, *Perillus bioculatus* (Fabricius) (Heteroptera: Pentatomidae), is predaceous and native to North America (Knight, 1952). Under laboratory and field conditions, *P. bioculatus* predation has been recorded on 31 host species representing four insect orders (Saint-Cyr & Cloutier, 1996). About half of these prey species are chrysomelid beetles, including the Colorado potato beetle, *Leptinotarsa decemlineata* (Say). *Perillus bioculatus* is an important natural enemy of *L. decemlineata*, with potential for biological control (Hough-Goldstein et al., 1996; Weber, 2008). We observed nymphal and adult *P. bioculatus* feeding on larval and adult *D. carinulata* at several saltcedar infestations in northern Nevada, USA.

Previous work with *D. carinulata* characterized the retention of immunoglobulin (IgG) labels under laboratory and field conditions (Williams et al., 2011). In that study, rabbit and chicken IgG labels showed >80% retention on adult beetles for ca. 14 days after marking. In addition, IgG labels externally applied to *D. carinulata* eggs were retained by emerging larvae; label retention declined as larvae aged. That study also revealed that the larvae retained rabbit IgG to a greater extent than chicken IgG. This study investigates the effect of DMSO on retention time of IgG solutions applied to *D. carinulata*, and whether DMSO-IgG labels can be transferred from a prey insect (*D. carinulata*) to a predator (*P. bioculatus*). Here, we applied rabbit or chicken immunoglobulin IgG with and without DMSO internally to adult *D. carinulata* by allowing beetles to either feed on labeled plant foliage or externally by immersing beetles into labeled solution. We then determined the duration of label retention for the beetles. In the predation study, we fed DMSO-rabbit IgG-marked *D. carinulata* adults to adult *P. bioculatus* to determine whether the IgG label could be transferred to a predator after one predation event.

Materials and methods

Insect collection and rearing

Adult *D. carinulata* were collected with a sweepnet from *Tamarix ramosissima* Ledeb. near the Walker River, ca. 65 km south of Fallon, Nevada (38°53′03.23″N, 118°47′42.66″W, 1 237 m a.s.l.) in the spring and summer of 2009. Beetles were sorted into groups of ca. 300 and held at 25 ± 1 °C, 50–70% r.h., and L16:D8 photoperiod in Plexiglas cages (26 × 26 × 20 cm) until experimentation. Beetles were provided with *T. ramosissima* stems in plant nutrient solution (OASIS® Clear Solution™; Smithers-Oasis, Kent, OH, USA) as a food source. Foliage was replaced every 3 days.

Perillus bioculatus adults and nymphs were captured by sweepnet or by hand on *T. ramosissima* at several sites in Reno, Nevada, in the summer of 2009. At the time of collection, *P. bioculatus* were commonly observed feeding on *D. carinulata*. Bugs were held individually in ventilated 45-ml plastic vials under the environmental conditions given above, and provided with three or four unmarked *D. carinulata* adults per week until experimentation.

Insect marking procedures

Prey marking study – Effect of DMSO and application method. The study was setup as a randomized complete block design with four treatment factors. The first treatment factor was the protein label, which consisted of rabbit IgG (Sigma, St. Louis, MO, USA), chicken IgG (Rockland Immunochemicals, Gilbertsville, PA, USA), or ddH_20 (untreated control). The second treatment factor was method of label application, which was either immersion of beetles in IgG solution, or feeding beetles IgG-marked foliage. The third treatment factor was the inclusion, or not, of DMSO (Sigma) in the IgG mixture. The fourth treatment factor was the post-marking interval, which was assessed 0, 1, 3, 5, 7, 14, and 21 days after marking. Three replicates were set up for each treatment combination. The treatment factors were fixed, replication was a random factor, and post-marking interval (time) was a repeated measure.

Protein labels (5.0 mg ml^{-1}) were mixed in either ddH_20 or a 1:1 mixture of DMSO:ddH_20. Each of these mixtures was then used in the following immersion or feeding application treatments. For the immersion treatment, the marks were applied to groups of 100 beetles by submerging them in 3 ml of solution for 1 min with intermittent vortexing. Beetles were then air dried in a fume hood at 23 °C for 15 min. For the feeding treatment, the IgG mixture was applied to *T. ramosissima* foliage. This was accomplished by cutting a 20-cm length of the apical portion of the plant and submersing the apical portion (ca. 15 cm) of the plant in 3 ml of solution. After label application, foliage was air dried in a fume hood at 23 °C for 15 min. Stems were enclosed individually using cages (17 cm length) constructed from tree seedling protector tubes, nylon hosiery, and a florist's waterpick (Williams et al., 2012). One hundred beetles were added to each cage and the caged plants were hung vertically in an environmental chamber under the conditions described above for 24 h. For both immersion and feeding treatments, untreated control beetles were treated in the same manner with either ddH_20 or a 1:1 mixture of DMSO:ddH_20 only, depending on whether the treatment included DMSO or not. After immersion or the 24-h feeding period, groups of beetles (100) were released into each of 36, 1-m organdy sleeve cages with unlabeled *T. ramosissima* branches (70–90 cm long) and held in one of three environmental chambers (one replicate, i.e., 12 sleeve cages per chamber) under the conditions described above. Cut ends of the branches were submerged in plant nutrient solution (see above), and branches were replaced every 5 days. At each designated post-marking interval given above, ca. 15 live beetles were removed from each replicate-cage and frozen immediately at −20 °C until the ELISA was performed (see below).

Predation study – Transfer of label from marked prey to predator. A laboratory study was conducted to determine whether rabbit IgG-marked prey (adult *D. carinulata*) could be detected in adult *P. bioculatus* after a predation event. The predators (n = 68 adults) were preconditioned by holding them individually in ventilated 45-ml plastic vials for 24 h without food or water at 25 °C and L16:D8 photoperiod. This study was setup as a completely randomized design. Rabbit IgG (5 mg ml^{-1}) was tested using the two application treatments described above: feeding treatment [IgG + DMSO:ddH_20 (1:1) applied to saltcedar foliage after which beetles were allowed to feed for 24 h], and immersion treatment [beetles submerged in IgG + DMSO:ddH_20 (1:1) for 1 min with intermittent vortexing]. Controls for both treatments were a 1:1 mixture of DMSO:ddH_20 only. Beetles were allowed to air dry in a fume hood at 23 °C for ca. 15 min after treatment.

Each feeding assay was conducted by placing a marked *D. carinulata* in a ventilated 45-ml plastic vial with an adult *P. bioculatus*. Vials were held on a laboratory bench (fluorescent overhead lighting, 25 °C) for observation (at least 12 times every hour). All assays were conducted on a single day from 08:30 to 17:30 hours. For each assay, we recorded start time of trial (the time when prey and predator were placed into a vial), start time of the predation event (the time when a predator subdued and inserted its mouthparts into a prey individual), and end time of the predation event (the time when a predator withdrew its mouthparts from a prey individual and began grooming and/or walked away from the prey). Latency period was the time from the start of a trial to start of a predation event. Handling time was the time from start of a predation event to the completion of the predation event. When a predation event was finished, both insects (i.e., predator and prey) were held individually at −20 °C until ELISA (see below).

Double antibody sandwich ELISA

An anti-rabbit or anti-chicken double antibody sandwich ELISA was performed on each *D. carinulata* and

P. bioculatus individual as described by Hagler (1997). Each well of a 96-well ELISA microplate was coated with either 100 µl of anti-rabbit IgG (developed in goat) or anti-chicken IgG (developed in rabbit) (both Sigma) diluted 1:500 in ddH_20 and incubated for 1 h at 23 °C. The IgG antibodies were discarded and 260 µl of 1% non-fat dry milk in ddH_20 was added to each well for 30 min at 24 °C to block any remaining non-specific binding sites on the plates. Each insect was assayed twice: (1) a rinsate sample was collected to test for the presence of external label, after which (2) the insect was macerated with a tissue grinder and assayed to give the total label content (external + internal). For each insect, the ELISA optical density (OD) from the rinsed sample was subtracted from the OD yielded from the macerated sample to give the OD value for internal label. The following procedure was used for rinsate and macerated samples. Each insect sample was placed in a microcentrifuge tube containing 500 µl of tris-buffered saline (TBS). The samples were then vortexed for 10 s and placed on a plate rocker for 1 h. A 100-µl aliquot of rinsate was placed in a well of the pre-treated assay plate. The insect was then macerated with a pestle, and vortexed for 10 s and placed back on the plate rocker for 1 h. A 100-µl aliquot of the macerated insect sample was placed in a well of a different pre-treated assay plate. Plates containing samples were then incubated overnight at 4 °C. Insect samples were then discarded and each well was rinsed three times with tris buffered saline (TBS) Tween 20 (0.05%) and two times with TBS. Aliquots (50 µl) of either anti-rabbit IgG conjugated to horseradish peroxidase or anti-chicken IgG conjugated to horseradish peroxidase (both Sigma) diluted to 1:1 000 in 1% non-fat milk, were added to each well for 1 h at 24 °C. The secondary antibodies were discarded and the plates were again washed as described above and 50 µl of substrate was added (TMB One Component HRP Microwell Substrate No. TMBW-1000-01; BioFX Laboratories, Owings Mills, MD, USA). Plates were held at ca. 23 °C and the ELISA OD was measured at 10, 30, 45, and 60 min with a microplate reader set at 655 nm. Effectiveness of substrate incubation varied between the rabbit and chicken IgGs. For this reason, data from the optimal incubation time were used for each IgG label (rabbit, 30 min; chicken, 60 min).

Data analysis

The percentage of positive ELISA values was determined for each mark-treatment combination by calculating the percentage of insects with OD values ≥three times SD above the mean OD yielded by the respective negative control insect treatment (i.e., insects treated with ddH_20 or a 1:1 mixture of DMSO:ddH_20 only; Hagler, 1997). ELISA OD values were control-adjusted with the average value of negative control for the appropriate microplate, and expressed as proportional responses relative to the IgG positive control on that microplate. This procedure standardized OD values between microplates. Standardized OD data and percent positive data were then arcsine square root-transformed (Zar, 1996). For the prey marking study, repeated measures in Proc MIXED (SAS Institute, 2009) were used for the full factorial model, including comparison of levels of time (post-marking interval, days 0–14). Day 21 was not included in the analysis because of incomplete data at this time interval. Fisher's least significant difference (LSD) was used to identify significant differences between the treatments. Response variables were OD for external and internal IgG retention, and percent positives for external and internal IgG retention. Main (fixed) effects were DMSO, application method, time, and IgG label. Replicate was a random effect. Satterthwaite approximation was used to calculate appropriate degrees of freedom for F tests of fixed effects. For the predation study, a paired t-test was used to compare internal vs. external label retention for the feeding and immersion treatments. The untransformed data values are presented in results. None of the insect negative controls or blanks (ddH_20 or a 1:1 mixture of DMSO:ddH_20) was positive for either IgG label (mean ± SD = 0.040 ± 0.005, 0.062 ± 0.018, and 0.050 ± 0.012 for rabbit, chicken, and blanks, respectively).

Behavioral data were analyzed to determine whether the IgG labels affected the feeding behavior of the predators. Latency period and handling time of prey were square root transformed and subjected to a one-way ANOVA (Zar, 1996).

Results

Insect marking procedures

Prey marking – Effect of DMSO and application method. External label retention. Repeated measures analysis revealed a significant post-marking interval*label application method*DMSO interaction (Table 1), which was driven by differential response to DMSO and method of application over time; inclusion of DMSO in the IgG treatment resulted in greater label retention in the immersion application method, but reduced label retention in the feeding application method (Figure 1). Chicken label retention declined sharply during the first 3 days after application (Figure 1B), whereas retention of rabbit label showed a more gradual decline over time (Figure 1A). Mean OD values for rabbit IgG at day 0 ranged from ca. 0.017 (feeding with DMSO) to 0.83 (immersion with DMSO), and declined to almost 0 for all treatments at day 21. Mean OD values for chicken IgG at

Table 1 Results from linear mixed models repeated measures analysis (full factorial design) for immunoglobulin (IgG) retention on *Diorhabda carinulata*

Response variable	Covariance structure	Source of variation	d.f.	Type III F	P>F
External IgG retention optical density	Heterogeneous first order autoregressive	DMSO	1,21.5	0.45	0.51
		Application method	1,21.5	291.12	<0.0001
		Time	5,30.1	66.64	<0.0001
		IgG label	1,21.5	5.87	0.024
		Application method*DMSO	1,21.5	33.51	<0.0001
		Time*DMSO	5,30.1	7.36	0.0001
		IgG label*DMSO	1,21.5	3.24	0.086
		Time*application method	5,30.1	17.59	<0.0001
		IgG label*application method	1,21.5	12.84	0.0017
		IgG label*time	5,30.1	4.67	0.0028
		Time*application method*DMSO	5,30.1	3.43	0.014
		IgG label*application method*DMSO	1,21.5	1.76	0.20
		IgG label*time*DMSO	5,30.1	0.16	0.97
		IgG label*time*application method	5,30.1	1.47	0.23
		IgG label*time*application method*DMSO	5,30.1	0.93	0.48
Internal IgG retention optical density	Heterogeneous first order autoregressive	DMSO	1,25.7	0.71	0.41
		Application method	1,25.7	622.84	<0.0001
		Time	5,33.4	111.91	<0.0001
		IgG label	1,25.7	95.26	<0.0001
		Application method*DMSO	1,25.7	41.37	<0.0001
		Time*DMSO	5,33.4	8.46	<0.0001
		IgG label*DMSO	1,25.7	0.39	0.54
		Time*application method	5,33.4	20.34	<0.0001
		IgG label*application method	1,25.7	21.46	<0.0001
		IgG label*time	5,33.4	5.43	0.0009
		Time*application method*DMSO	5,33.4	1.62	0.18
		IgG label*application method*DMSO	1,25.7	8.78	0.0065
		IgG label*time*DMSO	5,33.4	0.71	0.62
		IgG label*time*application method	5,33.4	4.13	0.0050
		IgG label*time*application method*DMSO	5,33.4	0.47	0.79
External IgG retention percent positive	Heterogeneous Toeplitz	DMSO	1,21.5	22.36	<0.0001
		Application method	1,21.5	694.78	<0.0001
		Time	5,27.6	48.19	<0.0001
		IgG label	1,21.5	74.25	<0.0001
		Application method*DMSO	1,21.5	44.77	<0.0001
		Time*DMSO	5,27.6	3.33	0.018
		IgG label*DMSO	1,21.5	3.65	0.070
		Time*application method	5,27.6	15.14	<0.0001
		IgG label*application method	1,21.5	5.48	0.029
		IgG label*time	5,27.6	4.36	0.0047
		Time*application method*DMSO	5,27.6	1.60	0.19
		IgG label*application method*DMSO	1,21.5	0.03	0.86
		IgG label*time*DMSO	5,27.6	2.38	0.064
		IgG label*time*application method	5,27.6	8.17	<0.0001
		IgG label*time*application method*DMSO	5,27.6	1.59	0.20

Table 1 *(Continued)*

Response variable	Covariance structure	Source of variation	d.f.	Type III F	P>F
Internal IgG retention percent positive	First order ante- dependence	DMSO	1,32.2	16.59	0.0003
		Application method	1,32.2	339.89	<0.0001
		Time	5,23.9	117.39	<0.0001
		IgG label	1,23.9	56.96	<0.0001
		Application method*DMSO	1,23.9	21.62	<0.0001
		Time*DMSO	5,24.5	3.47	0.017
		IgG label*DMSO	1,23.9	5.30	0.028
		Time*application method	5,24.5	94.49	<0.0001
		IgG label*application method	1,23.9	29.41	<0.0001
		IgG label*time	5,24.5	12.79	<0.0001
		Time*application method*DMSO	5,24.5	4.91	0.0031
		IgG label*application method*DMSO	1,23.9	2.67	0.11
		IgG label*time*DMSO	5,24.5	6.55	0.0006
		IgG label*time*application method	5,24.5	10.40	<0.0001
		IgG label*time*application method*DMSO	5,24.5	3.91	0.0099

Response variables were optical density and percent positives for external and internal IgG retention. Main (fixed) effects were dimethyl sulfoxide (DMSO) (with or without), application method (external or internal), time (duration in days of post-marking interval), and IgG label (rabbit or chicken). Random effect was replicate. Satterthwaite's approximation was used to calculate appropriate degrees of freedom for F tests of fixed effects.

day 0 ranged from ca. 0.03 (feeding with DMSO) to ca. 1.12 (immersion with DMSO), and also declined to almost 0 for all treatments at day 21.

A significant IgG label*post-marking interval*label application method interaction (Table 1) was due to a differential effect of time (i.e., days 7–14 after application) on retention of the two labels; more than 95% of the insects treated with rabbit IgG as an immersion tested positive 14 days after label application (Figure 1C), whereas more than 90% of the beetles immersed in chicken IgG tested positive 5 days after application (Figure 1D). For the immersion treatments, retention of rabbit IgG was greater than chicken IgG at days 5 (99 vs. 69%; Fisher's LSD: $P = 0.030$), 7 (99 vs. 40%; $P<0.0001$), and 14 (98 vs. 15%; $P<0.0001$) after application (Figure 1C and D). Label retention for the feeding treatments declined sharply during the first 3 days after treatment for both rabbit and chicken IgG, but they were still detectable at 14 days after treatment.

Internal label retention. Repeated measures analysis revealed significant interactions for IgG label*label application method*DMSO and IgG label*post-marking interval*label application method (Table 1), which were due to differential effects of time (days 0–1) for the rabbit IgG immersion treatments (Figure 2A) and time (days 0 and 7–14) for the chicken IgG immersion treatments (Figure 2B). Trends of label retention for rabbit and chicken IgG were similar to those observed for external label retention. Mean OD values for rabbit IgG at day 0 ranged from 0.29 (feeding with DMSO) to 1.32 (immersion without DMSO). Feeding treatments declined to 0 at day 14, but immersion treatments were still detectable at day 21. Mean OD values for chicken IgG at day 0 ranged from ca. 0.10 (feeding with DMSO) to 0.70 (immersion without DMSO), and declined to 0 for all treatments at day 21. The effect of DMSO inclusion was similar to that noted in the external label retention; addition of DMSO led to significantly greater retention on days 1–14 after application (Figure 2A). For chicken IgG, retention in the DMSO immersion treatment was greater at day 5 (Figure 2B).

A significant IgG label*post-marking interval*label application method*DMSO interaction was noted (Table 1), due to variable effects of DMSO on IgG label over time, and to variable effects of label application method over time for the two labels. All of the insects treated with rabbit IgG as an immersion tested positive 14 days after label application (Figure 2C). For beetles immersed in chicken IgG, 68% tested positive 14 days after application (Figure 2D). For the immersion treatments, retention of rabbit IgG was greater than retention of chicken IgG at day 14 (100 vs. 68%; Fisher's LSD: $P = 0.014$). For chicken IgG feeding treatments, label retention declined sharply during the first 3 days after treatment, but was still detectable at 14 days after treatment. Label retention in the feeding treatments with rabbit IgG declined more slowly.

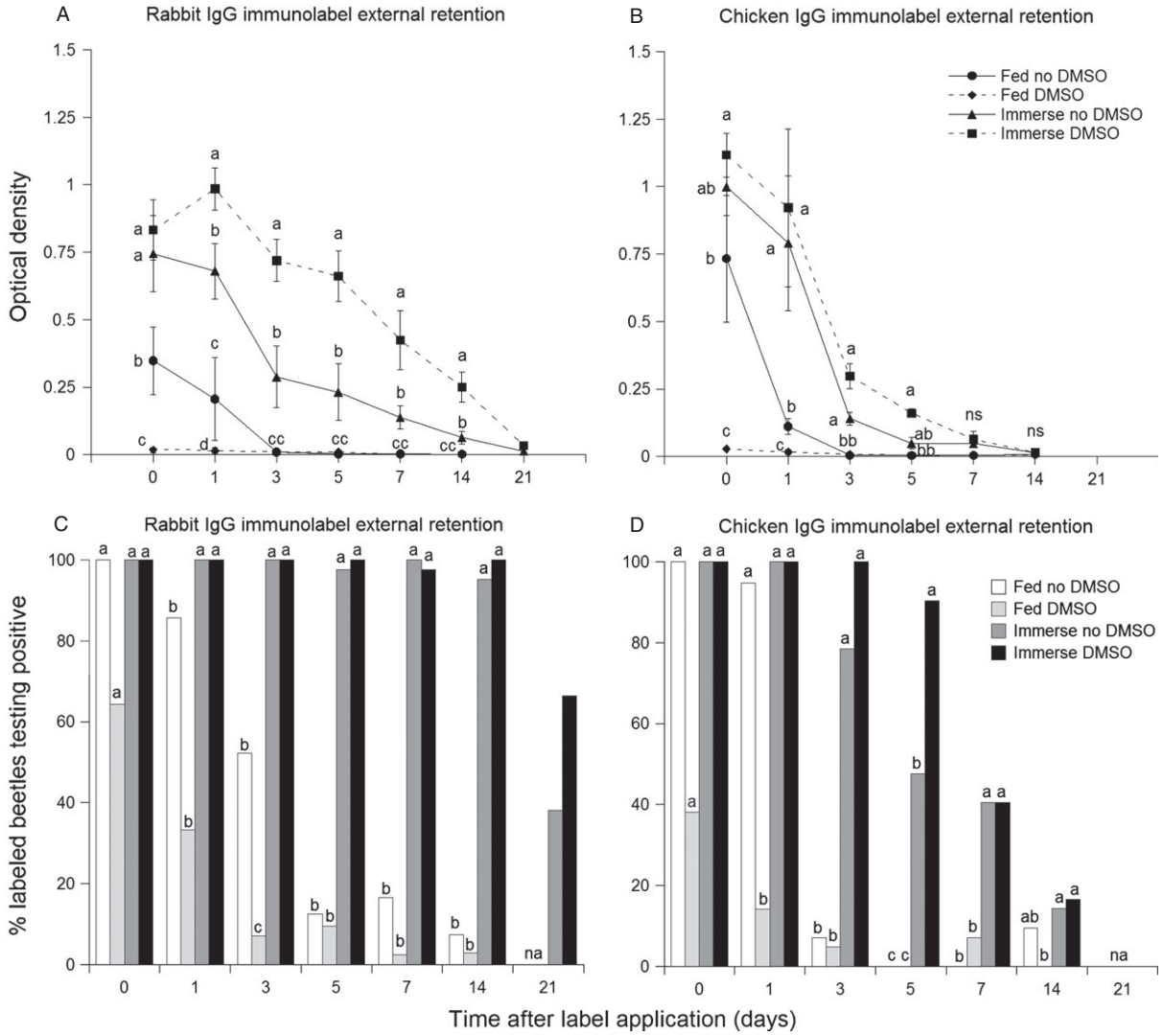

Figure 1 (A, B) Mean (± SE) optical density detected by immunoglobulin (IgG)-specific external enzyme-linked immunosorbent assays and (C, D) percentage of adult *Diorhabda carinulata* scoring positive for presence of rabbit or chicken IgG labels either alone or mixed with dimethyl sulfoxide (DMSO). Different letters indicate significant treatment differences for each post-marking interval (Fisher's LSD test: $P<0.05$). ns, not significant; na, data not available. Day 21 was not included in the repeated measures analysis because of incomplete data at this time interval.

Predation trial – Transfer of DMSO-IgG label to predator. Internal label retention was greater than external retention for both prey and predator (paired t-test: $P<0.05$). Mean OD values ranged from 0.07 to 0.88 for *D. carinulata* (marked prey), and from 0.01 to 0.21 for *P. bioculatus* that consumed a marked prey (Figure 3A). ELISA OD values for prey marked by immersion were >seven-fold higher than prey marked by feeding (Figure 3A). Mean percentages scoring positive for rabbit IgG label ranged from 57 to 100 for *D. carinulata* (marked prey), and from 7 to 83 for *P. bioculatus* that consumed a marked prey (Figure 3B). There was no significant difference in feeding behavior (latency or prey handling time) during the study (Table 2).

Discussion

This study showed that IgG-specific labeling was enhanced for a prey and predator by the addition of DMSO to the marking mixture. Retention of rabbit and chicken IgG labels on adult *D. carinulata* was comparable to that reported by Williams et al. (2011) who demonstrated that rabbit or chicken IgG are effective external labels of *D. carinulata* adults and eggs, that

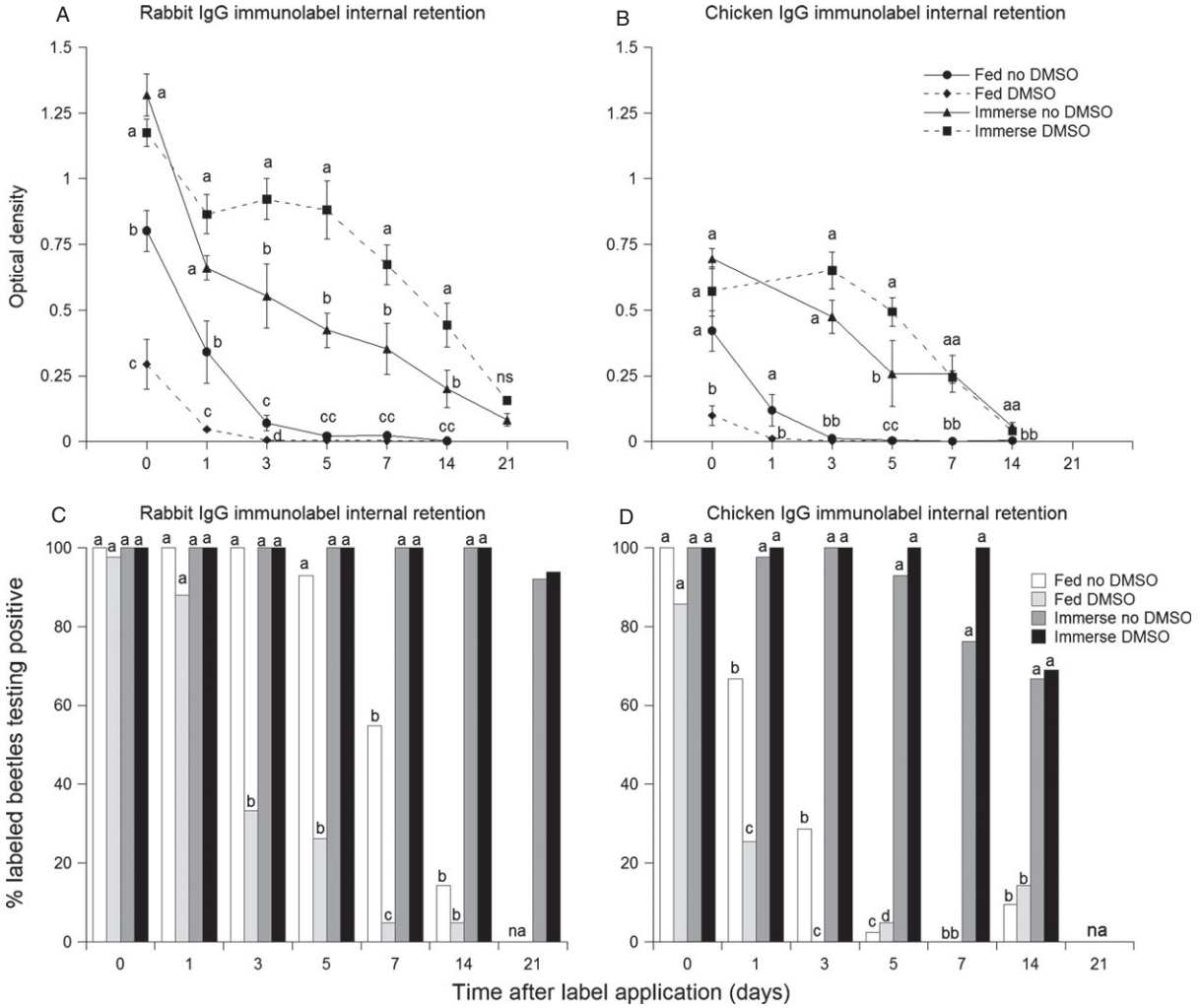

Figure 2 (A, B) Mean (± SE) optical density detected by immunoglobulin (IgG)-specific internal enzyme-linked immunosorbent assays and (C, D) percentage of adult *Diorhabda carinulata* scoring positive for presence of rabbit or chicken IgG labels either alone or mixed with dimethyl sulfoxide (DMSO). Different letters indicate significant treatment differences for each post-marking interval (Fisher's LSD test: $P<0.05$). na, data not available. Optical density data for chicken day 1 immersion treatments were not available (panel B). Day 21 was not included in the repeated measures analysis because of incomplete data at this time interval.

these labels were retained at >80% for ca. 14 days after marking under laboratory and field conditions, and that these labels can be transferred to successive life stages. Optical density and percent positive values in this study were similar to those obtained by Williams et al. (2011). Hagler (1997) showed that rabbit and chicken IgG were detected in the convergent lady beetle, *Hippodamia convergens* Guérin-Méneville, several weeks after external treatment with the labels. *Heterotermes aureus* (Snyder) and *Anaphes iole* Girault retained rabbit IgG protein as an internal label for >35 and 8 days after ingestion, respectively (Hagler & Jackson, 1998; Hagler et al., 2009).

We observed an interaction between DMSO and label application method. For the immersion application method, the addition of DMSO led to longer label retention. This effect was stronger for rabbit IgG than for chicken IgG, for both external and internal label retention. This might be due in part to limited uptake of the label by the beetle's digestive system. Comparison of external vs. internal label retention supports the idea that DMSO increased transfer of label into the insect. For the feeding application method inclusion of DMSO led to shorter label retention for both IgG labels. This may be due to a repellent or feeding deterrent effect by DMSO. Specifically, during the 24-h feeding period we observed that beetles in

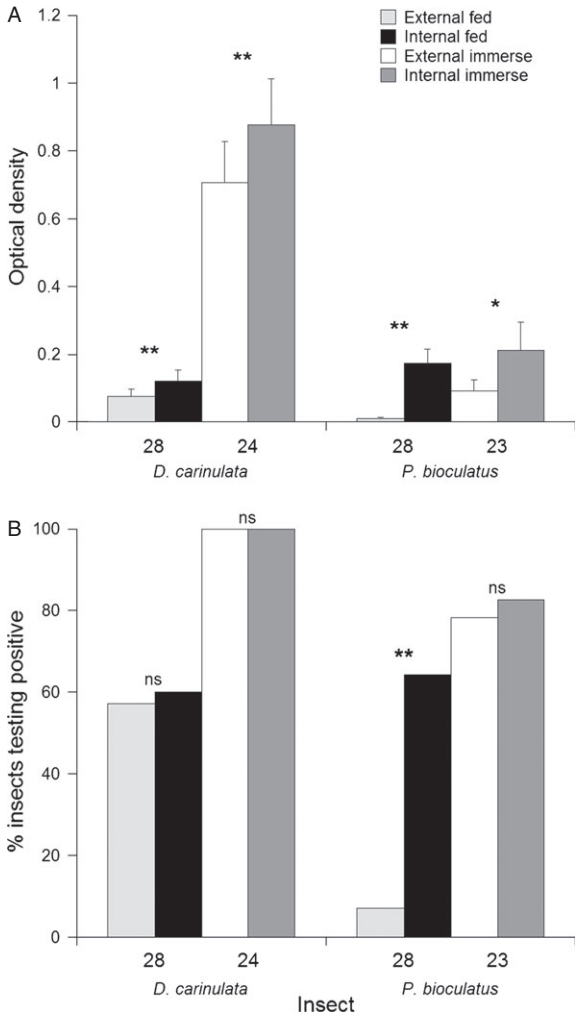

Figure 3 Effect of label application method on transfer of dimethyl sulfoxide (DMSO)-rabbit immunoglobulin (IgG) label from prey (*Diorhabda carinulata*) to predator (*Perillus bioculatus*). (A) Mean (+ SE) optical density detected by enzyme-linked immunosorbent assays, and (B) percentage of insects scoring positive for the presence of label. Comparisons of external vs. internal label retention for each label application method are based on paired t-tests: *0.01>P>0.001; **P<0.001; ns, not significant. The numbers below the paired bars indicate the numbers of insects tested for each comparison.

the DMSO treatment appeared to spend more time on the cage than on the plant. Moreover, at the end of the feeding period there was less frass in the DMSO cages than in the non-DMSO cages.

In the predation study, the DMSO internal label retention was greater than the external retention for both prey and predator. This effect was especially evident in the immersion treatment, and suggests that a portion of the IgG label moved into the insect,

Table 2 Comparison of feeding behavior (mean ± SE) in predation trial with *Diorhabda carinulata* and *Perillus bioculatus*

Treatment	Latency period (min)[2]	Handling time (min)[3]	n
Composite controls[1]	44.27 ± 25.98	202.55 ± 18.03	11
Feeding	60.82 ± 25.28	180.24 ± 14.96	17
Immersion	73.19 ± 28.08	181.69 ± 15.17	16

[1]Controls from feeding and immersion treatments were pooled after initial analyses showed no difference. Controls were a 1:1 mixture of dimethyl sulfoxide (DMSO):dd$H_2$0 only.
[2]Latency period was the time from start of a trial to start of a predation event ($F_{2,41}$ = 0.34, P = 0.71).
[3]Handling time was the time from start to completion of a predation event ($F_{2,41}$ = 0.64, P = 0.53).

whereas some remained on the insect surface. A single predation event was sufficient to transfer DMSO-rabbit IgG label from prey to predator. IgG label transferred from prey could be detected in both internal and external samples of the predator, but label retention was greatest in the internal samples for both feeding and immersion application methods. This was especially clear in the OD data, and was expected, because *P. bioculatus* has piercing-sucking mouthparts and feeds primarily on the internal tissues of its prey. Our results are consistent with those of Kelly et al. (2012), who demonstrated transfer of rabbit and chicken IgG labels from internally labeled *Manduca sexta* L. larvae to a predaceous stink bug, *Podisus maculiventris* (Say). When measured immediately after the predation event, 80% retention of rabbit IgG was observed for *P. maculiventris* (Kelly et al., 2012); in this study we observed ca. 65% internal label retention on *P. bioculatus* provisioned with *D. carinulata* fed on DMSO-rabbit IgG label. Predators with chewing mouthparts potentially ingest both external and internal labels, whereas predators with piercing-sucking mouthparts might ingest more internal label than external label. Thus, labeling prey simultaneously with internal and external labels may be desirable for future predation studies (Hagler, 2011). Presence of label in the external samples of *P. bioculatus* probably reflects contamination of tarsi and mouthparts during capture and feeding. Feeding behavior of *P. bioculatus* was not significantly influenced by application method of DMSO-IgG label; however, the possible effect of DMSO-IgG labels on the predatory behavior of *P. bioculatus* deserves further study.

Our studies demonstrated that addition of DMSO to IgG protein labels enhances external and internal uptake of the label when applied via immersion. In addition,

DMSO-IgG label was readily transferred from prey to predator after a single predation event. This was the case for prey that had been labeled either internally by feeding on marked food or by direct immersion in the marking solution. These results suggest that DMSO-IgG labels will be useful for studying predator–prey interactions via gut content analysis. Label retention for prey was 2–3 weeks, which is adequate for conducting predation studies on marked *D. carinulata*. Inclusion of DMSO in IgG will lead to a cost reduction because DMSO is relatively inexpensive (30 USD for 100 ml) and thus should require smaller amounts of costly IgG labels. Future studies are needed to determine how to optimize DMSO in protein marking studies. It appeared that DMSO inhibited feeding by *D. carinulata* in the prey marking study; thus future study to identify DMSO:label ratios that do not negatively affect insect feeding behavior are warranted. Identifying the internal tissues that are labeled by DMSO-IgG proteins would also be useful, for instance by histological studies using fluorescent-labeled secondary antibodies. Future studies characterizing the transfer of IgG label to other predators (e.g., chewing vs. piercing-sucking type predators), and the time decay of label retention in predators are also necessary for detailed ecological studies of predator–prey associations.

In addition to our evaluation of DMSO effects on IgG retention, we recorded a native insect, *P. bioculatus*, feeding on the exotic biological control agent *D. carinulata*, under field and laboratory conditions. Most of the known hosts of *P. bioculatus* are chrysomelid beetles (Saint-Cyr & Cloutier, 1996), which often aggregate on host plant patches (Pasteels et al., 1988). Herbivore-induced plant volatiles emanating from host plants provide host location cues for natural enemies. *Diorhabda carinulata* can form dense aggregations on saltcedar, causing the plants to produce a blend of volatile compounds (Cossé et al., 2006), some of which are the same as produced by Colorado potato beetle-infested potatoes (Bolter et al., 1997; Weissbecker et al., 2000). Several of the herbivore-induced plant volatiles common to the two plants are perceived by and attractive to *P. bioculatus* (Weissbecker et al., 1999, 2000; van Loon et al., 2000). Thus, the apparent pre-disposition of *P. bioculatus* to olfactory cues associated with chrysomelid beetle-host plant aggregations may have played an important role in the development of this new predator–prey association.

In summary, our findings suggest that vertebrate proteins can be used in predation studies with *D. carinulata*. Our earlier studies indicated that the IgG proteins are relatively photo- and heat-stable (Williams et al., 2011), and in this study we demonstrate that addition of DMSO enhances protein marking and that labels can be transferred to predators in one predation bout. The specificity, sensitivity, and persistence of the proteins and ELISA suggest that immunolabels can be used in future studies with *D. carinulata* on predator–prey interactions.

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References

Bolter CJ, Dicke M, van Loon JJA, Visser JH & Posthumus MA (1997) Attraction of Colorado potato beetle to herbivore-damaged plants during herbivory and after its termination. Journal of Chemical Ecology 23: 1003–1023.

Buczkowski G, Wang C & Bennett G (2007) Immunomarking reveals food flow and feeding relationships in the subterranean termite, *Reticulitermes flavies* (Kollar). Environmental Entomology 36: 173–182.

Choi M-Y, Groot A & Jurenka RA (2005) Pheromone biosynthetic pathways in the moths *Heliothis subflexa* and *Heliothis virescens*. Archives of Insect Biochemistry and Physiology 59: 53–58.

Cossé AA, Bartelt RJ, Zilkowski BW, Bean DW & Andress ER (2006) Behaviorally active green leaf volatiles for monitoring the leaf beetle, *Diorhabda elongata*, a biocontrol agent of saltcedar, *Tamarix* spp. Journal of Chemical Ecology 32: 2695–2708.

Dávalos A & Blossey B (2010) The effects of flooding, plant traits, and predation on purple loosestrife leaf-beetles. Entomologia Experimentalis et Applicata 135: 85–95.

DeLoach CJ, Moran PJ, Knutson AE, Thompson DC, Carruthers RI et al. (2008) Beginning success of biological control of saltcedars (*Tamarix* spp.) in the southwestern USA. Proceedings of the XII International Symposium on Biological Control of Weeds (eds. MH Julien, R Sforza, MC Bon, HC Evans & PE Hatcher et al.), pp. 535–539. CAB International, Wallingford, UK.

DiTomaso JM (1998) Impact, biology, and ecology of saltcedar (*Tamarix* spp.) in the southwestern United States. Weed Technology 12: 326–336.

van Driesche RG & Bellows TS Jr (1996) Biological Control. Chapman & Hall, New York, NY, USA.

Friedman JM, Auble GT, Shafroth PB, Scott ML, Merigliano MF et al. (2005) Dominance of non-native riparian trees in western USA. Biological Invasions 7: 747–751.

Goeden RD & Louda SM (1976) Biotic interference with insects imported for weed control. Annual Review of Entomology 21: 325–342.

Hagler JR (1997) Field retention of a novel mark-release-recapture method. Environmental Entomology 26: 1079–1086.

Hagler JR (2006) Development of an immunological technique for identifying multiple predator-prey interactions in a complex arthropod assemblage. Annals of Applied Biology 149: 153–165.

Hagler JR (2011) An immunological approach to quantify consumption of protein-tagged *Lygus hesperus* by the entire cotton predator assemblage. Biological Control 58: 337–345.

Hagler JR & Durand CM (1994) A new method for immunologically marking prey and its use in predation studies. Entomophaga 39: 257–265.

Hagler JR & Jackson CG (1998) An immunomarking technique for labeling minute parasitoids. Environmental Entomology 27: 1010–1016.

Hagler JR, Cohen AC, Bradley-Dunlop D & Enriquez FJ (1992) New approach to mark insects for feeding and dispersal studies. Environmental Entomology 21: 20–25.

Hagler JR, Baker PB, Marchosky R, Machtley SA & Bellamy DE (2009) Methods to mark termites with protein for mark–release–recapture and mark–capture type studies. Insectes Sociaux 56: 213–220.

Hough-Goldstein J, Janis JA & Ellers CD (1996) Release methods for *Perillus bioculatus* (F.), a predator of the Colorado potato beetle. Biological Control 6: 114–122.

Hunt-Joshi TR, Root RB & Blossey B (2005) Disruption of weed biological control by an opportunistic mirid predator. Ecological Applications 14: 861–870.

Julien MH & Griffiths MW (1998) Biological Control of Weeds. A World Catalogue of Agents and their Target Weeds, 4th edn. CABI Publishing, Wallingford, UK.

Kelly JL, Hagler JR & Kaplan I (2012) Employing immunomarkers to track dispersal and trophic relationships of a piercing-sucking predator, *Podisus maculiventris* (Hemiptera: Pentatomidae). Environmental Entomology 41: 1527–1533.

Knight HH (1952) Review of the genus *Perillus* with description of a new species (Hemiptera: Pentatomidae). Annals of the Entomological Society of America 45: 229–232.

van Loon JJA, de Vos EW & Dicke M (2000) Orientation behaviour of the predatory hemipteran *Perillus bioculatus* to plant and prey odors. Entomologia Experimentalis et Applicata 96: 51–58.

Lovich JE & de Gouvenain RC (1998) Saltcedar invasion in desert wetlands of the southwestern United States: ecological and political implications. Ecology of Wetlands and Associated Systems (ed. by SK Majumdar, EW Miller & FJ Brenner), pp. 447–467. Pennsylvania Academy of Science, Easton, PA, USA.

Mansfield S, Hagler JR & Whitehouse MEA (2008) A comparative study on the efficacy of a pest-specific and prey-marking enzyme-linked immunosorbent assay for detection of predation. Entomologia Experimentalis et Applicata 127: 199–206.

Mathes RL, Reber AJ, Hurley DJ & Dietrich UM (2010) Effects of antifungal drugs and delivery vehicles on morphology and proliferation of equine corneal keratocytes in vitro. American Journal of Veterinary Research 71: 953–959.

Moran PJ, DeLoach CJ, Dudley TL & Sanabria J (2009) Open field host selection and behavior by tamarisk beetles (*Diorhabda* spp.) (Coleoptera: Chrysomelidae) in biological control of exotic saltcedars (*Tamarix* spp.) and risks to non-target athel (*T. aphylla*) and native *Frankenia* spp. Biological Control 50: 243–261.

Morisette JT, Jarnevich CS, Ullah A, Cai W, Pedelty JA et al. (2006) A tamarisk habitat suitability map for the continental United States. Frontiers in Ecology and the Environment 4: 11–17.

Pasteels JM, Rowell-Rahier M & Raupp MJ (1988) Plant-derived defense in chrysomelid beetles. Novel Aspects of Insect-Plant Interactions (ed. by P Barbosa & DK Letourneau), pp. 235–272. Wiley, New York, NY, USA.

Paynter Q, Fowler SV, Gourlay HA, Groenteman R, Peterson PG et al. (2010) Predicting parasitoid accumulation on biological control agents of weeds. Journal of Applied Ecology 47: 575–582.

Rowley SD (2009) Hematopoietic stem cell cryopreservation: a review of current techniques. Journal of Hematotherapy 1: 233–250.

Saint-Cyr J-F & Cloutier C (1996) Prey preference by the stinkbug *Perillus bioculatus*, a predator of the Colorado potato beetle. Biological Control 7: 251–258.

Santos NC, Figueira-Coelho J, Martins-Silva J & Saldanha C (2003) Multidisciplinary utilization of dimethyl sulfoxide: pharmacological, cellular, and molecular aspects. Biochemical Pharmacology 65: 1035–1041.

SAS Institute (2009) SAS/STAT User's Guide, Release, 9.2 edn. SAS Institute, Cary, NC, USA.

Shafroth PB, Cleverly JR, Dudley TR, Taylor JP, van Riper C et al. (2005) Control of *Tamarix* in the western United States: implications for water salvage, wildlife use, and riparian restoration. Environmental Management 35: 231–246.

Southwood TRE (1978) Ecological Methods, vol. 2nd. Chapman and Hall, London, UK.

Tracy JL & Robbins TO (2009) Taxonomic revision and biogeography of the *Tamarix*-feeding *Diorhabda elongata* (Brullé, 1832) species group (Coleoptera: Chrysomelidae: Galerucinae: Galerucini) and analysis of their potential in biological control of tamarisk. Zootaxa 2101: 1–152.

Weber DC (2008) Colorado potato beetle. Encyclopedia of Entomology, 2nd edn (ed. by JL Capinera), pp. 324–328. Springer, Dordrecht, The Netherlands.

Weissbecker B, van Loon JJA & Dicke M (1999) Electroantennogram responses of a predator, *Perillus bioculatus*, and its prey, *Leptinotarsa decemlineata*, to plant volatiles. Journal of Chemical Ecology 25: 2313–2325.

Weissbecker B, van Loon JJA, Posthumus MA, Bouwmeester HJ & Dicke M (2000) Identification of volatile potato sesquiterpenoids and their olfactory detection by the two-spotted stinkbug, *Perillus bioculatus*. Journal of Chemical Ecology 26: 1433–1445.

Williams L III, Hagler JR & Tonkel KC (2011) Retention of immunolabels by *Diorhabda carinulata* (Coleoptera: Chrysomelidae), a biological control agent of saltcedar. Entomologia Experimentalis et Applicata 141: 154–162.

Williams L III, Zhu YC, Snodgrass GL & Manrique V (2012) Plant-mediated decisions by an herbivore affect oviposition pattern and subsequent egg parasitism. Arthropod-Plant Interactions 6: 159–169.

Zar JH (1996) Biostatistical Analysis, 3rd edn. Prentice-Hall, Upper Saddle River, NJ, USA.

Zavaleta E (2000) The economic value of controlling an invasive shrub. Ambio 29: 462–467.