Journal of Chemical Ecology
https://doi.org/10.1007/s10886-020-01176-4

Establishing *Diorhabda carinulata*: Impact of Release Disturbances on Pheromone Emission and Influence of Pheromone Lures on Establishment

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Received: 31 December 2019 / Revised: 6 March 2020 / Accepted: 23 March 2020

Abstract

Before weed biocontrol insects are transported and released in a new area, they are commonly collected into small paper containers, chilled, and kept under dark conditions. This process can be termed a pre-release protocol. The influence of a pre-release protocol on establishment success of a gregarious biological control agent was assessed using the northern tamarisk beetle, *Diorhabda carinulata* (Desbrochers), and its exotic, invasive host plant saltcedar (*Tamarix* spp.). Pre-release protocol impacts on aggregation pheromone production by *D. carinulata* were characterized under controlled conditions. Additional experiments were undertaken to determine if deployment of aggregation pheromone lures might enhance the agent's persistence at release sites. Adults that experienced the pre-release protocol produced less aggregation pheromone compared to undisturbed adults. Olfactometer bioassays indicated that a cohort of adults subjected to the pre-release protocol were less attractive to other adults than a control cohort. Efficacy of aggregation pheromone-based lures to retain adults at release sites was evaluated by comparing capture numbers of adult beetles at paired treatment and control release sites, 10–14 days after the release of 300, 500, or 1000 individuals. A greater number of adult *D. carinulata* were captured where the pheromone lures had been deployed compared to control release sites. Application of aggregation pheromone when a new release of *D. carinulata* is planned should allow biological control practitioners to increase retention of beetles at a release site.

Keywords Aggregation · Biological control · Release protocol · Biological invasion · Weed control

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10886-020-01176-4) contains supplementary material, which is available to authorized users.

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Introduction

The beetle *Diorhabda carinulata* (Desbrochers) (Coleoptera: Chrysomelidae) was introduced to North America for classical biological control against the invasive weedy complex of the shrub *Tamarix* spp. The beetle aggregates in response to a male-produced aggregation pheromone (Cossé et al. 2005, 2006; Gaffke et al. 2018, 2019). Production of aggregation pheromones is thought to be a critical component in the successful establishment and spread of *D. carinulata* and the closely related *D. elongata* Brullé, which is also used as a *Tamarix* biocontrol agent (Cossé et al. 2006; Hudgeons et al. 2007). Males of *D. carinulata* and *D. elongata* are hypothesized to be the 'pioneers' that leave an aggregation and search for suitable hosts (Cossé et al. 2006). Dispersing individuals alight on host plants and initiate pheromone production, which rapidly induces nearby conspecifics to form an aggregation (Bartelt et al. 2001; Cossé et al. 2006).

Diorhabda carinulata is multivoltine, typically having 2–3 generations per year (Lewis et al. 2003). Reproductive males

produce two aggregation pheromone compounds, (2*E*, 4*Z*)-2,4-heptadien-1-ol and (2*E*, 4*Z*)-2,4-heptadienal, which are attractive to both males and females. Reproductive males produce the compounds during the day, but do not respond to the pheromone at night (Cossé et al. 2005). Males produce ca. 3.1 ng.hr.$^{-1}$ of (2*E*, 4*Z*)-2,4-heptadienal and 2.08 ng.hr.$^{-1}$ of (2*E*, 4*Z*)-2,4-heptadien-1-ol. The alcohol component of the pheromone blend is the primary compound that stimulates aggregation; it is equally attractive as a single component or in a blend and is more attractive than the aldehyde (Bean et al. 2013; Cossé et al. 2005). The aldehyde component is behaviorally active but only capable of initiating very small aggregations (Cossé et al. 2005). Thus, its precise role is unknown. Production of the aggregation pheromone could be critical to maintaining high densities when insects disperse over short or long distances, as observed for other species (Fernandez and Hilker 2007; Grevstad and Herzig 1997; Wood 1982). Adults enter a reproductive diapause in the fall, initiated when day length drops below a critical threshold (Bean et al. 2007).

The deployment of a *D. carinulata* pheromone in conjunction with new releases of the insect could result in improved retention of this insect at release sites (Hudgeons et al. 2007). Field deployment of a *D. carinulata* aggregation pheromone component has resulted in increased densities of the insect on pheromone-baited host plants compared to on untreated control plants (Gaffke et al. 2018, 2019). This strategy may allow biological control practitioners to maximize the number of successful releases that could be made, by increasing the probability of establishment through a greater number of small releases (Grevstad et al. 2011). Preliminary trials conducted in 2004 demonstrated this principal. When three releases of 100 adults were compared with and without aggregation pheromone lures, *D. carinulata* were recovered only from sites where releases were made in conjunction with the aggregation pheromone (T. Dudley, unpublished data).

One possible explanation for pheromone-assisted releases enhancing establishment is that successful colonization of new locations by field populations of *D. carinulata* is governed by semiochemicals, chiefly the aggregation pheromone (Bartelt et al. 2008; Cossé et al. 2005, 2006; Hudgeons et al. 2007). If, during the initial release, too little pheromone is produced by males to maintain an aggregation, it is possible that beetles will disperse, and the release will fail to establish (Hudgeons et al. 2007). Because pheromone production in *D. carinulata* is closely associated with active feeding by males, we hypothesized that standard pre-release protocols may negatively impact aggregation pheromone production in *D. carinulata.* Before biocontrol insects are released in the field, they are commonly packaged into small paper cups with host plant material, chilled and stored without light (Winston et al. 2014). The agents can be kept under these conditions for a few hours to several days. This process of packaging and holding insects is what we term the pre-release protocol. Therefore, we investigated the possibility that the pre-release protocol may impact aggregation pheromone production in *D. carinulata*, which, in turn, could make new releases susceptible to dispersal. Further, we tested for the effect of lure-based applications of *D. carinulata* aggregation pheromone to retain populations within a release area.

Methods

Lures and Chemicals *Diorhabda carinulata* aggregation pheromone, (2*E*, 4*Z*)-2,4-heptadien-1-ol, was synthesized (Restoration Science, Santa Barbara, California, USA) for the purposes of this study according to modification of the methods of Petroski (2003). The alcohol was used for field applications, as it is more attractive alone than the aldehyde or a blend of the two (Cossé et al. 2005; Gaffke et al. 2018, 2019). Aggregation pheromone lures were prepared for field deployment by formulation into ISCA® technologies (Riverside, California, USA) wax-based formula SPLAT® (Specialized Pheromone & Lure Application Technology). SPLAT has successfully delivered *D. carinulata* aggregation pheromone in field experiments (Gaffke et al. 2018, 2019). Treatment lures consisted of SPLAT impregnated with the aggregation pheromone (2.17% active ingredient). Lures placed in control patches consisted of SPLAT without active ingredient. The formulation of SPLAT used in this current study is a flowable matrix that was applied using an 80 ml syringe. Cattle ear tags (Y-Tex Corporation, Cody, WY, USA) were used as the application substrate for dollops. The ear tags were attached to plants and enclosed with mesh cages to minimize animal encounter with the dollops.

The release rate of compounds from SPLAT has been characterized and can be accurately controlled by the volume of SPLAT applied (Mafra-Neto et al. 2013). Lures at two dosages of aggregation pheromone were studied for potential manipulation of *D. carinulata* in the field, delivered in 1 g or 4 g aliquots of SPLAT (Gaffke et al. 2018, 2019). A single 4 g application (5 ml) of SPLAT was used to investigate the potential for pheromone lures to increase the likelihood of establishment of the insect. Release rates of the *D. carinulata* aggregation pheromone from 4 g dollops of SPLAT were reported by Gaffke et al. (2019). Specifically, they release approximately 17,514 ng.hr.$^{-1}$ of pheromone during the first day of lure deployment, which decreases after the first few days but stabilizes to a mean rate of ca. 228 ng.hr.$^{-1}$ for up to 30 days. The emission of the 4 g dollop in the first day is roughly equivalent to the production rate of 3300 adult *D. carinulata* males while between days 10–31 it is roughly equivalent to the hourly emission of 60 adult males.

Insect Source Laboratory colonies of *D. carinulata* were started using field-collected individuals from southern

Montana, USA. This population is presumed to have dispersed north from the initial release site of the *D. carinulata* ecotype from Fukang, China near Lovell, WY (DeLoach et al. 2003; Lewis et al. 2003). Repeated releases of *D. carinulata* occurred in Montana when the agent became available, but multiple years of post-release monitoring conducted by United States Department of Agriculture Animal and Plant Health Inspection Service and Agricultural Research Service personnel did not detect establishment of *D. carinulata* in Montana (G. Adams, J. Gaskin, personal communication). The greenhouse-based continuous culture of *Diorhabda carinulata* was sustained on greenhouse-based potted *Tamarix* plants enclosed in insect rearing sleeve cages (MegaView Science Co., Ltd., Taichung, Taiwan). *Tamarix* plants used to mass rear *D. carinulata* were collected from the sites intended for future releases. Adult beetles were induced into diapause and retained in a plant growth chamber (model E30B, Percival Scientific Inc., Perry, Indiana, USA) set at 22 °C and under a 12:12 h light/dark cycle to maintain diapause (Bean et al. 2007). Diapausing adults were removed from the growth chamber and caged on potted *Tamarix* plants in a greenhouse at 25 °C and a 16:8 h light/dark cycle for two weeks before field release. Adult beetles rapidly transition to the reproductive phase when exposed to 15 or more hours of daylight (Bean et al. 2007).

Volatile Compound Collection Volatile collections to assess for the effects of the pre-release protocol on aggregation pheromone production were conducted on adults exposed to a pre-release protocol or adults left undisturbed as a control. All adults were maintained on caged plants in a greenhouse at 30 °C with a 16:8 h light/dark cycle. Supplemental lighting was provided by GE MVR1000/C/U multi-vapor® quartz metal halide bulbs (100,280 Lumens) (General Electric Company, Cleveland, Ohio, USA). Each volatile collection consisted of 100 individuals, 1–4 weeks old. Fourteen collections of each treatment were made, with new adults used in each replication. The sex ratio of adults was not fixed so as to mimic actual releases. However, insects were randomly sourced from populations in which the sex ratio was 1:1.

Individuals included in the pre-release protocol treatment were aspirated from host plants, placed in 47 ml paper containers (Sealright Company, Inc., De Soto, Kansas, USA), with sprigs of freshly cut *Tamarix*, then retained in a 4 °C refrigerator for 12 h, to emulate typical operational conditions for biocontrol agents collected for re-distribution (Winston et al. 2014). Packaging agents in small individual release containers, which are then chilled to decrease metabolic expenditure, and subsequently stored for days prior to release, is standard practice in biological control. This protocol generally places the insects in high density, cold, dark conditions over a few hours to a few days. Adults were aspirated from the paper cups at the end of the chilling/darkness treatment and placed directly into volatile collection chambers. On the morning of the volatile collections, control individuals were aspirated from caged host plants and placed in volatile collection chambers in the same greenhouse. Aerations of adults began within 15 min of placement in the chamber.

Volatile collections were made using glass collection chambers (i.d. 95 mm, length 625 mm) (Analytical Research Systems, Gainesville, Florida, USA) that contained a single, healthy potted *Tamarix*. Two collection periods were used to determine the effects of the pre-release protocol on *D. carinulata*: for the first six (0–6) and the last six (24–30) hours of a 30 h total period.

Volatile collection traps containing 30 mg of super-Q (Alltech Associates, Inc., Deerfield, IL, USA) adsorbent were placed at the apical opening of collection chambers, with purified air flowing through the chambers at 100 $ml.min^{-1}$. Collected volatiles were eluted from the traps into vials with methylene chloride (200 µl) and the samples spiked with 10 µl of a 0.84 $ng.\mu l^{-1}$ solution of 1-octanol in methylene chloride as an internal standard. Compounds were analyzed using an Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, California) coupled to an Agilent 5973 mass selective detector (GC/MS).

Bioassays A four-chambered 30 cm × 30 cm × 2.5 cm olfactometer (Sigma Scientific LLC, Micanopy, FL, USA), with a two-chamber adapter, was used to determine the responses of adult *D. carinulata* to conspecifics subjected to the pre-release protocol treatment. Purified air was supplied to the olfactometer at 200 $ml.min^{-1}$. The olfactometer had a central area and two lateral arms leading into odor source chambers. The bioassays were conducted in a bioassay room located at the Rocky Mountain Research Station's Bozeman Forestry Sciences Laboratory (USDA Forest Service, Bozeman, Montana, USA).

Adult beetles obtained from the greenhouse culture were stored individually in 1.7 ml microcentrifuge vials (Axygen, Inc., Union City, California, USA) at 4 °C for 12 h prior to assays. These conditions mimicked the conditions of the pre-release protocol. Adults were kept individually, to minimize sorting disturbances, before testing. Locomotory responses to three types of volatile cues were tested in the olfactometer: aerations of 100 adults subjected to the pre-release protocol, aerations of 100 adults aspirated from caged host plants, which acted as a positive control, and an empty odor chamber with purified air, which acted as a negative control.

In the first set of bioassays, the responses of individual adults to the positive control versus the negative control, and the pre-release protocol treatment versus the negative control, were conducted. The second set of bioassays compared the positive control to the pre-release protocol treatment. Each responding adult was allowed three min to respond to the odor sources. When an individual entered one arm of the

olfactometer, it was removed and the result recorded. If the individual did not enter an arm of the olfactometer within three min, it was recorded as non-responding and was not included in the subsequent analysis. After each individual was tested, the Teflon olfactometer was cleaned with hot, soapy water and dried. After half the bioassay subjects were tested, placement of the odor treatments in the chambers was reversed.

Field Site Selection and Setup A total of 24 release sites for *D. carinulata* were selected across Montana: 10 in 2016, eight in 2017, and six in 2018. Within each site, two patches of *Tamarix* were identified to receive releases. All release sites were assessed for the presence of *D. carinulata* using sweep nets before beetles were released. No *D. carinulata* were detected prior to field releases. Release sites were located a minimum distance of 200 km from known *D. carinulata* populations. The *Tamarix* patches in Montana have been identified as *T. ramosissima* or *T. ramosissima* X *T. chinensis* hybrid (Gaskin and Kazmer 2009). Patches were spaced a minimum of 250 m apart within the same study site. Within each of the study patches, five plants were identified as permanent monitoring plants: a central release plant and four plants surrounding the central plant. The four plants surrounding the central plant corresponded to cardinal directions and were located approximately 5–10 m from the central plant. At each of the study sites, one of the central release plants was treated with a 4 g dose of pheromone-impregnated SPLAT, the other paired central release plant was treated with a 4 g dose of SPLAT with no active ingredient.

Field Release Protocol Releases consisted of newly eclosed, and post diapause reproductive adults from a greenhouse-based continuous culture (Bean et al. 2007). Adults that undergo diapause and transition into an active reproductive state are well documented to respond to aggregation pheromone (Cossé et al. 2005, Gaffke et al. 2018, Gaffke et al. 2019). These adults were pooled before field release to randomize against potential effects of age on the overall success of individual releases. Field releases of 300 individuals per site occurred at 10 locations from 19 to 21 July 2016. In 2017, 500 individuals were released at eight locations on 19 or 21 June. In 2018, releases of 1000 individuals at six locations occurred on 12 or 27 June. Adults, packaged in a 240 ml paper container were placed in the branches of the release plant, and the container lid removed to allow the adults to exit and crawl into the canopy of the plant. This protocol was used to minimize immediate disturbance and rapid dispersal of beetles from the plant that received insects. Adults were released only on the central plant and were not distributed throughout the patch.

Field Release Monitoring Sites were assessed for the presence of *D. carinulata* by sweep net sampling 10–14 d after release. The five designated monitoring plants in each study patch were sampled using five 1 m passes of a net through the foliage. After each plant was swept, the contents of the net were examined and *D. carinulata* present were tallied and placed back onto the same plant. Subsequent surveys following the initial post-release survey were made at the expected time of emergence of subsequent generations of *D. carinulata* and were conducted to assess the persistence of populations from the releases (Lewis et al. 2003).

Statistical Analyses Welch's t-test was used to analyze for differences in pheromone production between beetles subjected to the pre-release protocol and the control group. The proportion of *D. carinulata* adults responding to a positive control, a negative control, and the pre-release protocol treatment (volatiles produced by 100 conspecifics stored under cold, dark conditions) were compared using χ^2 tests. The effect of the aggregation pheromone lure on the number of *D. carinulata* sampled from the release patch 10–14 d post-release was analyzed using a Wilcoxon rank-sum test. The rank-sum test was used due to the small sample size and highly variable data. The effect of the aggregation pheromone on persistence of a new release of *D. carinulata* was analyzed using a generalized linear regression best fit with a Poisson distribution. This distribution is commonly used for count data and is the most appropriate for counts of rare events.

Results

Volatile collections. GC/MS comparisons of volatiles from adults subjected to the pre-release protocol and those of undisturbed adults (control) confirmed that the pre-release protocol caused differences in the amounts of the two-component aggregation pheromone (Fig. 1). In the first six hours of collections, adults subjected to the pre-release protocol emitted less of the primary behaviorally active component, (2*E*,4*Z*)-2,4-heptadien1-ol, than the control group (2.6 ng.hr.$^{-1}$ vs.18.2 ng.hr.$^{-1}$, respectively; $t = -3.61$ on 19.53 d.f., $P = 0.002$) (Fig. 1a). A difference was also detected in the amount of (2*E*,4*Z*)-2,4-heptadienal emitted by adults exposed to the pre-release protocol compared to control adults (24.4 ng.hr.$^{-1}$ vs 12.8 ng.hr.$^{-1}$, respectively; $t = -2.25$ on 21.328 d.f., $P = 0.03$) (Fig. 1a).

For the six-hour samples, between hours 24–30 of the collection period, there was no difference in the amount of (2*E*, 4*Z*)-2,4-heptadien1-ol between control adults and those that underwent the pre-release protocol ($t = 1.01$ on 24.76 d.f., $P = 0.32$) (Fig. 1b). However, there was a difference in the amount of the less behaviorally active component, (2*E*, 4*Z*)-2,4-heptadienal (85.1 ng.hr.$^{-1}$ vs 150.4 ng.hr.$^{-1}$, respectively; $t = 2.38$ on 16.69 d.f., $P = 0.03$)(Fig. 1b).

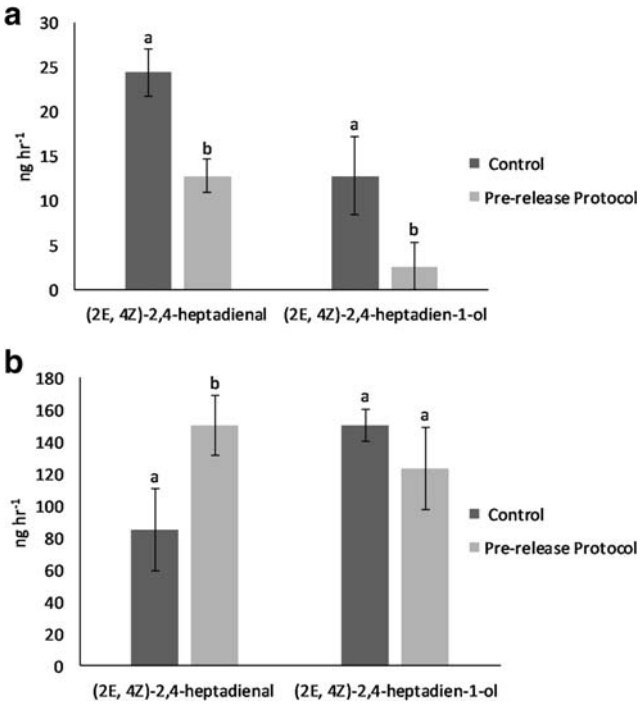

Fig. 1 Volatile emissions (ng.hr.$^{-1}$ ± SE) of (2E, 4Z)-2,4-heptadienal and (2E, 4Z)-2,4-heptadien-1-ol from 100 adult *Diorhabda carinulata* at two time points: a) first 6 h and b) last 6 h of a 30-h collection time. Adults either underwent a pre-release protocol or were kept under normal rearing conditions (control) ($n = 14$). Different letters above bars denote statistical differences between treatments

Behavioral Bioassays Males and females demonstrated a preference for the positive control over the volatiles emitted by adults that underwent the pre-release protocol (Fig. 2a). Some 70% of females and 76% of males preferentially orientated to the positive control (respectively, $\chi^2 = 7.52$, $P = 0.006$, $n = 100$, $\chi^2 = 13.4$, $P < 0.001$, n = 100), with 10 females and 12 males classified as non-responders. Responses of male and female *D. carinulata* to the odor chamber containing adults that underwent the pre-release protocol Vs the negative control of purified air were equivocal. Some 55% of females and 45% of males responded to the negative control compared to volatiles from adults that underwent the pre-release protocol (respectively, $\chi^2 = 0.32$, $P = 0.57$, n = 100, $\chi^2 = 0.32$, $P = 0.57$, n = 100) (Fig. 2b), with 18 females and 25 males classified as non-responders. Individuals given the choice of responding to olfactory cues from the negative control or the positive control demonstrated a preference for the positive control; 72% of females and 79% males preferred the positive control (respectively, $\chi^2 = 8.81$, $P = 0.003$, n = 100, $\chi^2 = 14.8$, $P < 0.001$, n = 100) (Fig. 2c). Of the tested individuals, 4 females and 14 males were classified as non-responders.

Field Release Monitoring In 2016, two weeks after initial releases of 300 adults were made at each of the study sites, *D. carinulata* was detected by sweep net at four of the five release sites with an aggregation pheromone lure. Adult and larval *D. carinulata* were found together at two of the five release sites with an aggregation pheromone lure. No *D. carinulata* were detected at sites where a release was made without an aggregation pheromone lure. Samples at sites with a pheromone lure averaged 1.8 individuals per 25 sweeps compared to 0 at release sites without a pheromone lure (Wilcoxon rank sum test, $P = 0.02$) (Fig. 3a).

In 2017, two weeks after initial releases, sweep samples at field sites that received 500 adults had no difference in *D. carinulata* abundance between release sites with and without an aggregation pheromone lure (Wilcoxon rank sum test, $P = 0.25$) (Fig. 3b). Release sites without a pheromone lure averaged 0.25 individuals per 25 sweeps while release sites with a pheromone lure averaged 2.0 individuals per 25 sweeps.

In 2018, two weeks after initial releases, field sites that received releases of 1000 adults and an aggregation pheromone lure had higher numbers of *D. carinulata* compared to the release sites without an aggregation pheromone lure (Wilcoxon rank sum test, $P = 0.02$). Release sites without an aggregation pheromone lure averaged 0.33 individuals per 25 sweeps while release sites with an aggregation pheromone lure averaged 9 individuals per 25 sweeps (Fig. 3c).

There was no detectable effect of the aggregation pheromone lure on the establishment and persistence of *D. carinulata* populations originating from releases of 300 individuals made in 2016 ($P = 0.99$) (Table 1). Three of the releases receiving 300 adults (two releases with, and one release without, a pheromone lure) were lost to herbicide application after monitoring the 1st generation.

Releases made with 500 individuals in 2017 had more adults per 25 sweep samples in sites with a pheromone lure compared to sites without a pheromone lure ($P = 0.002$) (Fig. 4b). The number of generations passed after the initial release was made was also a significant factor, with an average reduction of 0.59 individuals for each additional generation across all the sites ($P = 0.03$).

Releases made with 1000 individuals in 2018 also resulted in more adults per 25 sweeps across three generations compared to sites that did not receive a pheromone lure ($P < 0.001$) (Table 1). The number of generations after the initial release was a significant factor, with an average reduction of 0.28 adults per 25 sweeps for each additional generation ($P = 0.01$).

Discussion

Adults of the biological control agent *Diorhabda carinulata* released in the field tend to fly from release areas, likely due to handling disturbances, resulting in populations vulnerable to Allee effects. Our study attempted to elucidate the effects of a pre-release protocol on pheromone production and determine

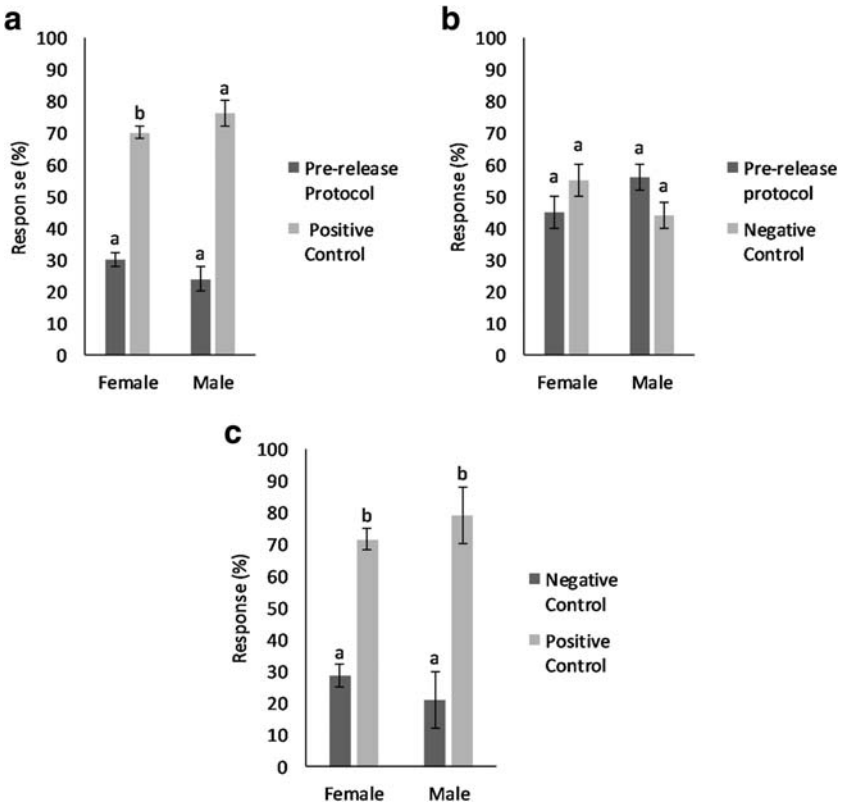

Fig. 2 Binary choice responses (%) of individual reproductive adult *Diorhabda carinulata* in a two-choice olfactometer to volatiles emitted by 100 adults that underwent different treatments: (**a**) a pre-release protocol Vs a positive control, (**b**) a pre-release protocol Vs a negative control, and (**c**) a positive control Vs a negative control, ($n = 100$). Different letters above bars denote statistical differences between treatments

the impact that supplementary synthetic aggregation pheromone had when applied in conjunction with releases of *D. carinulata*. We demonstrated that (i) storage of the insects for 12 h under cool, dark conditions, for the purpose of reducing transportation stress, resulted in a reduction in the initial amount of aggregation pheromone emitted by adults, and (ii) adults can be retained at release sites through application of synthetic aggregation pheromone.

The 12-h holding period used in this study is relatively short compared to that used for most biocontrol agents packaged for release. Typically, insects are collected in the field, sorted and packaged in a laboratory the next day, before being shipped overnight and released into the field a day later. The reduction in aggregation pheromone production after holding adults in dark, cool conditions for just 12 h may be more pronounced with longer holding times.

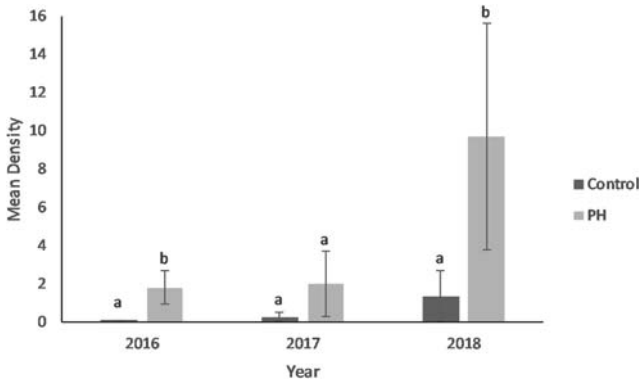

Fig. 3 Sampling for *Diorhabda carinulata* adults for dispersal 10–14 d after an initial release. Mean adult density (numbers ± SE per 25 sweeps) for releases of 300 adults in 2016, 500 adults in 2017, or 1000 in 2018. Releases were made at sites with an aggregation pheromone lure (PH) or without an aggregation pheromone lure (Control)

Once released, male *D. carinulata* need to warm, become active, and initiate feeding before releasing aggregation pheromone (Cossé et al. 2005). Male *D. carinulata* vary emission of aggregation pheromone, which is produced in conjunction with feeding (Cossé et al. 2005). If newly released males start to disperse rather than feed, then production of the aggregation pheromone will be delayed. If an adequate quantity of aggregation pheromone is not being emitted, then newly released adults will not be retained at the release site. Consequently, this will make the population more susceptible to Allee effects, which has been observed at numerous research release sites (DeLoach et al. 2004). The emission of pheromone was reduced during the first collection period for adults subjected to a pre-release protocol relative to control adults. The effect of this reduction was apparent in the behavioral bioassays, with adults that underwent a pre-release protocol only being as attractive as purified air. These results indicate that adult *D. carinulata* subjected to a typical pre-release protocol are likely incapable of attracting or retaining conspecifics at a release site immediately following release.

Reduced emission of aggregation pheromone was ephemeral, with emission of both components being higher during

Table 1 Poisson regression analysis for *Diorhabda carinulata* persistence, based on mean adult numbers per 25 sweeps, after initial release

Year	Release size	Parameter	Coefficient	Standard error	Z value	*P*
2016	300	treatment	19.32	1923.4	0.010	0.99
		generation	0.038	0.12	0.25	0.80
2017	500	treatment	1.87	0.61	3.01	0.002
		generation	−0.59	0.27	−2.17	0.03
2018	1000	treatment	1.39	0.23	6.00	<0.001
		generation	−0.28	0.11	−2.55	0.01

the second (24–30 h) collection period. During this period, adults exposed to the pre-release protocol released similar amounts of the attractive alcohol component as did control insects. However, pre-release protocol insects released more of the aldehyde component than control insects. The aldehyde component is only a minor attractant and it is not known what effect, if any, increased emission of this compound has on aggregation formation and retention. The increased emission during the second collection period suggests that impacts of the pre-release protocol are not permanent, and the insect can recover once exposed to ambient field conditions. This is important, as it suggests there may not be any long-term impact of the pre-release protocol. It is worth noting that the emission rates of both components of the aggregation pheromone were lower during the first sampling for both groups of adults (pre-release protocol and control) compared to the second volatile sampling. This was likely due to disturbing the insects when aspirating them for addition to the collection chambers. However, even with reduced emission, control adults were still able to attract individuals in the bioassay trials, in contrast to the pre-release protocol adults.

The effect of reduced emission of aggregation pheromone and the consequent loss of attraction in pre-release protocol insects can be ameliorated through deployment of synthetic *D. carinulata* aggregation pheromone. Manipulation of *D. carinulata* in the field by deployment of the aggregation pheromone resulted in higher numbers of adults on plants treated with pheromone (Gaffke et al. 2018, 2019). In our trials, the effect of field application of the aggregation pheromone with a new release of insects was especially apparent in subsequent generations, with more individuals detected at release sites with synthetic aggregation pheromone than at sites without. This suggests that a single application of the aggregation pheromone during the initial release may have ongoing

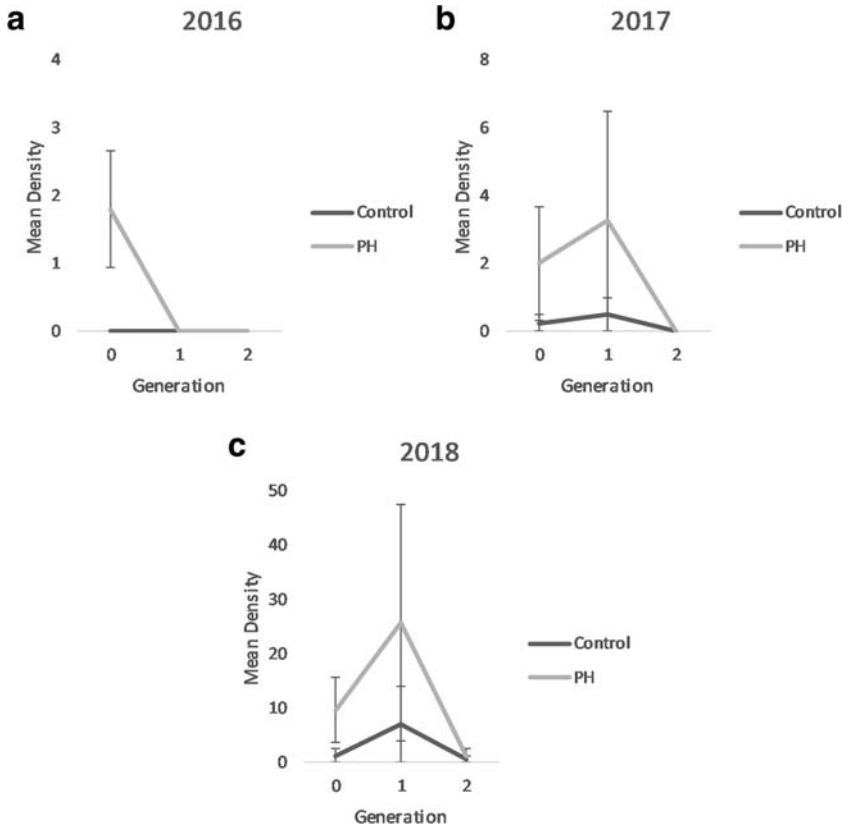

Fig. 4 Sampling for *Diorhabda carinulata* persistence after the initial release. Mean adult density (numbers ± SE per 25 sweeps) for release of (**a**) 300 adults in 2016, (**b**) 500 adults in 2017, and (**c**) 1000 adults in 2018. Releases were made at sites with an aggregation pheromone lure (PH) or without an aggregation pheromone lure (Control). Post-release monitoring at 10–14 days after the initial release is designated as generation 0 on the x-axis

beneficial effects and impact population growth. Increased population growth increases the likelihood that biocontrol releases will persist (Dennis 2002; Grevstad 1999). Use of an aggregation pheromone, especially at the time of release of a biological control agent, may provide a tool for scientists and biological control practitioners to limit dispersal of the agent from release locations.

Releases of 5000 *Diorhabda* spp. on *Tamarix* are recommended to optimize the likelihood of establishment (Knutson and Muegge 2008). However, we used substantially smaller numbers in this study, representing 6, 10, and 20% of the recommended release size (DeLoach et al. 2011). Despite this, we were able to detect *D. carinulata* in the field after release. The results of our study support observations made by Hudgeons et al. (2007), who documented an increased probability of detection of *D. elongata* after release at a new site, not with larger release sizes but with the nearby presence of caged conspecifics. The negative relationship we observed between *D. carinulata* numbers in our samplings and number of generations suggests that the numbers of individuals used in releases for this study may not have been sufficient to result in persistent, long-term establishment of the agent. Thus, a combination of aggregation pheromone and greater numbers of individuals in releases could improve establishment success.

This study demonstrates that a pre-release protocol results in reduced amounts of aggregation pheromone emitted by *D. carinulata*, at least initially following release. The effects of this reduced emission can be mitigated through use of a synthetic aggregation pheromone, which helps to retain individuals at release sites. In turn, this may result in larger field populations of the insect at release sites in subsequent generations.

Acknowledgements Many people provided critical support for this project. A special thanks must be provided to Patricia Gilbert, Amy Adler, and Jennifer Kramer for identifying release locations. Thanks also to the Montana Saltcedar Team for assisting in coordination of the project and helping to find additional collaborators. Megan Hofland and Norma Irish offered helpful advice on the mass rearing process.

Funding Information This project was provided through the Montana Noxious Weed Trust Fund and USDA Forest Service's Forest Health Technology and Enterprise Team - Biological Control of Invasive Native and Non-Native Plants (FHTET-BCIP) competitive grant program.

Data Availability All data analyzed during this study is included in Supplementary Information files.

Compliance with Ethical Standards

Conflict of Interests T.D. and J.R. are sole owners of Restoration Science LLC, which developed and provided the pheromone compound, as used in this research. This product is not currently commercially available. A.M. is the CEO of ISCA Technologies, the company that formulated the pheromone in their proprietary carrier matrix, SPLAT, for this research. This product formulation is not currently commercially available. A.G., S.S., D.B., R.P., and D.W. declare that they have no conflicts of interest.

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