

# Orientation behavior of predaceous ground beetle species in response to volatile emissions identified from yellow starthistle damaged by an invasive slug

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Received: 22 March 2014 / Accepted: 21 July 2014 / Published online: 7 August 2014  
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**Abstract** We investigated indirect defense in the yellow starthistle (*Centaurea solstitialis*)–grey garden slug (*Deroceras reticulatum*)–ground beetle (*Pterostichus melanarius* and *Scaphinotus interruptus*) system. In this host plant/herbivore/predator system, the ground beetles are the primary predator of *D. reticulatum*, the dominant herbivore of the highly invasive weed, *C. solstitialis*. The aim of our study was to examine the behavioral responses of two species of ground beetle to olfactory stimuli emitted from yellow starthistle damaged by *D. reticulatum*. The beetle *P. melanarius* showed a significant preference for the odor of damaged yellow starthistle relative to the odor of intact plants, while *S. interruptus* did not. Volatiles from *D. reticulatum*-damaged yellow starthistle were collected and identified as *trans*- $\beta$ -farnesene, germacrene D, bicyclogermacrene, and 1,5,9-trimethyl-1,5,9-cyclododecatriene. No quantitative relationship was observed between beetle plant choice or decision time and the level of herbivory. Similarly, there was no relationship between volatile compound relative abundance and level of herbivory, suggesting that our range of leaf damage produces either

undetectable semiochemicals or no variation in volatile emission.

**Keywords** Volatile organic compound · *Centaurea solstitialis* · Indirect defense · *Pterostichus melanarius* · *Scaphinotus interruptus* · *Deroceras reticulatum*

## Introduction

Nearly, all biogenic volatile organic compounds (VOCs) serve multiple ecological functions (Sharkey et al. 2001; Dudareva et al. 2006), such as in plant communication, and interest in their plant signaling role has recently increased (Tholl et al. 2006; Yuan et al. 2009). On a tri-trophic level, leaf consumption by herbivores may induce emission or up-regulation of plant volatiles, serving as a signal to natural predators and parasitoids that a plant is under attack. By attracting the natural predators and parasitoids of the plant herbivore, VOCs serve as an indirect inducible plant defense and offer competitive advantage (Turlings et al. 1990; Dicke et al. 1990; Vet and Dicke 1992; Heil 2004).

Tritrophic plant–herbivore–carnivore systems occur widely throughout nature (Price et al. 1980). Yellow starthistle (YST), *Centaurea solstitialis* L. (Asteraceae), is a winter annual native to the Mediterranean region that established in North America around the 1850s (Wilson et al. 2003; DiTomaso et al. 2006). It is a highly competitive and invasive weed with a strong potential for displacing native species and reducing biodiversity. YST emits a suite of volatile compounds (Buttery et al. 1986; Binder et al. 1990), including those implicated in indirect defense in other systems (Arimura et al. 2004; Beck et al. 2008), a mechanism which may be especially beneficial

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Handling Editor: Jarmo Holopainen.

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**Fig. 1** The tritrophic system.  
**a** *Centaurea solstitialis*,  
**b** *Deroceras reticulatum*,  
**c** *Pterostichus melanarius*,  
**d** *Scaphinotus interruptus*.  
 Photographs by R. Hernandez  
 and M. Oster



during the early growth stages of YST when physical defense is low. Its herbivore, *Deroceras reticulatum* (Müller) (Limacidae), or the grey garden slug, is indigenous to the Western Palearctic region (South 1992) and has been accidentally introduced from Europe into most parts of the world (Getz 1959; Quick 1960). This species preferentially feeds on young leaves and seedlings (Hanley et al. 1995) and is implicated as a major herbivore of YST, consuming the weed in its early growth stages with high rates of sublethal damage or complete consumption (Woods et al. 2000; Oster et al. unpublished data). The third trophic level examined in this system is occupied by the ground beetles *Pterostichus melanarius* (Illiger) and *Scaphinotus interruptus* (Menetries) (Coleoptera: Carabidae)—dominant natural enemies and predators of the grey garden slug in the field (Ingram 1946; Cornic 1973; Oberholzer and Frank 2003; Tulli et al. 2009). Both species are nocturnal and take cover during the day (Kavanaugh 1992). *P. melanarius* was accidentally introduced from Europe and now maintains a Holarctic range and broad distribution (Lindroth 1961; Spence and Spence 1988). In contrast, *S. interruptus* is a beetle native to North America, occurring almost exclusively west of the Rocky Mountains (Weber and Kavanaugh 1992). In addition to slugs, it has been documented to feed on snails (Thiele 1977; Ingram 1946), but relatively little else is known about its biology.

To our knowledge, damaged YST has not been studied in the context of indirect defense, and correspondingly, no olfactometer work has been done with *S. interruptus*. The robustness of *D. reticulatum* as a natural enemy of *C.*

*solstitialis* may depend on the sensitivity of slug detection by the above carabid species via indirect signaling. The aims of our study were to examine the behavioral responses of two species of ground beetle, *P. melanarius* and *S. interruptus*, to olfactory stimuli emitted from YST herbivorized by *D. reticulatum* (Fig. 1), and to identify the herbivore-induced volatiles.

## Materials and methods

### Tritrophic system

We collected adult beetles (*P. melanarius* and *S. interruptus*), slugs (*D. reticulatum*), and YST (*C. solstitialis*) seeds in June 2012 from Stanford University's Jasper Ridge Biological Preserve located in San Mateo County, CA. The laboratory housed both species of ground beetles obtained from pitfall traps individually in 20 × 15 × 5 cm rectangular plastic containers with snap-on lids and 20 needle perforations to provide ventilation. Controlled temperature chambers kept the beetles at 17 °C, 12:12 h L:D photoperiod. The photoperiod was reversed, with the scotophase starting at 6:00 am, so that experiments with the nocturnal animals could be conducted in the dark during the daytime. Beetles received 10 mg of fresh slug once 10 days before each trial so that each beetle experienced the same number of starvation days before olfactometer testing. The total number of replicates performed for each beetle was 33 for *S. interruptus* and 73 for *P. melanarius*.

The same conditions applied to slugs obtained from underneath old boards with the addition of a sealed plastic bag surrounding the container to maintain moisture. Slugs fed for 24 h on cut YST leaves once every 2 weeks. Feeding was suspended 14 days prior to a slug's placement on a YST plant for herbivory. YST plants were grown from seed collected at Jasper Ridge in a greenhouse and watered every 2 days. The study used three-week-old plants, 17–20 cm in height. Undamaged plants had no contact with slugs.

#### Experiment 1: olfactometry

Two slugs were placed on each plant and enclosed by a mesh covering for 24 h to produce damaged plants and then removed immediately prior to olfactometer use. The leaf amount eaten ranged from 26 to 299 mm<sup>2</sup>, representing approximately 2–25 % of the total area of each damaged leaf. The olfactometer experiment used a total of 7 damaged and 7 undamaged plants. Approximately 17 beetles were tested per day. Only one pair of damaged and undamaged plants was utilized each day so beetles could be tested with the same plant on a given day. Each plant pair was tested together, without being switched around. The Y-tube olfactometer testing beetle response was designed as follows: One whole damaged and one whole undamaged YST plant were each placed in a sealed one-gallon glass jar in a lit room for at least 4 h before a trial at 20 °C. The lid of each jar with YST contained two tubing outlets for in/out airflow. Medical grade air was released at a flow rate of 250 ml/min and split to each plant jar. Lastly, each arm of the glass Y-tube connected to one of the two jars through tubing and a glass bulb that fit into each arm. Given the nocturnal activity of both the carabids and gastropod, a 120 V R30 red incandescent bulb hung above the center of the Y-tube. The experiment took place in a windowless laboratory room while the lamp allowed for observation of activity in conditions perceived by the beetle as dark (Fig. 2a). Two Y-tubes were alternated between runs and washed with ethanol, dried, and cooled to room temperature. Reversing the position of the arms accounted for arm bias while alternating Teflon<sup>®</sup> tubes connected to each jar ensured that there was no difference in beetle response between one air type going through Arm 1 or Arm 2 (i.e., air from damaged plant flowing through Arm 1 had the same beetle response as when flowing through Arm 2). Recording of beetle behavior occurred every 30 s. The beetle was given a maximum of 15 min to make a decision. A choice was recorded when the beetle reached the end of an arm (where the arm joined the glass bulb) and

remained there for at least 30 s. If no decision was made within 15 min, it was deemed no choice.

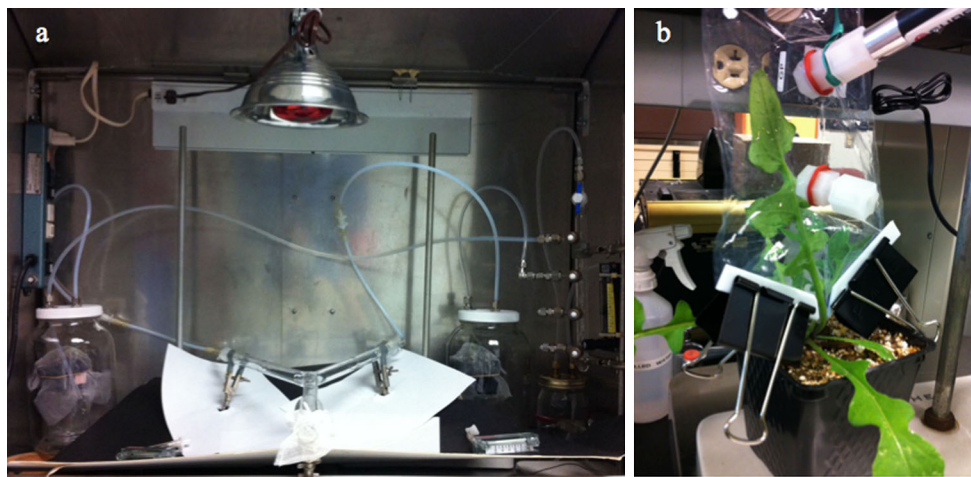
#### Experiment 2: volatile collection after herbivory

The in situ volatile collection experiments were performed as similarly described (Beck et al. 2008) and occurred during morning hours with lighting present in the same laboratory conditions as Experiment 1 at 20 °C. Prior to volatile collection, slugs were placed on the leaves of an intact plant enclosed inside a 2,000-ml glass beaker covered by mesh for 3.5 h, producing 9 damaged plants for volatile collection (2 additional damaged plants were utilized for further compound verification via a second column). Leaf amounts eaten per plant ranged from 40 to 410 mm<sup>2</sup>. Control experiments were performed to determine the background volatile profiles of the slug trail ( $n = 5$ ), plants with undamaged leaves ( $n = 7$ ), and empty bags ( $n = 5$ ) using the same collection protocol as the slug-damaged plants.

A 10.2 × 25.4 cm customized Teflon<sup>®</sup> bag (Welch Fluorocarbon, Dover, New Hampshire) (Beck et al. 2008) was utilized for solid phase micro-extraction fiber (SPME) insertion. A SPME (Supelco, Bellefonte, PA; 100 μm, polydimethylsiloxane fiber) needle was inserted through the port septum and allowed to adsorb volatiles for an exposure period of 2 h (Fig. 2b). A laboratory stand (30.5 cm) with two horizontal rods was positioned next to each plant. The plastic connection of the top outlet port was held up via a twist tie to the lower rod. The Teflon<sup>®</sup> bag was secured over the plant by folding the base of the bag over a rectangular piece of Teflon<sup>®</sup> (6.35 × 1.59 × 0.16 cm) on each side of the stem and secured with a large binder clip.

Collected volatiles were directly injected and thermally desorbed on either a J & W Scientific (Folsom, CA) DB-Wax column (60 m × 0.32 mm i.d. × 0.25 μm) installed on a HP-6890 N gas chromatograph (GC) coupled to HP-5975B mass selective detector (MSD) (MS; Palo Alto, CA) or a J & W Scientific DB-1 column (60 m × 0.32 mm i.d. × 0.25 μm) installed on a HP-6890 GC coupled to HP-5973 MSD. Desorbed volatiles were analyzed using methods previously published (Beck et al. 2008). NIST, Wiley, and internally generated databases were used for fragmentation pattern identification. The retention indices (RIs) were calculated using a homologous series of *n*-alkanes on DB-Wax and DB-1 columns. Volatile identifications were verified by comparison to authentic sample retention times and fragmentation patterns. Chemical standards were obtained as follows: *trans*-β-farnesene (Bedoukian); germacrene D (isolated by previous ARS researchers); 1,5,9-trimethyl-1,5,9-cyclododecatriene (Sigma-Aldrich). Bi-





**Fig. 2** **a** Y-tube olfactometer setup for behavioral assay, **b** volatile collection of *C. solstitialis* using a Teflon® bag and SPME. Photographs by M. Oster

cyclogermacrene was identified based on fragmentation pattern and retention indices on two columns.

## Data analysis

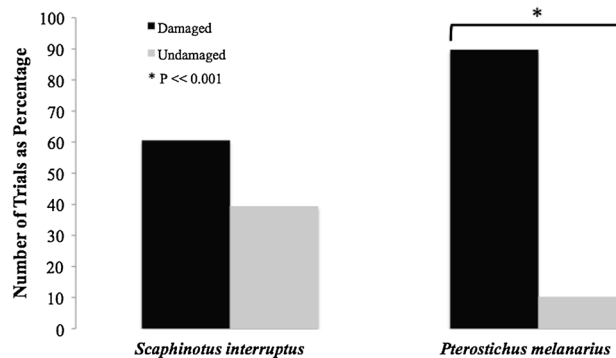
### Experiment 1

We performed the Pearson's chi-square test by predator species to determine significance in preference for damaged versus undamaged YST, analyzing the proportion of trials in which the insect chose odor A or odor B. Our null hypothesis stated that predators had equal preference for the two odor sources. Trials in which no choice was made were not utilized in the statistical analysis. The chi-square goodness-of-fit test was used to determine whether the response of *P. melanarius* differed from that of *S. interruptus*. Logistic regression (R version 2.13.0) was performed to determine correlation between the proportion of damaged choices (beetle choice of damaged rather than undamaged YST) and the amount of leaf area eaten. A linear regression (log-transformed) tested whether the decision time for each beetle was affected by the amount of leaf area eaten. We used Kendall's tau coefficient two-tailed test to assess presence of a trend. An ANOVA tested for interaction between pairs of the following variables: species, choice, and amount eaten.

### Experiment 2

Data from GC–MS analyses were transferred to Microsoft Excel for comparison of retention times and compound identification for same-column analysis. Calculated retention indices were used to assist in compound identification: Samples were run on the DB-Wax column, while the DB-1

### Behavioral Response of Beetle Species



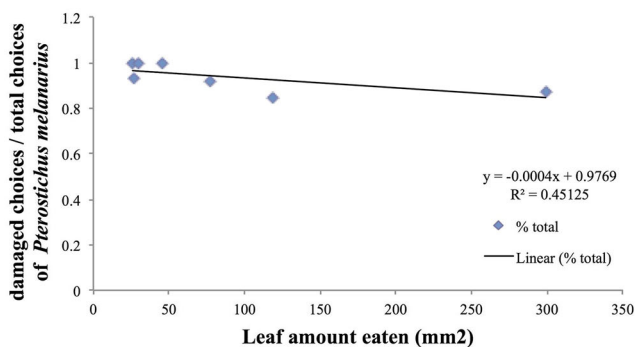
**Fig. 3** Behavioral response of two beetle species in a Y-tube olfactometer. 'Damaged' signifies a beetle choosing the Y-tube arm attached to the damaged plant. 'Undamaged' signifies a beetle choosing the Y-tube arm attached to the undamaged plant. A choice was recorded when the beetle reached the end of an arm (where the arm joined the glass bulb) and remained there for at least 30 s within a 15 min trial

column was used for peak resolution and compound confirmation. We used Kendall's tau for testing association between volatile compound emission for each compound or for the sum of compounds and amount eaten.

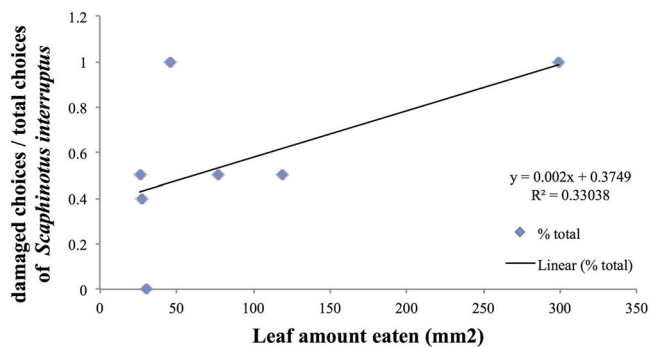
## Results

### Experiment 1

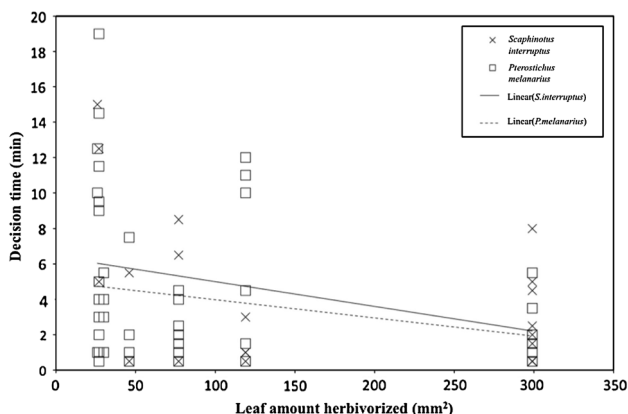
*P. melanarius* showed a significant preference (89.7 %) for the odor of damaged versus undamaged YST in the Y-tube olfactometer ( $\chi^2 = 42.88$ ,  $P \ll 0.001$ , 73 trials: 61 for damaged, 7 for the undamaged plant, Fig. 3). *S. interruptus* did not



**Fig. 4** The fraction of damaged YST choices as percentage made by each beetle species in response to the level of herbivory by *D. reticulatum* (*P. melanarius*  $P = 0.31$ ; *S. interruptus*  $P = 0.097$ ).



Fraction is defined as the ratio of trials in which a beetle species chose a damaged plant to the total number of trials in which the beetle made a decision



**Fig. 5** Relationship between beetle response time and level of herbivory by *D. reticulatum* ( $P > 0.05$  for both beetle species). Decision time is the interval beginning when a beetle was placed at the entrance of the Y-tube and ending when the beetle made a choice for damaged YST

show a significant preference for damaged YST (60.6 %;  $\chi^2 = 1.48$ ,  $P = 0.22$ , 33 trials: 20 for damaged, 13 for undamaged). A goodness-of-fit test ( $\chi^2 = 11.49$ ,  $P \ll 0.001$ ) confirmed that the species behaved differently. There was no significant difference between the two arms of the Y-tube with regard to *P. melanarius* ( $\chi^2 = 2.36$ ,  $P = 0.12$ ) and *S. interruptus* ( $\chi^2 = .004$ ,  $P = 0.95$ ) choice. Air from the damaged plant attached to Arm 1 elicited the same beetle response as when the attachment was switched to Arm 2.

We also analyzed the behavioral responses of both carabids to levels of YST herbivory. The proportion of damaged YST choices over the total number of trials was plotted against the leaf area eaten (Fig. 4), revealing no relationship for either *P. melanarius* ( $P = 0.31$ ) or *S. interruptus* ( $P = 0.097$ ). Logistic regression also showed no correlation between probability of a damaged choice by *P. melanarius* and the level of herbivory ( $P = 0.38$ ), but a positive relationship for *S. interruptus* ( $P = 0.032$ ). However, when the trials for one plant with the highest level of herbivory were excluded, neither correlation

was significant (*P. melanarius*,  $P = 0.25$ , *S. interruptus*,  $P = 0.70$ ). There was no correlation for either species between decision time and the level of herbivory, based on linear regression ( $P > 0.05$  for both, Fig. 5) and Kendall's tau coefficient two-tailed test ( $P > 0.05$ ). Decision time was also shown to not be affected by the individual plant used ( $P = 0.067$ , 0.083) and time since plant damage ( $P = 0.81$ , 0.56) for *P. melanarius* and *S. interruptus*, respectively. The majority of decisions occurred within 5 min, although the longest beetle decision times were observed for plants with the least herbivory. An ANOVA confirmed the absence of interaction effects ( $P > 0.05$  for all variables).

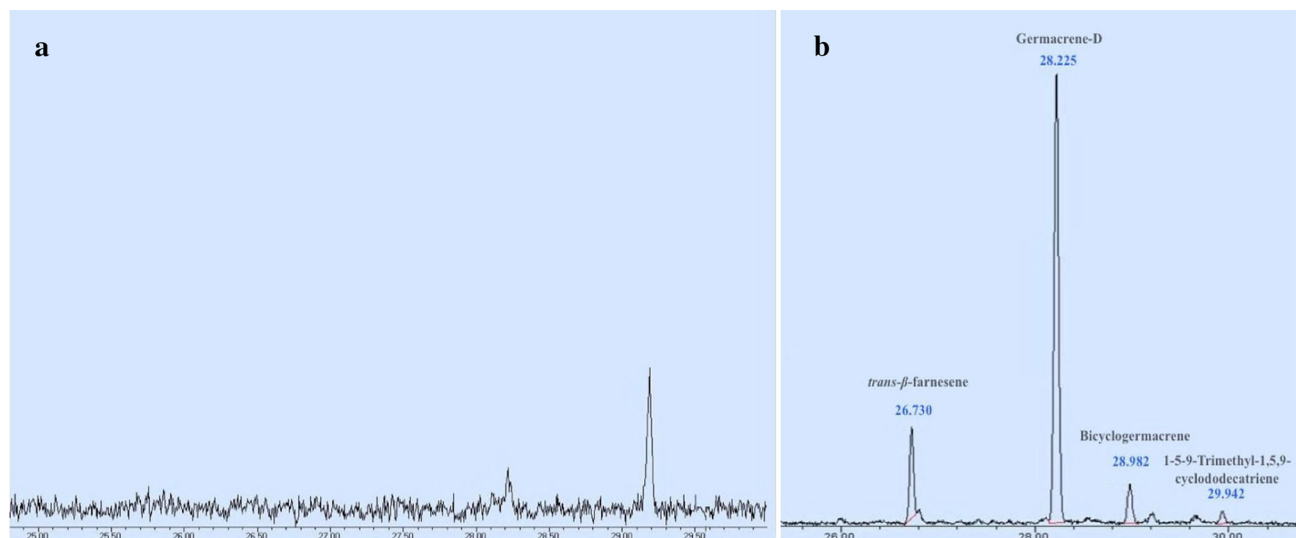
## Experiment 2

No VOCs from slug trail, plants with undamaged leaves, or empty bags were detected. Compounds unique to *D. reticulatum*-damaged YST confirmed by authentic standard and identified by comparison to the library or similar column were *trans*- $\beta$ -farnesene, germacrene D, bicyclogermacrene, and 1,5,9-trimethyl-1,5,9-cyclododecatiene (Fig. 6). Compounds and their average relative abundances are listed in Table 1. There was no statistically significant relationship between amount eaten and volatile relative abundance for each compound or for all four compounds taken as a single signal.

## Discussion

### Volatiles and beetle choice

No volatiles were detected from the undamaged YST plants in our study. This lack of volatile emissions from undamaged, intact YST has been noted in other reports (Beck et al. 2008). It is possible that the difference in plant age (and thus size) between studies plays a role: Our study utilized 3-week-old plants, while the other study collected



**Fig. 6** Volatile compound chromatograms for **a** intact, undamaged YST pre-herbivory (visible peak is GC-MS contaminant), **b** damaged YST post-herbivory

**Table 1** Identified YST volatiles

RI	Compounds pre-herbivory	Compounds post-herbivory	Fragment pattern	Relative abundance	SEM
1662	nd	<i>trans</i> -β-farnesene	69(100), 41(80), 93(55), 67(25), 79(20)	365,694	82,357
1707	nd	germacrene D	161(100), 105(55), 91(50), 41(40), 81(37)	1,031,914	295,268
1732	nd	bicyclgermacrene	121(100), 93(90), 41(55), 92(50), 107(47)	206,393	46,305
1801	nd	1,5,9-trimethyl-1,5,9-cyclododecatriene	68(100), 67(40), 93(37), 107(30), 189(27)	446,136	113,383

RI = calculated retention indices relative to *n*-alkanes on a DB-wax column; nd = not detected; compounds pre-herbivory = undamaged YST ( $n = 7$ ); compounds post-herbivory = slug-damaged YST ( $n = 9$ ); fragment pattern = five largest fragments ( $m/z$ ) with relative percentages to that of the base peak (100) shown in parentheses; relative abundance = average per compound; SEM = standard error mean of each compound post-herbivory

volatiles from plants at 3–5 months of age. In our olfactometry assays, *P. melanarius* showed a significant preference for the odor of slug-damaged YST, while *S. interruptus* did not. Volatile analysis of the odor attracting *P. melanarius* showed to be comprised of four identified sesquiterpene compounds, three of which prominently figure in semiochemical activity (Gibson and Pickett 1983; Kielty et al. 1996; Weissbecker et al. 2000). Since isolated volatiles were not individually tested for beetle response, *P. melanarius* may be attracted to one or a combination of the identified volatiles. Germacrene D consistently had the largest relative abundance when compared to the other detected volatiles: 1,5,9-trimethyl-1,5,9-cyclododecatriene at approximately 45 %, *trans*-β-farnesene at 35 %, and finally bicyclgermacrene at 20 %. Germacrene D was also the most abundant compound in the case of mechanical YST damage (Beck et al. 2008). In other studies, the compounds germacrene D, *trans*-β-farnesene, and bicyclgermacrene were part of the volatile blend attractive to

the predaceous stinkbug when emitted by beetle-damaged potato plants (Weissbecker et al. 2000), suggesting indirect defense activity. The compound *trans*-β-farnesene is the main constituent of the aphid alarm pheromone and has been shown to attract *P. melanarius* to its aphid prey (Kielty et al. 1996). Further studies have shown it to be emitted by plants as an allomone to repel aphids themselves (Gibson and Pickett 1983). The compound 1,5,9-trimethyl-1,5,9-cyclododecatriene has a high retention index (1801), which suggests a low volatility/vapor pressure. This reduces the likelihood of long-range travel in sufficient concentrations and thus requires that the beetle be in close proximity for detection. In addition, Beck et al. (2008) did not detect or report an unknown near this retention time with the described fragment pattern for mechanically damaged YST. The compound 1,5,9-trimethyl-1,5,9-cyclododecatriene may be a newly identified semiochemical which, to our knowledge, has not been previously detected in plant volatile emission and thus

warrants further investigation. However, the response of *P. melanarius* to the compounds should be tested both as a blend and individually to conclusively identify semiochemical behavior in this system.

Our results show that unlike *S. interruptus*, *P. melanarius* responded to volatile stimuli from slug-damaged YST. *P. melanarius*, *D. reticulatum*, and *C. solstitialis* originated from Europe (Getz 1959; Quick 1960; Lindroth 1961; Spence and Spence 1988; Wilson et al. 2003), whereas *S. interruptus* is native to North America (Ingram 1946; Weber and Kavanaugh 1992). Thus, all three players in this tri-trophic system (plant–herbivore–predator) have been introduced from overseas where they may have had time to co-evolve an indirect defense mechanism. This may provide a mechanistic explanation for the often absent natural enemy pressure on introduced plants (Maron and Vila 2001; Agrawal and Kotanen 2003). For example, other studies have found that introduced plants experience a shift in natural enemies from specialist to generalist (Müller-Schärer et al. 2004), which may cause a lack of response from native herbivores or predators to novel volatile blends emitted from the introduced plant species (Wheeler and Schaffner, 2013).

Both carabid species are predators that consume slugs (Ingram 1946; Thiele 1977; Symondson et al. 1996). *D. reticulatum* has exhibited predator avoidance behavior toward *P. melanarius*, suggesting that *P. melanarius* may be an effective predator of *D. reticulatum* (Armsworth et al. 2005). *S. interruptus* appears to be physically adapted to prey on other food items such as snails (Ingram 1946, Thiele 1977), and slugs may be a novel prey item for this species. Compared to *P. melanarius*, *S. interruptus* has a larger body size, a thicker carapace, larger mandibles (Krumbiegel 1960), and spoon-shaped palpi (Thiele 1977), giving it the ability to break through snail shells (Ingram 1946; Thiele 1977). In contrast, *P. melanarius*, which eats slugs and aphids (Symondson et al. 1996), has a stout body with shorter appendages (Lindroth 1961). Visual cues apparently do not influence prey detection for these beetles. Both carabid species are nocturnal and do not demonstrate orientation toward prey when it is presented solely visually (Wheater 1989). *D. reticulatum* leaves trails of mucus that can be followed by conspecifics for up to 6 h after being produced (Cook 1979; Wareing 1986). However, we observed that both species of beetles did not appear to respond to presence of the slug trail mucus. Additionally, *P. melanarius* has not shown a preference for or attraction to slug mucus when presented on a plate at the outlet of a container in which the beetle was housed (Wheater 1989).

#### Trends in leaf amount eaten

After excluding trials for one plant with an extremely high level of herbivory (299 mm<sup>2</sup>), we detected no relationship

for either species between the fraction of damaged choices made and the level of herbivory. Thus, this extreme point carries much influence, and more data would be necessary to confirm this relationship. Previous work performed on YST by others demonstrated that when varying degrees of mechanical damage were applied, an increase in the amount of damage to YST did not result in a significant increase in volatiles (Smith and Beck 2013). We observed no linear relationship between the amount of time beetles took to decide and the amount of leaf area damaged by *D. reticulatum*, which suggests that once YST has been damaged, the degree of herbivory does not play a significant role, possibly because there is not a significant difference in VOC concentrations. Furthermore, Experiment 2 showed that volatile levels did not correlate with the mm<sup>2</sup> eaten by the slug.

It seems unlikely that the concentration in emissions was affected by the total plant size or by the amount of total plant area that remains after damage because both possibilities were controlled for: damaged and undamaged plants were the same age (3 weeks) and approximately the same size, in addition to there being no evidence of a plant-wide response or of individual plant effects (Smith and Beck 2013; Oster et al. unpublished data). Furthermore, the damaged leaves themselves were chosen to be very close in size (average surface area of 11.25 cm<sup>2</sup>), while damage only represented 2–25 % of a single leaf. Finally, our result shows that beetle decision time was unaffected by time since plant damage is further supported by an experiment with mechanically damaged YST, where an immediate increase in volatiles after leaf puncture remained at consistently high levels over 48 h (Smith and Beck, unpublished data).

Both *D. reticulatum* and *P. melanarius* may serve as natural enemies to help control important pests: the slug as a control for YST within our system, and *P. melanarius* as a control for the slug, a common pest for other plant species. Thus, it may be important to assess future climate change effects on YST volatile emissions (McFrederick et al. 2009) and to monitor *D. reticulatum* and *P. melanarius* populations in order to better predict the future success of this invasive weed. Our study demonstrates the role of plant volatiles in recruiting a predator to its prey and has laid the groundwork for future *P. melanarius* and *S. interruptus* olfactometer studies.

**Acknowledgments** We would like to express gratitude to Nona Chiariello of Jasper Ridge Biological Preserve at Stanford University and Todd Tobeck of the Carnegie Institution for Science for providing field advice, to Robert Furrow for statistical expertise, and to Kara Yeung for aid in presentation and design. We also thank Wai Gee of the USDA Agricultural Research Service for providing technical knowledge. This Jasper Ridge Global Change experiment was supported by the National Science Foundation (Grant 091817), the Carnegie Institution for Science, and Stanford University.



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