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SHORT COMMUNICATION

# Volatile emissions from the flea beetle *Altica litigata* (Coleoptera: Chrysomelidae) associated with invasive *Ludwigia hexapetala*

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**Abstract** The flea beetle *Altica litigata* (Chrysomelidae) is an insect herbivore to plants within the families Lythraceae and Onagraceae, including ornamentals such as crape myrtle, *Lagerstroemia* spp. This insect is important both as a pest species and as a naturally occurring biological control agent due to its aggregate feeding behavior, which typically results in severe defoliation of the host plant. Despite the negative economic impact to ornamentals and contrary benefits as a biological control agent, there are few reports on the semiochemical communication of this family of insects. Uruguayan primrose-willow (*Ludwigia hexapetala*) is an invasive aquatic weed in California and serves as a host to *A. litigata*. To better characterize this association, the volatile emissions of *A. litigata* were collected while the flea beetles were: in containers by themselves, in containers with *L. hexapetala* leaves, in situ on *L. hexapetala* leaves in a growth chamber, and in situ on *L. hexapetala* leaves in the field. For comparison, the volatile emissions of *A. litigata* associated with two subspecies of creeping water primrose (*L. peploides*) were also evaluated. Two himachalene-type sesquiterpenes, showing the same carbon skeleton as compounds previously reported from *Aphthona flava* and *Epitrix fuscula*, were detected as volatiles from *A. litigata*.

**Keywords** *Altica litigata* · Coleoptera · Flea beetle · Himachalene-type sesquiterpenes · *Ludwigia* · Primrose-willow · Water primrose

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## Introduction

Uruguayan primrose-willow, *Ludwigia hexapetala* (sect. Oligospermum, family Onagraceae), is an invasive aquatic plant in California freshwater wetlands (Wagner et al. 2007; Okada et al. 2009; Hoch and Grewell 2011). Once established, the rapid growth of *L. hexapetala* readily dominates native vegetation (Fig. 1a), resulting in altered water flow, increased sedimentation, anoxic water conditions, and encroachment upon the habitats of fish and wetland-dependent wildlife (Dandelot et al. 2005).

Owing to their voracious herbivory, large densities, and subsequent defoliation abilities (Fig. 1b), the use of Chrysomelidae as potential biological control agents of invasive aquatic *Ludwigia* species has been considered (Nayek and Banaerjee 1987; McGregor et al. 1996; Center et al. 2002). Five *Aphthona* flea beetle species have been released as a biocontrol agent for the invasive weed leafy spurge (Gassmann et al. 1996) in addition to other related genera (*Agasicles* and *Longitarsus*) used to help control alligator weed and tansy ragwort, respectively (Coombs et al. 2004).

The water primrose flea beetle, *Altica litigata* Fall (Coleoptera: Chrysomelidae), is a known insect pest to

**Fig. 1** Collections being performed at the Delevan NWR (Butte County, CA) site: **a** navigating through a stand of *Ludwigia hexapetala*, **b** observed aggregation of *Altica litigata* on *L. hexapetala*, and **c** in situ volatile collections using SPME and Teflon collection bags

several nursery plants including crape myrtle (Cabrera et al. 2008; Pettis et al. 2004; Pettis and Braman 2007), but is also known to aggregate and feed on *L. hexapetala* (Fig. 1b) (Center et al. 2002; observations by R.I. Carruthers). Recent molecular studies have revealed that a composite of genetic types, or potentially different *Altica* species, is involved in the host specificity and feeding patterns that may separate pest flea beetles from those potentially valuable for biological control of key pest weeds such as *L. hexapetala* (Jenkins et al. 2009, Jenkins personal communications). Despite several reports of pheromone identification and aggregate behavior of other Chrysomelidae genera (Peng et al. 1999; Bartelt et al. 2001, 2011; Soroka et al. 2005; Toth et al. 2005; Zilkowski et al. 2006), little is known about the systematics and chemical communication of the water primrose flea beetle *A. litigata* from California. This and other research are ongoing to clarify this situation.

The objectives of this specific study were: collect the volatile emissions of California populations of *A. litigata* associated with invasive *L. hexapetala* in both laboratory and natural settings to identify potential signaling volatiles (i.e. aggregation pheromone); and, if identified, evaluate the feasibility of using the volatiles to augment biological control of *L. hexapetala* in California wetlands. Moreover, two subspecies of *L. peploides* were used for comparison purposes since field observations noted significant herbivory on *L. peploides* (ssp. *peploides*) and water managers are interested in management strategies.

## Materials and methods

### Flea beetle capture and maintenance

Flea beetles were collected on various dates and locations in California from different populations of *Ludwigia* spp. Collection #1 (10/20/09), Delevan National Wildlife Refuge (NWR) (Colusa County, CA) on *L. hexapetala*; collection #2 (11/3/09) Gray Lodge Wildlife Area (Butte County, CA) on *L. peploides* ssp. *peploides*; and, collection #3 (11/4/09) Lake Hennessey (Napa County, CA) on *L. peploides* ssp. *montevidensis*. Flea beetle taxonomical identification was performed by Alexander Konstantinov (USDA-ARS Systematic Entomology Laboratory Beltsville, MD, USA) and molecular characterizations were further conducted by Tracie Jenkins (Dept. of Entomology, The University of Georgia) in an ongoing study. Flea beetle colonies were maintained in a Percival Scientific incubator, 16:8 light/day cycle (temperature ramp: 4 a.m. lights off, 18.5°C; 4:30 a.m. lights on, 20°C; 3:00 p.m. lights on, 35°C; 8:30 p.m. lights off, 25°C), Philips F32T8/TL741 fluorescent lights, and 78% average relative humidity. Colonies were kept in 53 × 46 × 43 cm rearing cages and fed either *L. hexapetala* or *L. peploides* ssp. *peploides* and *L. peploides* ssp. *montevidensis* depending on the *Ludwigia* species they were collected from. Smaller containers (Farbi-Kal polypropylene PK32T 32 oz) were used for egg laying and larval development. Egg laying: adults (20–30) were placed in containers with *L. hexapetala* sprigs in nine dram (26 × 67 mm) plastic vial for 1–5 days. Adults were removed when a sufficient number of eggs were laid. Larval development: once eggs were laid on *L. hexapetala* sprigs, approx. 5 cm of play sand was added to the containers. *L. hexapetala* sprigs and water were added as necessary to feed developing larvae. Play sand was kept moist to provide optimal conditions for pupation. Newly emerged adults used for studies were placed in a separate cage. For this study adult flea beetles were defined as greater than 3 days old and having showed signs of egg laying. Younger flea beetles used during collection of volatiles were 1−2 days old.

### Plant collection and maintenance

*Ludwigia* taxa were grown in 2-L pots lined with mesh and filled with a 2:1 ratio of play sand/SuperSoil®. One to two pots were placed in 64- to 80-L tubs on a bench in a USDA-ARS greenhouse and water was added and maintained at a level above the rims of the pots. Greenhouse

lights were turned on above plants from 6 to 9 a.m. and again from 4 to 8 p.m. to ensure a total of 14 h light. *L. hexapetala* collected from Delevan NWR on 10/20/09 were planted and fertilized on 10/21/09; *L.* ssp. *peploides* collected from Gray Lodge Wildlife Area on 11/3/09 were planted and fertilized on 11/9/2009; *L. peploides* ssp. *montevidensis* collected from Lake Hennessey on 11/4/09 were planted and fertilized on 11/10/09. One tablet of plant fertilizer (Pond Care Aquatic Plant Food tablets, 20N-10P-5K) was added to each 2-L pot. After initial plantings all pots were fertilized simultaneously on 12/1/09, 2/24/10, and 6/10/10. Identity of *Ludwigia* taxa at each study site was confirmed through morphological evaluation (Hoch and Grewell 2011) and somatic chromosome counts that were used to determine ploidy level (Raven and Tai 1979; Zardini et al. 1991) with modifications adapted from a similar protocol (Singh 2003) to improve accuracy.

## Collection of volatiles

*Containerized flea beetles*. Twenty *A. litigata* (non-sexed) collected from *L. peploides* ssp. *peploides* in the Gray Lodge Wildlife Area on 11/3/2009 were immediately placed in a short, clear glass, wide-mouth septa jar with a 125 mL volume (VWR International) upon arrival to the laboratory (Lacey et al. 2004). A second experiment with identical number of flea beetles was performed concurrently. Volatile collections were performed at room temperature via solid-phase microextraction (SPME) 100 µm polydimethylsiloxane fibers (Supleco) with the following parameters (Beck et al. 2008, 2009): $P$, permeation of volatiles within the container = 3 h; $E$, exposure of SPME fiber to volatiles = 1 h; $S$, storage time of adsorbed volatiles = 1 min; and $T$, thermal desorption of volatiles on SPME fiber onto GC column = 10 min. Air exchange for the flea beetles was allowed via an 18 gauge needle placed in the septum overnight. The following morning a sprig of *L. peploides* ssp. *peploides* (ca. 8 leaves) was added to each sample jar containing the flea beetles and the volatile collections repeated using $P = 0$ min, $E = 2$ h, $S = 1$ min, and $T = 10$ min. Sprig stems were placed in a small lidded container with water and a hole in the lid to pass the stems through. Similarly, sample jars containing only sprigs of *L. peploides* ssp. *peploides* were analyzed for volatile content using $P = 0$ min, $E = 2$ h, $S = 1$ min, and $T = 10$ min. An identical set of experiments was performed with flea beetles collected from *L. peploides* ssp. *montevidensis* in the Lake Hennessey area on 11/4/09.

## Growth chamber in situ collection of volatiles

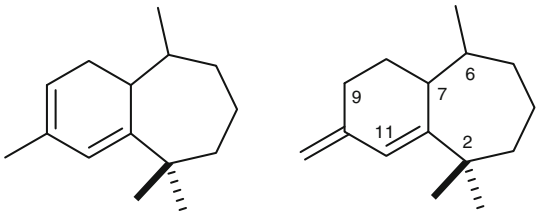
Two intact sprigs of *L. hexapetala* (ca. 8–10 leaves each) in small sealed containers with water were placed in a 1.9-L glass (canning) jar fitted with a lid possessing two holes for SPME sampling and lined with Teflon gasket. Added to the glass jar containing the *L. hexapetala* leaves were ca. 185 reared flea beetles that had food withheld for 1–3 days prior to the volatile analysis and the system placed in the growth chamber for 24 h (see growth chamber parameters noted previously). Volatiles were analyzed via two SPME fibers inserted into the canning jar lid ($P = 0$ h, $E = 24$ h, $S = 1$ min, and $T = 10$ min). A similar experiment with ca. 205 reared flea beetles was performed 1 week later for confirmation via this method.

## Field in situ collection of volatiles

Volatiles were collected in situ from *L. hexapetala* in their natural habitat (Delevan NWR) both with and without *A. litigata* present using custom Teflon collection bags and SPME fibers (Beck et al. 2008, 2009) in early August 2010 (Fig. 1a–c). For *L. hexapetala* without *A. litigata* present collection bags were placed over 3–6 mature leaves of *L. hexapetala*, sealed (late afternoon/early evening), two SPME fibers inserted, and volatiles collected overnight. For *L. hexapetala* with flea beetles present, leaves infested with numerous flea beetles were quickly bagged to include at least 20–30 flea beetles, sealed, and volatiles collected overnight ($P = 0$ h, $E = 12$–$14$ h, $S = 4$–$9$ h, and $T = 10$ min); or, flea beetles (20–30) were tapped into a collection bag, and the bag then placed over the *L. hexapetala* leaves. Additional in situ volatile collections, both with and without flea beetles, were performed the following morning ($P = 0$, $E = 1.5$–$2$ h, $S = 8$–$9$ h, and $T = 10$ min). After field volatile collections each SPME cartridges was placed in a 25 × 200 mm PTFE culture tube (VWR International), capped, sealed with Teflon tape and parafilm, and placed in a cooler with dry ice until GC–MS analyses.

## Analysis of volatiles

For experiments where two SPME fibers were run concurrently in the same collection bag/sample jar, the adsorbed volatiles from one SPME fiber were thermally desorbed on an HP-6890 gas chromatograph (GC) coupled to an HP-5973 mass selective detector with a J&W Scientific DB-Wax column (60 m × 0.32 mm i.d. × 0.25 µm) installed. The second SPME fiber was desorbed on a second, similar GC–MS, but with a J&W Scientific DB-1 column (60 m × 0.32 mm i.d. × 0.25 µm) installed. The GC–MS parameters were identical to previously described programs (Beck et al. 2009). A third GC–MS (Agilent 6890N coupled to a 5975B) with an Astec Chiraldex B-DM enantioselective column (Supelco) installed was used for further compound verification and attempts to separate




A B

**Fig. 2** Relative configurations of the himachalene-type sesquiterpenes detected from *Altica litigata*. Compound A = 2,2,6,10-tetramethylbicyclo[5.4.0]-undeca-1(11),9-diene; Compound B = 2,2,6-trimethyl-10-methylene-bicyclo[5.4.0]-undec-1(11)-ene

stereoisomers of Compound A (Fig. 2). Other experiments (ca. 15) were performed in duplicate in order to perform injections on both the DB-Wax and DB-1 columns. The retention times, linear retention indices (RIs), and fragmentation patterns were verified with authentic compounds for all compounds listed in Table 1. Compounds A and B were verified by a racemic synthetic standard (6R,7S and 6S,7R for both A and B) provided by one of the authors (AAC).

## Results

The containerized volatile collection experiments (Fig. 3a–c, for example) (Lacey et al. 2004) resulted in the detection of a single component, Compound A (Fig. 2), albeit in relative minor amount, from *A. litigata* when the flea beetles were analyzed without the presence of a food source (Table 1). Aside from typical SPME fiber contaminants and column degradation peaks, no other volatiles were detected from only *A. litigata* during the containerized experiments.

Experiments utilizing a growth chamber allowed for analyses of the volatile emissions of laboratory-reared, hungry flea beetles on host plant material and under mimicked environmental conditions. These patterns of emitted volatiles were then compared to the emissions of intact host plants without flea beetle presence. Compound A was again detected in the experiments including *A. litigata*. A second sesquiterpene Compound B (Fig. 2), similar in structure to Compound A, along with *β*-cyclocitral and 2-tridecanone was detected (Table 1); the latter three compounds were transient in both appearance and amount.

**Table 1** Volatiles detected from *Altica litigata* and *Ludwigia* species

| No. | Identity | DB-Wax RI | | DB-1 RI | | FB | FB + plant | LUPE PE | LUPE MO | LUHE |
|---|---|---|---|---|---|---|---|---|---|---|
| | | calcd | auth | calcd | auth | | | | | |
| **1** | Myrcene | 1158 | 1158 | 985 | 984 | | | | | + |
| **2** | Limonene | 1195 | 1196 | 1023 | 1023 | | | | | + |
| **3** | (*Z*)-Ocimene | 1227 | 1230 | 1030 | 1030 | | | | | ++ |
| **4** | (*E*)-Ocimene | 1248 | 1248 | 1042 | 1042 | | | | | +++ |
| **5** | (3*Z*)-Hexenyl acetate | 1314 | 1315 | 989 | 990 | | | | | + |
| **6** | (3*Z*)-Hexenol | 1384 | 1385 | 840 | 841 | | | tr | + | + |
| **7** | α-Copaene | 1489 | 1488 | 1374 | 1375 | | | ++ | ++ | |
| **8** | *β*-Caryophyllene | 1592 | 1592 | 1414 | 1415 | | | + | + | + |
| **9** | Compound A | 1600 | 1600 | 1420 | 1420 | det | det | | | |
| **10** | *β*-Cyclocitral | 1615 | 1615 | 1194 | 1194 | | | | | tr |
| **11** | alloaromadendrene | 1641 | 1640 | 1453 | 1458 | | | + | + | |
| **12** | *γ*-Muurolene | 1684 | 1684 | 1468 | 1469 | | | ++ | ++ | |
| **13** | Germacrene D | 1703 | 1701 | 1473 | 1474 | | | + | + | |
| **14** | Compound B | 1709 | 1709 | 1479 | 1479 | | det | | | |
| **15** | Bisabolene | 1723 | 1724 | 1497 | 1499 | | | tr | tr | + |
| **16** | (*E*,*E*)-α-Farnesene | 1745 | 1745 | 1496 | 1499 | | | tr | tr | ++ |
| **17** | *δ*-Cadinene | 1753 | 1751 | 1512 | 1512 | | | ++ | ++ | tr |
| **18** | 2-Tridecanone | 1806 | 1805 | 1476 | 1476 | | | | | + |
| **19** | Geranyl acetone | 1851 | 1850 | 1430 | 1430 | | | | | + |

Compound identification by calculated (calcd) retention indices (RI) relative to *n*-alkanes on DB-Wax and DB-1 columns, retention times, and authentication (auth); *det*, detected but in varying relative amounts; *FB*, flea beetles (*Altica litigata*); *LUPE PE*, *L. peploides* ssp. *peploides*; *LUPE MO*, *L. peploides* ssp. *montevidensis*; *LUHE*, *L. hexapetala*; *tr*, trace and/or transient; +, minor; ++, moderate; +++, major

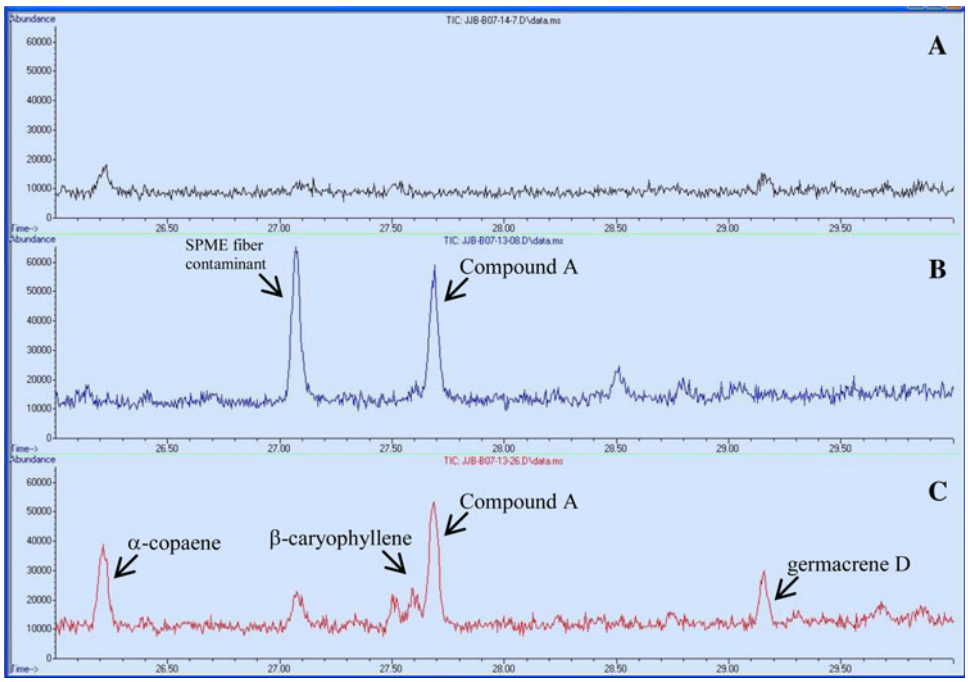



**Fig. 3** DB-1 column total ion chromatograms (TICs) of the volatile analysis of **a** leaves of *L. peploides* ssp. *peploides*, **b** flea beetles, and **c** flea beetles on *L. peploides* ssp. *peploides*

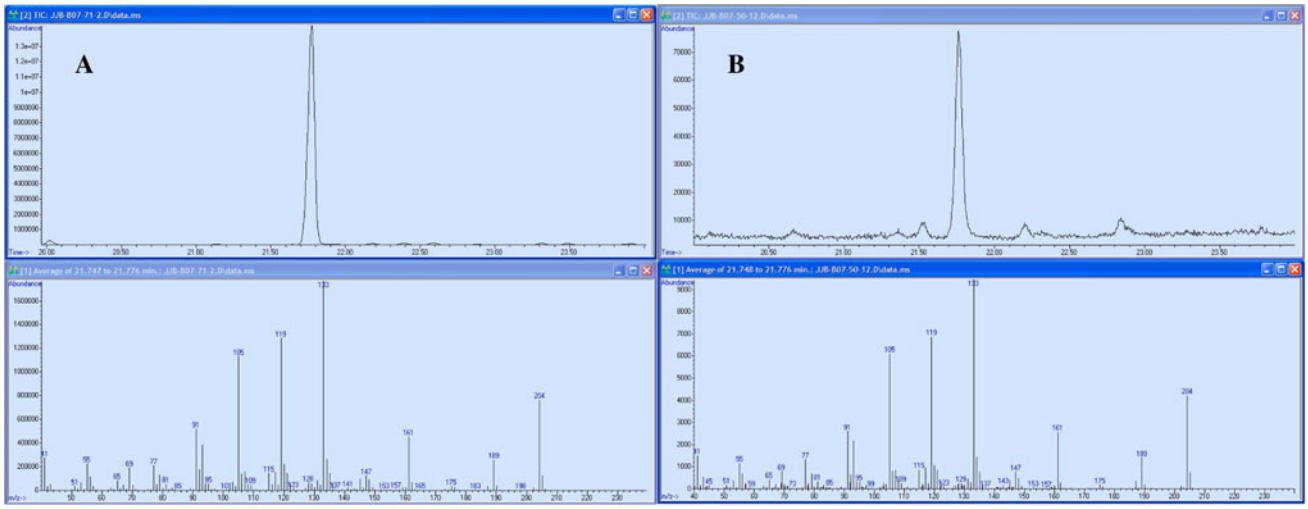



**Fig. 4** DB-Wax column TICs and corresponding mass fragmentation patterns of **a** synthetic Compound A and **b** detected Compound A from flea beetles on *L. hexapetala*

In situ field volatile collection experiments resulted in several instances of the detection of Compound A and only one instance of detection of Compound B.

Table 1 lists the volatiles detected from the host plants used for this study: *L. peploides* ssp. *peploides*, *L. peploides* ssp. *montevidensis*, and *L. hexapetala*. The profiles of volatiles of the subspecies of *peploides*, primarily sesquiterpenes, were similar in both composition and relative amounts. The volatile profile of *L. hexapetala* provided a number of monoterpenes in relatively large amounts in addition to several sesquiterpenes in lower amounts.

## Discussion

Over the course of five separate experiments where *A. litigata* were analyzed by themselves (no host plant food source), only one experiment detected the presence of Compound A, albeit in a relatively large amount. The successful detection of Compound A was performed with *A. litigata* brought in directly from the field and immediately placed in analysis containers. A similar containerized experiment utilizing 2-day old laboratory-reared *A. litigata* by themselves either did not produce sufficient amounts for detection or there were simply no volatiles emitted by the

flea beetles. The ontogenetic variation of the reared flea beetles was not specifically explored during the course of this study and thus its role in emission composition is unknown.

The detection of Compound A from *A. litigata*, both laboratory-reared and collected from the field, was reproducible over the two different host plants, *L. hexapetala* and *L. peploides* ssp. *peploides*. However, the detection of Compound B was more transient than that of Compound A and appeared in only three of the experiments. It should be noted that detection of Compound A occurred over a range of ages of the flea beetles (2 days to more than 5 days) but the occurrence of Compound B detection was only from adult flea beetles and during the collection of volatiles overnight. Due to the transient detection of Compound B and a varying ratio of Compound A to B (24:1, 1:1, and 2:3) the comparison of Compound A to B could not be used at this point for species identification. Bartelt et al. (2001) were able to use the ratios of several similar pheromone sesquiterpenes for identification of *Phyllotreta* and *Aphthona* flea beetles.

The retention times on three different columns and the mass fragmentation patterns of Compounds A and B were verified with their synthetic counterparts (Fig. 4). However, the enantiomers of Compound A could not be separated by enantioselective GC despite the use of a 60 m column and low ramp temperatures. Similarly, Bartelt et al. (2001) failed to resolve Compound A.

The level of herbivory appeared to parallel Compound A and B emission. An increase in herbivory of the *L. hexapetala* host plant was typically associated with the detection of these compounds. No remarks can be offered regarding the time of day for optimal emission since experiments run overnight and experiments run during the day both contained Compound A and/or B.

The observed volatile emissions of the different host plants used in this study were surprisingly different. It should be noted that little is known of the volatile emission and chemical composition of *Ludwigia* spp. (Shilpi et al. 2010). The volatiles in Table 1 are being offered as a point of reference and not intended as in-depth investigation of the composition and relative amounts. The two subspecies of *L. peploides* were similar in their emission profiles with a total of eight sesquiterpenes, of which α-copaene, γ-muurolene, and δ-cadinene were fairly prominent and the other volatiles present in trace to minor amounts. *L. hexapetala* displayed more variety in the types of compounds producing four monoterpenes, with (*E*)- and (*Z*)-ocimene in relatively large amounts, and five sesquiterpenes in trace to minor amounts. (*E*,*E*)-α-Farnesene was detected in moderate amounts. Terpenoid volatile profiles from vegetative sources have been shown to vary within a genus and even among cultivars (Roitman et al. 2011). In addition, many terpenoids have demonstrated semiochemical behavior (El-Sayed 2011), thus the difference in volatile emission between the *Ludwigia* spp. may play an important role in the chemical ecology of this invasive species.

Of the volatiles emitted from both *Ludwigia* spp., there are several that warrant investigation for semiochemical activity—(3*Z*)-hexenol, β-caryophyllene, (*E*,*E*)-α-farnesene, and δ-cadinene—all of which show various semiochemical activity for other Coleoptera (El-Sayed 2011). While the presence of the himachalene-type sesquiterpenes and these noted semiochemicals are compelling as potential attractants for *A. litigata*, it should be noted that these are offered as conjecture only and would need to be proven in thorough field bioassays.

Though transient or present in small amounts, the compounds β-cyclocitral and 2-tridecanone were detected in *L. hexapetala*. β-Cyclocitral has known origins from β-carotene (Lewinsohn et al. 2005), which is known to be present in *Ludwigia* spp. An interesting result regarding β-cyclocitral was its close association with Compound A and/or B. β-Cyclocitral was detected in seven different instances and under varying conditions, of those seven instances six were in conjunction with Compound A and the seventh was with Compound B. All of these instances were also in the presence of *L. hexapetala*. The plant volatile 2-tridecanone, also known as an aggregation pheromone for *Drosophila* spp. (Skiba and Jackson 1993), was detected in experiments with the host plant and the flea beetle present on the host plant. Finally, a mention should be made regarding the highly transient detection of ethyl- and butyl-isothiocyanate. Because these compounds were detected only twice and on only one column, they are not included in Table 1. If these isothiocyanates were legitimately detected instances they pose an interesting result and would be of particular interest since a similar compound allyl isothiocyanate, a host plant metabolite, was found to increase the attractiveness of sesquiterpene pheromones for *Phyllotreta cruciferae* (Soroka et al. 2005; Toth et al. 2005).

A total of 39 experiments performed under varying conditions, collection techniques, and analyses demonstrated the presence of the himachalene-type Compounds A and B from the flea beetle *A. litigata*. Compounds A and B were shown to have the same carbon skeleton as components of aggregation pheromones isolated from other Chrysomelidae genera (Bartelt et al. 2001, 2011). In addition, an analysis of the volatile emissions of three known host plants of *A. litigata*, *L. hexapetala*, *L. peploides* ssp. *peploides*, and *L. peploides* ssp. *montevidensis* provided several candidates for potential attractants. Unfortunately, the enantiomeric composition of the natural Compound A or B was not determined, and due to limited availability bioassays to determine the efficacy of Compounds A and B as attractants for *A. litigata* were not performed. Though a synthesis of Compounds A and B has

been reported (Muto et al. 2004), the synthetic scheme is complex and involves numerous steps, thus efforts into obtaining sufficient amounts for field trapping studies were not justified at this time. If Compounds A and B were commercially available an in-depth field trapping study would be warranted to delineate the role of each compound, and whether both compounds are necessary for attracting *A. litigata*. Further investigations of the host plant volatiles and their potential role as kairomones are necessary to determine if they enhance the efficacy of Compounds A and B. Finally, until the correct enantiomeric composition of Compounds A and B is known and both are readily accessible, the feasibility of using these volatiles to augment biological control of *L. hexapetala* in California wetlands is low.

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