Altered host plant volatiles are proxies for sex pheromones in the gall wasp *Antistrophus rufus*

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We describe a previously uncharacterized function for changes in plant chemistry induced by phytophagous insects: to provide cues for mate location. Larvae of the gall wasp *Antistrophus rufus* Gillette (Hymenoptera: Cynipidae) feed within inconspicuous galls inside the flowering stems of the prairie perennials *Silphium laciniatum* L. and *Silphium terebinthinaceum* Jacquin (Asteraceae). Adult male *A. rufus* emerge before females and are challenged with locating mates that are sequestered within dead plant stems that occur in a matrix of dead vegetation. Allozyme studies revealed complete reproductive isolation between wasp subpopulations in the two plant species. In laboratory bioassays, males responded only to their natal plant species, antennating the stem surface. Males from *S. laciniatum* also responded to hexane extracts of *S. laciniatum* stems, and extracts contained much higher concentrations of monoterpenes (α-pinene, β-pinene, and camphene) than did *S. terebinthinaceum*. Ratios of “+” and “−” enantiomers of α- and β-pinene approximated 50:50 for nongalled *S. laciniatum* stems but strongly differed from 50:50 in galled stems, with “+” and “−” enantiomers strongly dominant in different plants. In bioassays, male wasps from *S. laciniatum* responded to a synthetic blend of the monoterpenes in enantiomeric ratios characteristic of galled stems. Male *A. rufus* rely entirely on olfaction to locate females within stems in a complex prairie habitat, and gall wasps themselves apparently influence the plant to modify ratios of monoterpene enantiomers. These plant volatiles serve as a signal for males, acting as a sex pheromone proxy for females concealed within plant tissues.

Phytophagous insects may induce release of plant volatile chemicals that relay information across trophic levels. For example, induced plant volatiles can attract natural enemies of herbivores (e.g., refs. 1 and 2), discourage oviposition by conspecific herbivores to prevent intraspecific competition between larvae (3), or serve both functions (4). Volatile compounds associated with insect-feeding damage also induce physiological resistance in conspecific plants (5) or across plant species (6). In this paper, we describe a previously uncharacterized function for changes in plant chemistry induced by phytophagous insects: to provide cues for mate location. Although earlier studies have revealed that plant volatiles play an important role in insect mate location (e.g., refs. 7 and 8), ours is to our knowledge the first to suggest that insects alter the chemical composition of plant volatile bouquets for purposes of mate location.

*Antistrophus rufus* larvae feed within galls inside the flowering stems of four species of *Silphium* that occur in prairies of the Midwestern United States (9, 10). These galls form entirely within the stem and are not discernable externally. *Silphium laciniatum* L. and *Silphium terebinthinaceum* Jacquin cooccur throughout the Midwest, including at our prairie study sites in central Illinois (J.F.T. and L.M.H., unpublished results); they are closely related (11) and occasionally hybridize (12). Stems of *S. laciniatum* bolt in mid-May, whereas those of *S. terebinthinaceum* appear about 1 mo later. Consequently, the period during which adult female *A. rufus* are ovipositing into stems shows a bimodal pattern across the two hosts, with ~1 mo between peaks (J.F.T. and L.M.H., unpublished results). The gall wasp larvae develop during summer. In fall, *Silphium* stems senesce, detach from their taproot, and fall to the ground, where they lie through the winter in a matrix of dead plants of various species, the remnants of the plant community.

Male *A. rufus* are protandrous, emerging in spring from the dead and desiccated plant stems, and show an allochronic emergence pattern corresponding to plant phenology: males emerge from *S. laciniatum* ~1 mo earlier than from *S. terebinthinaceum* but are present on both species for a period of ~30 d (J.F.T. and L.M.H., unpublished results). Males walk along the dead stems, rapidly drumming the tips of their antennae on the surface, and position themselves over sites where females will emerge. Males guard these sites, driving off rival males by charging and head-buttting (J.F.T. and L.M.H., unpublished results). After mating, females walk to nearby bolting stems (11). In fact, both sexes of adult *A. rufus* show a pronounced disinclination to take flight, usually walking on host stems or along the ground (J.F.T. and L.M.H., unpublished results). In hundreds of field observations, we have seen only occasional short hopping flights (<1 m distance) to adjacent plants. A disinclination to fly is adaptive for insects such as *A. rufus* that are small-bodied and live in windy habitats where they are subject to involuntary aerial dispersal (13) or occupy perennial late-successional habitats such as prairies, obviating the need to disperse (14).

Male *A. rufus* are challenged with locating mates that are sequestered within dead plant stems, searching through a three-dimensional matrix of dead vegetation for stems that contain females. The difficulty in locating a mate is compounded by the brief life span of *A. rufus* males, averaging <9 d for starved individuals and only 16 d when provided water and a sugar source in the laboratory (J.F.T. and L.M.H., unpublished data). Males are probably relatively short-lived in the field because they are not known to visit flowers of other plant species to feed and are active well before *Silphium* plants bloom. Location of mates is further complicated by interactions with spiders and other predaceous arthropods, resulting in male *A. rufus* dropping to the ground and recommencing their meandering search for host plants.

The purpose of our study was to determine the chemical basis for host plant species recognition and mate location in male *A. rufus*. To evaluate the degree to which males move between the two plant species in seeking mates, we conducted an allozyme analysis to evaluate the level of gene flow between *A. rufus* subpopulations on *S. laciniatum* and *S. terebinthinaceum*. That study revealed that the subpopulations were reproductively isolated, suggesting that males mate assortatively across plant species and are under selection to search for mates only on stems of their natal host species. We next demonstrated that males could discriminate between stems of the two *Silphium* species

Abbreviations: ArSl, *Antistrophus rufus* associated with *Silphium laciniatum*; ArSt, *A. rufus* associated with *Silphium terebinthinaceum*; LCP, Loda Cemetery Prairie Nature Preserve.

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and overwhelmingly preferred their natal host plant species. Analysis of plant volatile compounds revealed that stems of *S. laciniatum* produced three monoterpenes (α-pinene, β-pinene, and camphene) that were in very low abundance in stems of *S. terebinthinaceum*. Ratios of monoterpenene enantiomers may provide a cue for locating mates that persists long after the host plant dies.

**Materials and Methods**

**Source of Specimens.** To provide adult *A. rufus* for our experiments, we collected dead stems of *S. laciniatum* and *S. terebinthinaceum* during the winter of 2000–2001 from four tallgrass prairie sites: Meadowbrook Prairie (Champaign County, N 40° 04.72, W 88° 12.41); Buckley Prairie (Iroquois County, N 40° 33, W 88° 03); Loda Cemetery Prairie Nature Preserve (LCP; Iroquois County, N 40° 31.61, W 88° 04.71); and Prospect Cemetery Prairie Nature Preserve (Ford County, N 38° 55.47, W 95° 22.01). We also used plants of each plant species. Gall wasps had been reared from *S. laciniatum* and *S. terebinthinaceum* plants of each plant species. Gall wasps were collected in late May and were kept in covered paper cups, separated by host plant species, in an incubator at 15°C (15 h light/9 h dark) with honey for food and moistened cotton wicks for water or were stored at 4°C for later use.

We collected dead *Silphium* stems of both species from the same sites, as well as three other prairie sites, including Red Bison Prairie Corridor in central Illinois (Champaign County, N 40° 04.81, W 88° 14.83); Nardi Prairie in Indiana (Parke County, N 39° 38, W 87° 22); and Clinton Park Prairie in Kansas (Douglas County, N 38° 55.47, W 95° 22.01). We also used plants in a common garden of 4- to 5-yr-old *S. laciniatum* and *S. terebinthinaceum* plants (Missouri Wildflower Nursery, Jefferson City, MO) that we established in spring 2000 at the Landscape Horticulture Research Center of the University of Illinois (40° 05.43, W 88° 13.04). In spring 2001 and 2002, we manipulated the degree to which these common garden plants were galled by *A. rufus* by enclosing developing plant stems in organza cages and releasing mated female *A. rufus* of the appropriate subpopulations in cages, randomly assigning “galled” treatments (50 wasps) and “nongalled” treatments (0 wasps) to at least 15 plants of each plant species. Gall wasps had been reared from stems of *S. laciniatum* and *S. terebinthinaceum* stems from field sites LCP and Prospect Cemetery Prairie Nature Preserve.

**Gene Flow Between Subpopulations on Different Host Plants.** To evaluate the amount of mating between individuals of the two subpopulations, we screened 14 enzyme loci in larvae and adults from field sites LCP, Buckley Prairie, and Meadowbrook Prairie by using both starch and cellulose acetate electrophoresis [abbreviations and EC numbers: aconitase (ACON, 4.2.1.3); adenylyl kinase (AK, 2.7.4.3); aldolase (ALD, 4.1.2.13); fumarase (FUM, 4.2.1.2); glucose-phosphate isomerase (GPI, 5.3.1.9); glutamate-oxaloacetate transaminase (GOT, 2.6.1.1); glycerol-dehyde-3-phosphate dehydrogenase (G3PDH, 1.2.1.12); hexokinase (HEK, 2.7.1.1); isocitrate dehydrogenase (IDH, 1.1.1.42); malate dehydrogenase (MDH, 1.1.1.37); malic enzyme (ME, 1.1.1.40); NADH-dependent diaphorase (DIA-1, 1.6.2.2); peptidase A (PEP, 3.4.11); and phosphoglucomutase (PGM, 2.7.5.1)]. We homogenized *A. rufus* larvae or adults in 20 μl of grinding buffer and analyzed homogenates by electrophoresis on either 12% starch gels or Titan III cellulose acetate plates (Helena Laboratories, Beaumont, TX). Our method for starch gel electrophoresis was that of Berlocher and Smith (15). Cellulose acetate electrophoresis was performed for 30 min (GPI, G3PDH, MDH, ME) or 1 h (PGM, IDH) at 150 V, using Tris-citrate buffer (pH 8.6) with the gel apparatus chilled with ice. Enzyme staining for both electrophoretic techniques followed standard procedures (e.g., ref. 16).

**Identification of Volatile Chemical Cues.** We prepared hexane extracts (see Results and Discussion) of each of at least 20 *Silphium* stems of both plant species from field site LCP by the method already described. Because these hexane extracts did not yield sufficient concentrations of chemicals for resolving ratios of enantiomers in some cases (see Results and Discussion), we also extracted monoterpenes from stems by hydrodistillation: we collected dead stems of *S. laciniatum* and *S. terebinthinaceum* from our common garden (n = 5 per species). Stems of 20-cm sections were cut into small pieces (~3 mm) and boiled for 3.5 h in 500 ml of distilled water under reflux in an all-glass hydrodistillation apparatus with water-cooled...
condenser (18). We used 1 ml of hexane as the collection solvent.

To determine the source of variation in monoterpene content and enantionic ratios between *Silphium* species and galled and nongalled stems, we also studied volatile compounds released by bolting stems in our common garden. We trapped plant volatiles from three galled and nongalled plants by enclosing stems in a Reynolds oven cooking bag (made of inert materials) supported from three galled and nongalled stems by enclosing stems in a bolting stems in our common garden. We trapped plant volatiles nongalled stems, we also studied volatile compounds released by drawn out of the bag (90°C) through a column of the adsorbent SuperQ (Alltech Industries, Deerfield, IL) with a 1-hp vacuum cleaner on a variable power supply. We eluted the SuperQ with 2 ml of methylene chloride.

We identified volatile components of extracts in the lab of L.M.H. with a Hewlett–Packard 5973 mass spectrometer interfaced to a HP 6890 gas chromatograph, using a HP-5MS (cross linked 5% phenylmethyl siloxane) capillary column (30 m × 0.25 mm × 0.25 μm film thickness) in splitless mode with helium as the carrier gas (21). Enantiomeric composition of camphene was determined with a 50-m fused silica capillary column with heptakis(6-O-methyl-2,3-di-O-pentyl)-β-cyclodextrin (30% in polysiloxane OV 1701) at 40°C column temperature and 50 kPa hydrogen as a carrier gas (21). Enantionic composition of camphene was determined with a 50-m fused silica capillary column with heptakis(6-O-methyl-2,3-di-O-pentyl)-β-cyclodextrin (30% in polysiloxane OV 1701) at 40°C column temperature and 50 kPa hydrogen as a carrier gas (20). We compared the ratios of enantiomers of α- and β-pinene of galled and nongalled stems by using the nonparametric two-tailed Kolmogorov–Smirnov test (17, 19).

Verification of Response to Volatile Chemical Cues. To verify the response of male *A. rufus* to the three monoterpene standards, we diluted pure synthetic compounds in hexane, individually and in combination, to approximate one equivalent of a 30-cm section of plant stem with ratios of enantiomers characteristic of stems containing galls (≈70% “+” for both α- and β-pinene; see Results and Discussion). We also tested the response of males to standards of the monoterpenes in which ratios were maximally skewed (100% “+” α-pinene and 100% “−” β-pinene). The response of gall wasps was tested by using a bioassay similar to the previous study but improved to exploit the preference of the wasps to walk upside down on the Petri dish lid: We applied 3 ml of standards or pure solvent to two paper squares each (four total), stapled the squares to a disk of organdy (≈14 cm diameter) in a circular arrangement, secured the organdy over a plastic 9-cm-diameter Petri dish with a rubber band, and covered

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Sample sizes are in parentheses. The most anodal band was assigned the value of 100, with electrophoretic mobilities of other bands calculated relative to this value based on measurements of cellulose acetate gels (for starch mobilities, contact authors). Populations are named after their prairies (see Materials and Methods). BP, Buckley Prairie; MP, Meadowbrook Prairie.
the organdy with a second Petri dish to create a chamber. The apparatus was inverted on a ring stand; we released individual wasps into the chamber and observed with a mirror their behavior through the bottom dish.

Results and Discussion

Gene Flow Between Subpopulations on Different Host Plants. For both starch and cellulose acetate electrophoresis, eight of the loci we screened were invariant, but we found scoreable differences at six loci: GPI, PGM, G3PDH, MDH, ME, and IDH. For each of these six loci, there were consistent and fixed differences between gall wasp subpopulations on the two host plant species (Table 1): no alleles were shared between subpopulations, suggesting that the two subpopulations probably represent separate species (22). In our subsequent discussions, we label individuals from *S. laciniatum* as “*ArSl*” and those from *S. terebinthinaceum* as “*ArSt*” to reflect the probable species-level distinction between gall wasps associated with the two plant species.

The results of the allozyme study were consistent with mating trials we have conducted that confirmed that both *ArSl* and *ArSt* males attempted to mate only with females from their natal host species but showed no response whatsoever to females from the other host species (unpublished data). We therefore conclude that male wasps should be under strong selection to search for mates only on the *Silphium* species that was their natal host, further reducing the portion of the habitat where potential mates would be available.

Recognize the Host Plant Species. In the “Y” bioassay with plant stems, males from both plant species discriminated between *Silphium* species and showed a marked preference for their natal host species: 20 of 22 male *ArSl* responded to stems, with 18 choosing stems of *S. laciniatum* ($\chi^2 = 12.8, P < 0.001$); 20 of 24 male *ArSt* responded, with 15 choosing stems of *S. terebinthinaceum* ($\chi^2 = 5.0, P < 0.025$). When encountering stems of their natal host, all males actively antennated the stem surface in a manner identical to their behavior in the field but did not show this behavior on the other plant species.

Isolation of Volatile Chemical Cues. In Petri dish bioassays, male *ArSl* spent significantly more time on paper treated with the combined hexane and ethanol extracts of *S. laciniatum* stems (36.8 ± 10.7 sec) than on solvent-treated paper (10.3 ± 3.5 sec; $t = 2.36, P = 0.028$). All males responded to extract-treated paper as they did plant stems, antennating the surface, but did not display this behavior on solvent-treated paper. Males also responded to hexane extracts alone, spending 38.7 ± 8.6 sec on extract-treated paper compared with 12.2 ± 3.8 sec on solvent controls ($t = 2.84, P = 0.009$), with the typical antennating behavior. There was no significant response to the ethanol extract alone (21.9 ± 6.7 sec on extract-treated paper versus 16.6 ± 3.1 sec on control; $t = 0.72, P = 0.48$). These findings suggest that chemical cues by which male *ArSl* recognized host stems were contained in the hexane extract.

Identification of Volatile Chemical Cues. Hexane extracts of galled *S. laciniatum* contained $\alpha$-pinene, $\beta$-pinene, camphene, 3-hexanone, 2-hexanone, 3-hexanol, and 2-hexanol, but extracts of *S. terebinthinaceum* revealed only very minute quantities of $\alpha$- and $\beta$-pinene (Figs. 1 and 2; differences between plant species for all seven compounds were significant, with Kruskall–Wallis $P < 0.0001$, except $\beta$-pinene with $P < 0.03$). An independent study revealed similar differences between the plant species in the production of volatiles from developing stems in the common garden: 15-min aerations of *S. laciniatum* stems yielded significant amounts of monoterpenes, but *S. terebinthinaceum* stems produced such minute amounts that they were marginally detectable even when aeration periods were increased ∼15-fold (unpublished data).

In addition, enantiomeric ratios of monoterpenes differed...
between *S. laciniatum* stems that contained galls and those that did not (Fig. 3): for both α- and β-pinene, ratios of “+” and “−” enantiomers varied around 50:50 in nongalled stems, but these ratios were significantly skewed in stems that contained galls (α-pinene: Kolmogorov–Smirnov statistic = 0.14, \( P = 0.003; \) β-pinene: statistic = 0.24, \( P < 0.0001 \)). A preliminary study of volatiles of *S. terebinthinaceum* revealed that enantiomeric ratios of α-pinene show a similar shift with galling, but this effect was absent in β-pinene (unpublished data).

The variation in our data on enantiomeric ratios of monoterpenes of α- and β-pinene (Fig. 3) in galled stems may reflect differences between sections of plant stems in density of galls. The effect of gall wasps on enantiomeric ratios may be very localized within plants, inducing changes in adjacent tissue, rather than being a systemic effect on the whole plant, as is the case with the salivary secretions of gall wasps that induce gall formation (23). In that case, ratios of enantiomers (summed across plant sections) would be more highly skewed for stems with high densities of evenly distributed galls but would approach 50:50 for stems that lack galls in some sections. Future studies will evaluate the spatial aspects of induced changes in enantiomers with galling of stems.

**Verification of Response to Volatile Chemical Cues.** In Petri dish bioassays, male *ArSl* showed no response to α-pinene, β-pinene, and camphene tested separately in enantiomeric ratios representative of galled stems (mean time spent on paper squares: 21.3 ± 16.5, 51.0 ± 18.7, 50.3 ± 20.5, and 21.3 ± 16.5 sec, for α-pinene, β-pinene, camphene, and control, respectively; ANOVA *F*\(_{3,30}\) = 0.83, \( P = 0.49 \)). When the three compounds were combined with the same ratios of enantiomers as found in galled host material, however, male *ArSl* spent nearly twice as much time on paper treated with the monoterpenes (81.9 ± 11.8 sec) than on controls (41.7 ± 11.2 sec; \( f = 2.48, P < 0.020 \)) but, more importantly, responded to the synthetic blend by antennating the paper just as they did in searching the stems of host plants. Male wasps also significantly responded to the blend of 100% “+” α-pinene and 100% “−” β-pinene, remaining on monoterpene-treated paper more than four times as long as on solvent controls (23.1 ± 6.5 sec and 5.2 ± 1.0, respectively; \( f = 2.7, P = 0.013 \)). The considerably shorter times for the monoterpene treatment in this study (23.1 min) than in the previous study (81.9) suggest that males were more responsive to ratios simulating those of plants; however, the two bioassays were conducted independently, and their results therefore are not directly comparable. Our findings support the notion that male *ArSl* recognize stems of host plants that contained gall wasps by skewed ratios of monoterpene enantiomers.

We conclude from these findings that male *ArSl* are reproducively isolated from *ArSl* and rely on olfaction to locate females within the stems of their natal host plant species in a structurally and taxonomically complex prairie habitat. Chemical differences between plants in our common garden plot, where galling treatments were assigned randomly to plants, confirm that *S. laciniatum* and *S. terebinthinaceum* stems differ in the composition of their monoterpene profiles, and that gall wasps alter ratios of monoterpene enantiomers in both plant species. Thus, volatile bouquets of host plants serve as a signal for males that are searching for mates, and plant volatiles take on the functional role of sex pheromones for females that are sequestered within plant tissues. Our findings attest to the intimate and elaborate interactions characteristic of gall-forming insects and their host plants (24). Gall formers are known to manipulate host plant chemistry for their own benefit (25), but our results expand the inventory of host plant manipulation and show that gall formers influence plants in ways more extensive and subtle than previously reported.

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