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Electroantennogram responses of male *Sphinx perelegans* hawkmoths to floral and 'green-leaf volatiles'

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Abstract

Electroantennograms (EAGs) from field-collected male *Sphinx perelegans* hawkmoths were recorded in response to 10 individual floral scent compounds identified from *Clarkia breweri* (Onagraceae), 21 additional volatiles characteristic of other night-blooming flowers, and eight 'green leaf' volatiles. Measurable EAG responses were elicited to all compounds tested, but the most effective antennal stimulants were benzyl acetate, linalool, methyl salicylate and trans-2-hexenal. Mean, pooled EAGs to oxygenated terpenoids, aromatic esters and fatty acid derivatives were larger in magnitude than those in response to aromatic aldehydes/alcohols, monoterpenes and nitrogen-bearing compounds. The rank order of male *S. perelegans'* EAGs did not differ significantly from that of previously recorded responses of male *Hyles lineata* to the same scent compounds, and EAG magnitudes were generally larger for *S. perelegans* than for *H. lineata*. Both hawkmoth species are shown to have broad olfactory receptivities and could potentially respond to a wide array of plant volatiles as floral attractants.

Introduction

Hawkmoths (Sphingidae: Lepidoptera) are large, primarily nocturnal insects, with powerful, hovering flight and high metabolic demands (Heath & Adams, 1965; Heinrich, 1971; Bartholomew & Casey, 1978). Many hawkmoth species meet these demands by foraging for floral nectar; some are important pollinators across diverse temperate (Gregory, 1964; Miller, 1978; Grant, 1983) and tropical habitats (Silberbauer-Gottsberger & Gottsberger, 1975; Haber & Frankie, 1989; Nilsson et al., 1985).

Many hawkmoth-pollinated plants share a number of floral characters putatively associated with hawkmoth attraction, including nocturnal anthesis, pale coloration, sucrose-rich nectar and strong, sweet floral scent (Baker, 1961; Gregory, 1964; Faegri & van der Pijl, 1972; Miller, 1978; Grant, 1983; Knudsen & Tollsten, 1993). We have studied one plant, *Clarkia breweri* (Onagraceae), that appears to have evolved this suite of traits in conjunction with an evolutionary shift from bee- to hawkmoth-pollination (MacSwain et al., 1973; Raguso & Pichersky, 1995). Previous studies have focused on floral scent production in *C. breweri* and its evolution as a novel reproductive character in the genus *Clarkia* (Pichersky et al., 1994; Raguso & Pichersky, 1995; Raguso, 1995; Dudareva et al., 1996), yet its importance in hawkmoth attraction has not yet been determined. Floral scent is typically discussed as a long distance hawkmoth attractant (Tinbergen, 1958; Faegri & van der Pijl, 1979; Brantjes, 1973; 1978; Nilsson, 1983; Dobson, 1994), but experimental tests are few and results are equivocal. Chemical analyses show that hawkmoth-pollinated flowers emit a diverse array of terpenes, aromatics, fatty acid-derived and nitrogen-bearing compounds (Nilsson, 1983; Tollsten, 1993; Kaiser, 1993; Raguso & Pichersky, 1995), with low levels of chemical similarity between species (Knudsen & Tollsten, 1993). Brantjes (1973; 1978) conducted flight tunnel bioassays with two hawkmoth

species (*Deilephila elpenor* and *Manduca sexta*) and concluded that floral scent blends function as 'sign-stimuli' that release visual search behavior and upwind flight in hawkmoths. However, there have been no comprehensive surveys on physiological or behavioral responses of flower-feeding hawkmoths to different floral scent blends, and little is known about the relative importance of visual vs. olfactory cues in hawkmoth foraging (Knoll, 1925; Kugler, 1971; White et al., 1994).

One approach to studying hawkmoth-flower interactions is to measure the olfactory capabilities of different flower-visiting hawkmoth species. Previously, we recorded electroantennogram (EAG) responses of *Hyles lineata*, a generalist hawkmoth species that forages during daylight, dusk, and evening throughout North America, to different floral and vegetative odors (Raguso et al., 1996). *H. lineata* is an important pollinator of *C. breweri* (Raguso, 1995) and similar plants in central California (Grant, 1952; Gregory, 1964; Chase & Raven, 1975; Hodges, 1995) but visits an extremely broad range of flowering plants throughout its distribution (Fleming, 1970; Kislev et al., 1972). Not surprisingly, it is olfactorily responsive to a broad array of floral and vegetative volatiles including aromatics, fatty-acid derived compounds, and terpenoids, through a wide range of physiologically relevant concentrations (Raguso et al., 1996).

In contrast, the other important hawkmoth pollinator of *C. breweri*, *Sphinx perelegans*, is a strictly nocturnal species limited to western North America (Hodges, 1971), and appears to be a relative specialist in terms of larval hostplant use (S. Miller, C. Conlan, unpubl. data). In addition, adult *S. perelegans* moths have been observed foraging at relatively few plant species, including *C. breweri*, *Diplacus aurantiacus* (Scrophulariaceae) (Raguso, 1995), *Oenothera caespitosa* and *O. elata* (= *hookeri*, Onagraceae) (Gregory, 1963, 1964). Do the differences in distribution, life history and foraging patterns between these two hawkmoth species predict differences in olfactory receptivity to different plant volatiles, or should the antennae of these and other flower-visiting hawkmoths be expected to detect most/all classes of plant volatiles? Here we measure the EAG responses of male *S. perelegans* moths to a diverse array of 39 plant volatiles, including floral scent compounds from *C. breweri* and other night-blooming plants as well as vegetative volatiles.

Materials and methods

Insects. Adult male *S. perelegans* moths were collected at UV light traps in an oak/pine woodland two km east of Pinnacles National Monument, San Benito Co., CA in May, 1994, and were transported live to Albany, CA. Moths were held in 1 m^3 screen cages within the laboratory for 1–2 days (25 °C, L12:D12) and fed a 10% sucrose solution.

Olfactory stimuli. The compounds tested (Table 1) included 10 of the 12 major floral volatiles from *C. breweri* (Raguso & Pichersky, 1995), 21 aromatic, terpenoid, and nitrogen-bearing volatiles characteristic of other hawkmoth-pollinated flowers (Nilsson et al., 1985; Kaiser, 1991; Knudsen & Tollsten, 1993) and eight 'green leaf volatiles' (GLVs), C_6-C_8 aliphatic alcohols, aldehydes and esters that are ubiquitous in plant foliage (Visser et al., 1979; Light et al., 1993). Biological justification for our selection of odor stimulants was detailed by Raguso et al. (1996), and includes previous identification from the fragrance of hawkmoth or noctuid moth-pollinated flowers, or structural affinity to one of these compounds, with slight functional group variation. The GLVs appear to be important in insect-plant interactions (e.g. host finding and oviposition) and may provide strong structural contrast to the floral compounds used in our receptivity assays. The sources of test chemicals were Aldrich Chemical Co. and Robert Flath (USDA-ARS, WRRC). Chemical purities ranged from 97–99% (except amyl salicylate, 75%; (Z)-jasmone, 70%; farnesol, 95%). Test stimulants were prepared as 10% volumetric solutions in hexane. In the cases of indole, vanillin, and veratraldehyde, diethyl ether was used as a solvent. We prepared odor stimulants within 20 min prior to each experiment by pipetting c. 100 μg aliquots of each solution onto filter paper strips, and then allowing the solvent to evaporate for 30 s. The strips were then placed into Pasteur pipettes and stored at 5 °C until use.

EAG technique. EAG data were measured and recorded on a Tektronix 5113 storage oscilloscope as previously described by Light et al. (1988) and Raguso et al. (1996). We assembled electrodes by inserting silver chloride filaments within drawn-glass capillary tubes containing physiological saline solution (Raguso et al., 1996). In preparation for EAG recordings, live moths were mounted between a cardboard gasket and a grooved plexiglass block, in which the wings were immobilized beneath the cardboard, the body fit snugly

Table 1. Scent compounds used as antennal stimulants, EAG responses of *Sphinx perelegans*, and rank order values for both *S. perelegans* and *Hyles lineata* male hawkmoths

Compound Class	Chemical formula	*S. perelegans* EAG[1]	EAG Rank orders[2]		Changes
			S. perelegans	*H. lineata*	> 10
Aromatics					
amyl salicylate	$C_{12}H_{16}O_3$	0.45 ± 0.04	16	14	
benzaldehyde	C_7H_6O	0.78 ± 0.08	8	22	<
benzyl acetate[3]	$C_9H_{10}O_2$	0.90 ± 0.05	1	5	
benzyl alcohol	C_6H_6O	0.38 ± 0.04	20	36	<
benzyl benzoate[3]	$C_{14}H_{12}O_2$	0.26 ± 0.05	23	29	
benzyl salicylate	$C_{14}H_{12}O_3$	0.33 ± 0.04	21	26	
(E)-cinnamic aldehyde	C_9H_8O	0.31 ± 0.06	30	27	
eugenol[3]	$C_{10}H_{12}O_2$	0.36 ± 0.08	17	24	
indole[4]	C_8H_7N	0.33 ± 0.13	32	19	>
methoxy-2-methyl benzoate	$C_9H_{11}O_3$	0.41 ± 0.04	18	12	
methyl anthranylate[4]	$C_8H_9O_2N$	0.36 ± 0.06	29	13	>
methyl benzoate	$C_8H_8O_2$	0.66 ± 0.20	14	17	
methyl cinnamate	$C_{10}H_{10}O_2$	0.26 ± 0.03	33	16	>
methyl isoeugenol[3]	$C_{11}H_{14}O_2$	0.23 ± 0.04	22	25	
methyl salicylate[3]	$C_8H_8O_3$	0.89 ± 0.06	5	4	
phenylacetaldehyde	C_8H_8O	0.44 ± 0.11	19	9	>
2-phenyl ethanol	$C_8H_{10}O$	0.45 ± 0.05	27	15	>
vanillin[3]	$C_8H_8O_3$	0.05 ± 0.04	36	34	
veratraldehyde[3]	$C_9H_{10}O_3$	0.07 ± 0.02	37	30	
Fatty Acid Derivatives					
2-methyl butyraldoxime[4]	$C_5H_{12}ON$	0.43 ± 0.13	10	11	
hexanal	$C_6H_{12}O$	0.24 ± 0.05	34	31	
(E)-2-hexenal	$C_6H_{10}O$	1.05 ± 0.13	2	33	
hexan-1-ol	$C_6H_{14}O$	1.26 ± 0.25	35	2	>
(Z)-3-hexen-1-ol	$C_6H_{12}O$	0.79 ± 0.07	7	8	
(E)-2-hexen-1-ol	$C_6H_{12}O$	0.70 ± 0.15	13	7	
(Z)-3-hexenyl acetate	$C_8H_{14}O_2$	0.66 ± 0.21	9	3	
(E)-2-hexenyl acetate	$C_8H_{14}O_2$	0.36 ± 0.10	26	6	>
hexyl acetate	$C_8H_{16}O_2$	1.15 ± 0.29	4	23	<
(Z)-jasmone	$C_{11}H_{16}O$	0.43 ± 0.12	25	25	
methyl jasmonate	$C_{13}H_{20}O_3$	–	–	**	
Monoterpenes					
1,8 cineole	$C_{10}H_{18}O$	0.66 ± 0.15	6	35	<
geraniol	$C_{10}H_{18}O$	0.49 ± 0.08	24	21	
limonene	$C_{10}H_{18}O$	0.33 ± 0.06	31	28	
linalool[2]	$C_{10}H_{18}O$	0.92 ± 0.08	3	1	
linalool oxides (Z/E-furanoid)[3]	$C_{10}H_{18}O_2$	0.55 ± 0.14	11	10	
linalool oxide (Z-pyranoid)[3]	$C_{10}H_{18}O_2$	–	–	**	
myrcene	$C_{10}H_{16}$	0.53 ± 0.38	15	37	<
(E)-β-ocimene	$C_{10}H_{16}$	0.63 ± 0.02	12	32	<
allo-ocimene	$C_{10}H_{16}$	–	–	**	
Sesquiterpenes					
germacrene D	$C_{15}H_{24}$	0.39 ± 0.08	**	–	
farnesol	$C_{15}H_{26}O$	0.28 ± 0.06	28	18	>
nerolidol[3]	$C_{15}H_{26}O$	0.33 ± 0.04	**	–	

[1]Mean EAGs (absolute values of corrected mV responses) ± standard errors. [2]Tabulated from data expressed as % of standard stimulus. [3]Identified in floral headspace of *Clarkia breweri*. [4]Compounds bearing Nitrogen atoms. **Compound used in experiments with *Sphinx* or *Hyles*, but not with both species.

within the plexiglass groove, and the five terminal (right) antennal segments were excised. The recording electrode was inserted into the antennal cavity, while the ground electrode was inserted into the head at the base of the antenna. Antennae were bathed continuously in a filtered air stream and 1 s test stimulations were presented in randomized order as previously described, each stimulus followed by a 60 s purge period (Raguso et al., 1996). For each compound tested, EAGs were recorded from four male moths. Control stimuli (1 μL of hexane solvent per filter paper) and standard stimuli (1 μL of 1% linalool in hexane per filter paper) were interspersed about every fifth compound tested.

Treatment of EAG data. EAG responses (measured in mV) to test compounds were adjusted to compensate for solvent and/or mechanosensory artifacts by subtracting the accompanying control EAG, yielding corrected mV data (Reed et al., 1987; Light et al., 1988, Gabel et al., 1992). In order to examine relative antennal receptivity to all scent compounds, which varied two-fold in molecular weight and three-fold in volatility, we standardized the EAG data by expressing mean corrected mV EAG data as a percent of the standard stimulus, 1% linalool. In addition, we compared the EAGs of male *S. perelegans* with previously published data from male *H. lineata* (Raguso et al., 1996). We ranked EAG responses (% of standard) to the 37 scent compounds common to both studies in descending order of magnitude for each species, and then compared rank orders using Spearman's Rank Correlation Coefficient (Sokal & Rohlf, 1981).

Results

EAG responses. All test stimulants elicited measurable EAG traces that were generally similar in shape and greater than those observed for hexane controls. Of the ten *C. breweri* floral volatiles tested, the largest amplitude EAGs ($>$ 0.4 mV) were observed in response to linalool, methyl salicylate, benzyl acetate, and furanoid linalool oxide (Table 1). Vanillin, veratraldehyde, and methylisoeugenol were relatively poor antennal stimulants for male *S. perelegans* moths, as they were for male *H. lineata* in previous experiments (Raguso et al., 1996). Mean EAG responses to the 21 additional floral scent compounds varied from medium (0.25 mV, e.g., methyl cinnamate) to large amplitudes ($>$ 0.6 mV, e.g., 1,8 cineole, benzaldehyde) (Table 1). Large compounds of low volatility, such as germacrene D, nerolidol, and benzyl salicylate were relatively potent stimulants. Nearly all of the smaller, more volatile GLVs, elicited large amplitude EAGs, especially hexan-1-ol, hexyl acetate and trans-2-hexenal (Table 1). For comparative purposes, *S. perelegans'* EAG responses to different chemical classes of plant odorants are summarized (Figure 1) as pooled derived grand means (% of standard). Aromatic aldehydes/alcohols, monoterpenes, and nitrogen-bearing compounds evoked mean EAGs of moderate amplitudes, while aromatic esters, oxygenated terpenoids, and fatty-acid derivatives evoked generally large EAGs.

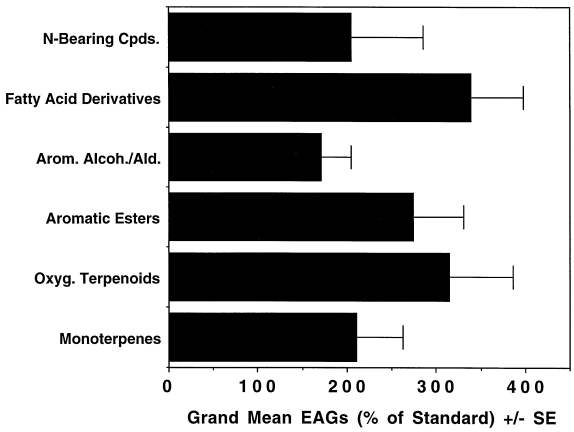

Figure 1. Summary of male *S. perelegans'* EAG responses to different chemical classes, as derived from pooling and averaging the mean EAG responses to each test compound belonging to a given chemical class. Relative responses are expressed as% of response to standard stimulus (1% v/v linalool in hexane).

EAG rank orders. Rank orders of mean EAGs, expressed as a percent of the standard stimulus, are listed in Table 1 along with previously published rank orders of male *H. lineata* EAGs for the same compounds (Raguso et al., 1996). EAG ranks for male *S. perelegans* and *H. lineata* were significantly correlated (Spearman's $\rho = 0.366$, P $= 0.026$, N $= 37$ compounds common to both experiments), but ranks differed by greater than 10 places for 38% of the test compounds (Table 1). Benzaldehyde, 1,8 cineole, myrcene, hexyl acetate, and trans-2-hexenal were potent antennal stimulants for *S. perelegans*, but poor stimulants for *H. lineata*. The converse was true for (E)-2-hexenyl acetate, 2-phenylethanol, indole and methyl anthranylate.

Discussion

The antennae of wild-caught adult male *S. perelegans* responded to a broad array of plant-derived olfactory stimulants representing numerous chemical moiety classes, variations in carbon skeleton, molecular weight and volatility. Given the overall similarity in EAG magnitudes and rank orders between male and female *H. lineata* observed in previous studies (Raguso et al., 1996), we suspect that male and female *S. perelegans* will have comparable olfactory receptivities to plant odorants. The results of this study establish that male *S. perelegans* hawkmoths can detect the volatile compounds emitted from flowers of *C. breweri*, which they visit and pollinate in central California (Raguso, 1995), and are consistent with the hypothesis that these fragrance compounds could function as olfactory attractants (MacSwain et al., 1973; Raguso & Pichersky, 1995). Behavioral bioassays will be needed to directly test this hypothesis. In addition, this study demonstrates that male *S. perelegans* moths can detect scent compounds that typify other night-blooming plant species, some of which are similar to those identified from *C. breweri* flowers, as well as small, aliphatic GLVs that are more characteristic of wounded vegetation (Visser et al., 1979; Light et al., 1993). The large EAG responses of male *S. perelegans* and *H. lineata* males to many of the 'green leaf' volatiles (Table 1), semiochemicals usually discussed as hostplant attractants for female herbivorous insects (Visser & Avé, 1978), underscore the potential importance of vegetative volatiles as modifiers of olfactory responses to sex pheromones (Dickens et al., 1993; Light et al., 1993) and nectar foraging behavior (Beker et al., 1989) in these phytophagous insects.

When combined with previously published data for wild *H. lineata* (Raguso et al., 1996) and laboratory-bred *Manduca sexta* (Light, unpubl. data), our results suggest that both specialist and generalist nectar-foraging hawkmoths have broad olfactory receptivities and are capable of responding to complex, multicomponent floral and vegetative odorant blends with constituents from diverse chemical classes. This is an intuitive result, given the spatial and temporal variation in floral resources encountered by hawkmoths (Kislev et al., 1972; Haber & Frankie, 1989) and the low levels of chemical similarity in the fragrances emitted by these plants (Knudsen & Tollsten, 1993; R. Raguso & M. Willis, unpubl. data). Our battery of odorant stimulants did not include the sulfur bearing compounds characteristic of nocturnal bat-pollinated flowers (Knudsen & Tollsten, 1995) nor the aliphatic esters and lactones that typify the odors of many ripe fruits (Fröhlich et al., 1989; Flath et al., 1990; McGrath & Karahadian, 1994). However, considering hawkmoths' opportunistic use of these additional food sources (Baker, 1961), it would not be surprising if their antennae also were generally receptive to the above classes of odorants.

S. perelegans and *H. lineata* are sympatric and share a subset of floral resources in central California, as is suggested by the similarity in their mean proboscis lengths (30–34 mm) and observed foraging behavior (Raguso, 1995). Although male *S. perelegans* and *H. lineata* differ somewhat in EAG rank orders (Table 1) and magnitudes (Raguso et al., 1996), it is unlikely that these differences bear any behavioral significance. Males of *S. perelegans* are significantly larger, on average, than those of *H. lineata*, (body mass and forewing length, $P < 0.005$) and some differences in EAG magnitude may be attributable to their larger size and longer antennae. Alternatively, incongruities in mean EAGs and rank orders may reflect differences in foraging experience, genetic endowment of antennal receptors, or the small sample sizes ($N = 4$ and 5 individuals) used in these studies. Future experiments within the *C. breweri* – hawkmoth system should address the behavioral function of floral scent and the connection between foraging experiences, associative learning, receptor endowment and olfactory physiology (e.g. Vet et al., 1990; Gerber et al., 1996).

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