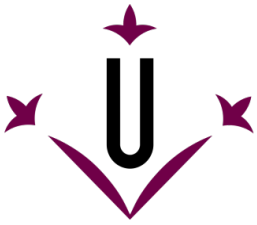

Day-Night and Phenological Variation of Apple Tree Volatiles and Electroantennogram Responses in *Cydia pomonella* (Lepidoptera: Tortricidae)

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ABSTRACT Volatile compounds from apple trees (variety Golden Smothee) were collected in the Þeldfromattachedapplebranchesenclosedinplasticbagsinthemorningandatduskandduringthree growth periods (after petal fall [APF], immature fruit [IF], and close-to-full ripening [CFR]). Collections were analyzed by gas chromatographyÐmass spectrometry (GC-MS) and gas chromatographyÐelectroantennographic detection (GC-EAD) using the antennae of *Cydia pomonella* males as biological detectors. Forty-four compounds were detected in the volatile collections. The most abundant compound in all treatments was (*Z*)-3-hexenyl acetate, a common green leaf volatile. Other abundant compounds were (*Z*)-3-hexenol, (*E*,*E*)--farnesene, hexyl acetate, 4,8-dimethyl-1,3,7-nonatriene, hexyl hexanoate, and germacrene D. Most of the compounds that showed signiÞcant differencesbetweenperiodswereemittedingreateramountsintheAPFand/orIFperiodsthaninthe CFR period. (*E*)--caryophyllene and an unidentiÞed compound were signiÞcantly more abundant during the day, whereas 2-hexanone, octanal, and (*Z*)-3-hexenol were signiÞcantly more abundant at dusk. GC-EAD responses were very weak and signiÞcantly higher than background noise only to hexyl acetate, 4,8-dimethyl-1,3,7-nonatriene, nonanal, (*Z*)-3-hexenol, hexyl butanoate, and (*E*,*E*)--farnesene. In further electroantennographic (EAG) assays with synthetic compounds, high responses by the antennae of both males and females were recorded to many of the compounds identiÞed. Males showed a response equal to or higher than females to all compounds except -myrcene.

KEY WORDS *Cydia pomonella*; host-plant volatiles, gas chromatography-mass spectrometry, gas chromatography-electroantennographic detection, electroantennograph

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THE CODLING MOTH, *Cydia pomonella* L. (Lepidoptera: Tortricidae), is a major pest in apple, pear, and walnut orchards worldwide. The larvae feed on the fruit and have endophytic behavior, making it necessary to spray intensively with insecticides for their control. The indiscriminate use of broad spectrum insecticides has generated the development of insecticide-resistant strains (Bouvier et al. 1998), which aggravate the unavoidable environmental problems associated with insecticide use. Alternative means of control are therefore necessary.

Since its description, the sex pheromone of *C. pomonella* (Roelofs et al. 1971) has been gradually introduced in management programs, Þrst as a monitoring tool and later to control populations with mating disruption (Howell et al. 1992, Trimble 1998) and attract-and-kill techniques (Charmillot et al. 2000). Presently, mating disruption is the most successful alternative to traditional chemical control and it is used worldwide (Calkins and Faust 2003). However, under mating disruption, pheromone traps are less effective at

detecting male presence (Gut and Brunner1996),reducing their use as monitoring tools.Plant volatiles, which are used by phytophagous insects as chemical cues to find host plants (Visser 1986), constitute an alternative source of attractants. Given that such chemicals also attract females, the population dynamics of both sexes can be monitored simultaneously.

In recent years there have been several studies on apple tree volatile emission and *C. pomonella* attraction to host-plant volatiles (Yan et al. 1999, Light et al. 2001, Hern and Dorn 2004, Knight et al. 2005, Knight and Light 2005, Vallat and Dorn 2005). The most effective compound is ethyl (*E,Z*)-2,4-decadienoate, the pear ester, a species-speciÞc and bisexual attractant, which is the only commercial kairomone for *C.pomonella.*Thepearesterwasdiscoveredbytesting compounds emitted by ripe Bartlett pears (Light et al. 2001). The efÞcacy of the pear ester in the Þeld depends on the species of fruit trees, as well as on the phenological state of the plants (Light et al. 2001, Knight and Light 2005). It is very effective in walnut orchards, but it has shown inconsistent results in European apple and pear orchards (Bosch and Avilla 2001). Moreover, the pear ester has been reported only in pear emissions, but neither in apple nor in walnut. All this suggests that other compounds should be key in the attraction of *C. pomonella* to its host plants.

Typically, volatile collections for the study of host-plant attractants for *C. pomonella* have been made under laboratory conditions, using plant parts (branches or fruits) that had been detached from the tree (Bengtsson et al. 2001, Hern and Dorn 2004). Mechanical damage can result in both quantitative and qualitative changes on the volatile emission profile of plants(Pare´ and Tumlinson1997, Agelopoulos et al. 1999, Bäckman et al. 2001, Vuorinen et al. 2005). Detaching, cutting, or chopping plant material should be avoided for volatile collection (Agelopoulos et al. 1999).

Most studies focusing on identification of the attractants for *C. pomonella* have been carried out during the photophase (Hern and Dorn 2002, Vallat and Dorn 2005) despite the fact that adult*C.pomonella*are crepuscular, and plants are known to release different blends of volatile compounds throughout the diel cycle (Staudt et al. 1997, 2000, Picone et al. 2002, Huber et al. 2005). Only in one previous study in apples were collections made at dusk and in situ (Ba¨ckman et al. 2001), but surprisingly, only (*E,E*)--farnesene, (*E*)-farnesene, and (*E*)--caryophyllene were detected in collections made under these conditions.

The aim of this study was to identify volatiles from apple trees that may be used by *C. pomonella* to locate host plants, as well as to compare their emission between day and dusk. For this we collected volatiles from apple trees in situ at dusk and in the morning and in different phenological development stages of the tree. Then we identified the volatiles that elicited antennal responses on male and female antennae of *C. pomonella.*

Materials and Methods

Insects. The colony was started in 1992 from insects collected in an abandoned apple orchard in Lleida (Spain), and it has been maintained on a semisynthetic diet (Pons et al. 1994) under a 16:8-h (L:D) photoperiod at 25 5C. Newly emerged adults were sexed every day and kept in small groups (up to 10 individuals) in plastic boxes (15 cm diameter by 7 cm height)andsuppliedwithwateruntilused.Testmales were never exposed to females, but test females were maintained with males to obtain mated individuals.

Solvents and Chemicals. Hexane, diethyl ether, and methanol (purities 95, 99.8, and 99.8%, respectively; Fluka Chemie, Buchs, Switzerland) were used as solvents. -farnesene

(95%) was purchased from Chemos (Regenstauf, Germany). (*Z*)-3-hexenol (98%), methyl salicylate (99%), ()-(*E*)--caryophyllene (99%), ()-linalool (97%), and myrcene (90%) were acquired from Fluka Chemie (Buchs, Switzerland).2-Cyclopentylcyclopentanone(95%), (*Z*)-3-hexenyl benzoate (97%), (*E*)-2-hexenal (98%), (*Z*)-3-hexenyl butanoate (98%), (*Z*)-3-hexenyl acetate (98%), and farnesol (racemic) were bought from Sigma-Aldrich Quõ´mica (Madrid, Spain). Octanal (99%), nonanal (95%), and decanal (95%) were purchasedfromAcrosOrganics(Geel,Belgium).6-Methyl-5-hepten-2-one (95%) and (*R*)-()-limonene were purchased from MERCK-Schuchardt (Darmstadt, Germany). Benzyl aldehyde was acquired from Probus(Badalona,Spain).Ethyl(*E*,*Z*)-2,4-decadienoate (88%) was a gift from Tre´ce´ (Adair, OK). Farnesene (racemic) was bought from TCI (Tokyo, Japan). Hexyl acetate, butyl hexanoate, hexyl hexanoate, hexyl butanoate, (*Z*)-3-hexenyl hexanoate,and hepty lacetate were synthesized (yields70% after distilling) following the method of Eras et al. (2002), and all had purities 95% after puriÞcation.

VolatileCollections. Volatile collections were made in the spring and summer of 2004 in a 1.1-ha apple orchard (variety Golden Smoothe), located in Gimenells (Lleida, Spain, 4137 N). A dynamic headspace system similar to that described by Ba¨ckman et al. (2001) was used for volatile collection. A 46 by 61-cm plastic oven bag (Pansaver; M&Q Plastic Products, Schuykill, PA) was placed over an apple branch and closed with a plastic clamp.A vacuum pump (NMP830 KNDC-12V; KNF Neuberger, Freibrug, Germany) pushed air through a stainless steel tube containing 1.3 g of activated charcoal (20/40 mesh; SKC, Dorset, UnitedKingdom),into the bag at 0.5ml/min. A second vacuum pump simultaneously extracted air from the bag at 0.45 ml/min through a glass trap containing 50 mg of Super-Q (80/100 mesh; Alltech Associates, Deerfield, IL) held between two layers of glass wool. Plastic bags were used only once to avoid contamination between samples.

Collections were made at three different periods of the season: (1) after petal fall (APF) between 7 and 17 May, over branches bearing leaves and one to four fruit clusters;(2)immature fruit(IF)between 30 June and 10 July, over branches bearing leaves and three or four fruit 4 cm diameter; and (3) close-to-full ripening (CFR) between 9 and 16 September, over branches containing two or three fruit 6 cm diameter. During each period, collections were made at two different times of the day over the same branch: morning (starting between 0900 and 1000 hours, local time GMT2), and dusk (beginning 30 min before dusk). A minimum of two blank samples were taken per day time and phenological stage from empty bags placed in the tree canopy.

Volatiles were collected for 2 h. Subsequently, Super-Q traps were taken to the laboratory and washed four times with 100 l of hexane to extract samples into conical-bottom vials. Fifty nanograms of heptyl acetate in 10 l hexane were added as an internal standard, and the vials were kept at 20C until analysis. Before being reused traps were rinsed with 2 ml of each hexane, diethyl ether, and methanol. Immediately before analysis, samples were reduced under a soft stream of nitrogen to 5 l.

The temperature inside the bag was measured every 30-45 min by an electronic thermometer. Average temperatures per sample ranged from 19.6 to 25.2C (APF-morning), 15 to 21.8C (APF-dusk), 23.8 to
30.4C (IF-morning), 21 to 29C (IF-dusk), 21.2 to 29C (CFR-morning), and 20.2 to 27.3C (CFR-dusk).

Gas Chromatography–Mass Spectrometry. Gas chromatography-mass spectrometry (GC-MS) analyses were carried out on an Agilent Technologies 6890N GC interfaced to an Agilent Technologies 5973 Network quadrupole MS (Agilent Technologies, Palo Alto, CA). Two microliters of the reduced sample was injected into the GC, and chromatographic

separation was performed on a DB-Wax (30 m by 0.25 mm by 0.25 m) capillary column (J&W Scientific, Folsom, CA). The injector temperature was 250C, and the split ratio was 1:5. The oven temperature started at 50C and was maintained for 2 min, increasing at 5C/min to 150C, held for 5 min, increased at 10C/min to 230C, and Þnally was kept at 230C for 10 min. The carrier gas was helium at a constant ßow rate of 1.5 ml/min. The MS operated by electron impact ionization at 70 eV, and scan range was from 40 to 400 m/z at 4 scan/s. The temperatures of transfer line and ionization source were 280 and 230C, respectively.

The samples were analyzed by GC-MS software (MSD-ChemStation version D.00.01; Agilent Technologies), spectra were compared with the available library(NISTlibrary75K),andidentiÞcationwasconÞrmed by injection of synthetic compounds when possible. Four to six volatile collections and at least oneblanksampleperdaytimeandseasonperiodwere analyzed by GC-MS. The amounts of all compounds that were not present in blanks were estimated as a percentage of the internal standard peak. Compounds absent in a sample were considered as missing values. Comparison of the emission of volatiles between the different day times and phenological periods was performed by an analysis of variance (ANOVA) for every single compound. Data were transformed to log(x 1) when necessary, and when signiÞcant differences existed, a DuncanÕs multiple range means separation test was performed.

Gas Chromatographic–Electroantennographic Detection. Gas chromatographicÐelectroantennographic detection (GC-EAD) analyses were made on an Agilent Technologies 6890N gas chromatograph coupled to an electroantennogram (EAG; Syntech, Hilversum, Holland).Acolumnßowsplitter(SGEEurope,Milton Keynes, United Kingdom) split GC efßuent in two 0.32-mm ID methyl-deactivated capillary columns (SGE Europe). Columns were equal in length (30 cm): one of them led to the ßame ionization detector (FID) and the other to the EAD preparation through a GC-EAD/single sensillum recording efßuent interface (Syntech). GC-EAD interface temperature was held at 230C by means of a TC-02 interface temperature controller (Syntech). Make-up nitrogen gas was added just before the split point to create a 30-ml/min ßow into each branch. Excised antennae of 2- to 3-doldmalesweresuspendedbetweentwoglasscapillary tubes containing 0.2 M KCl solution and gold electrodes. The electrodes were connected to a PR-05 probe (Syntech), which sent the signal to a computer for recording by GC-EAD software (Syntech). A CS-05 stimulus controller (Syntech) continuously passed humidiÞed air over the antenna at 1 liter/min. Three microliters of the reduced samples was injected in the GC, and chromatographic conditions were the same as for GC-MS except that the injector was set to splitless/split for 1 min after injection. Between 2 and 3 min before the solvent peak and 1 min aftertheendoftherun,theantennaewerechallenged with 1-g puffs of sex pheromone to check their responsiveness. Three to four volatile collections per diel and seasonal period were analyzed by GC-EAD. **EAGRecordingswithSyntheticCompounds.**EAGs wereconductedwiththosecompoundsidentiÞedthat were available as synthetics plus three compounds absent in our samples but reported in the literature as behaviorally active: ethyl 2,4-(*E*,*Z*)-decadienoate [the pear ester (Light et al. 2001)], butyl hexanoate (Hern and Dorn 2004), and farnesol (Coracini et al. 2004). Another compound was also tested, 2-cyclopentylcyclopentanone, which was emitted by the oven bags.

A given test stimulus was loaded onto a piece of Þlter paper (20 by 5 mm), which was subsequently insertedintoaPasteurpipette.Stimuliwereappliedas 0.1-sairpuffsthatpassedthroughthepipetteandwere released into a 1-liter/min humidiÞed air stream that passed over the antenna. Puffs were generated by a CS-05 stimuli controller

(Syntech). The quantity of each compound loaded onto Þlter paper amounted to 0.2mol (between 16.8 and 45.7g depending on the compound). Hexyl acetate (50 g, 0.35 mol) was used as a standard. In a previous study, we established a doseÐresponse relationship to this compound between 0.1 and 1,000 g with a saturation response of 3.4 mV (unpublished data). The pipettes were prepared a few minutes before recording. Excised antennaeof2-to3-d-oldmalesandvirginandmatedfemales were stimulated with 12 puffs, 30Ð40 s apart, in the following order: air (empty pipette), standard, hexane, three test compounds, standard, three test compounds, blank, and standard. The order of the test puffs was randomized among the antennae. A given compoundneverhadmorethanonereplicateoverthe same antenna, and 10Ð12 antennal recordings were made per compound and sex. After recordings, females were dissected to determine mating status.

The response to the closest hexane blank was subtracted from the response of the test compounds, and the response of the test compounds was calculated as a percentage relative to the average of the two closest standard responses. Data were transformed to log(x 1)beforeANOVAandDuncanÕsmultiplerangemeans separation test.

Results and Discussion

Emission of Volatiles from Apple Trees In Situ. Forty-four compounds were detected in the volatile collections from Golden Smothee apple branches in situ (Table 1). Of these, 10 could not be identiÞed and therefore are listed as "unidentiÞed 1Ð10." UnidentiÞedcompounds2Ð10aresesquiterpenes,withaverage

Table 1. Volatile compounds detected in apple trees headspace at three phenological stages in the morning and at dusk

Compound	Retention time (min)	Morning			Dusk		
		APF %IS ± SE[a]	IF %IS ± SE[a]	CFR %IS ± SE[a]	APF %IS ± SE[a]	IF %IS ± SE[a]	CFR %IS ± SE[a]
2-Hexanone	4.63		55.4	0.7 ± 0.4			78.9 ± 3.9
β-Pinene	4.93	33.7 ± 22.4	24.7 ± 14.0	2.8 ± 0.6	7.8 ± 1.9	16.2 ± 8.5	10.1 ± 11.1
3-Carene	5.73	14.0 ± 3.0	3.8		12.2 ± 4.2	58.7	
β-Myrcene	6.04	10.5 ± 7.2		3.9 ± 0.5	4.1 ± 1.1	4.2	6.2 ± 3.1
Pentyl acetate	6.26	276.8	140.1	20.9 ± 16.7			41.3 ± 36.6
Limonene	6.72	26.7 ± 13.0	42.3 ± 15.0	7.6 ± 2.2	15.8 ± 9.7	28.1 ± 4.8	9.9 ± 3.0
(*E*)-2-hexenal	7.15	9.2 ± 3.3	18.5 ± 1.0	13.3 ± 3.5	13.4 ± 5.8	40.0 ± 11.0	32.8 ± 17.2
(*E*)-β-ocimene	7.96	24.1 ± 6.3	0.4	2.2 ± 1.1	17.2 ± 5.7	3.1	1.8 ± 0.7
Hexyl acetate	8.46	25.2 ± 15.7	36.9 ± 17.6	187.8 ± 179.0	9.1 ± 3.8	21.5 ± 5.5	304.5 ± 308.8
Octanal	8.81	14.4 ± 2.4	7.9 ± 1.3	4.8 ± 0.9	15.2 ± 4.1	21.8 ± 4.3	6.1 ± 2.9
2-Methyl-6-methylene-1,7-octadien-3-one	9.26		39.7 ± 6.6			32.3 ± 27.0	
4,8-Dimethyl-1,3,7-nonatriene	9.33	87.5 ± 20.1	200.9	98.5 ± 79.7	152.2 ± 91.4	76.5 ± 11.6	26.7 ± 22.5
(*Z*)-3-hexenyl acetate	9.64	1817.4 ± 458.2	3632.9 ± 1852.8	4198.8 ± 1132.0	1493.3 ± 497.1	2801.5 ± 634.2	2722.9 ± 1030.1
6-Methyl-5-hepten-2-one	10.01	28.5 ± 19.5	26.7 ± 16.6	7.8 ± 2.4	11.1 ± 3.0	29.9 ± 5.3	19.5 ± 10.4
(*Z*)-3-hexenol	11.28	127.6 ± 80.4	71.7 ± 35.4	158.1 ± 60.7	153.6 ± 43.6	229.7 ± 70.0	253.9 ± 136.1
Nonanal	11.39	29.5 ± 5.2	60.7 ± 18.6	12.0 ± 1.8	33.0 ± 10.4	77.3 ± 16.1	18.2 ± 4.0
(*E*)-2-hexenol	11.83	3.3		3.4	3.8 ± 4.0	7.8 ± 2.8	16.6
Hexyl butanoate	11.97	42.0 ± 44.4	25.2	395.7	2.2 ± 1.2	14.0	69.0 ± 72.4
Hexyl 2-methylbutanoate	12.27	11.9		706.7			
Unidentified 1	12.40	33.8 ± 5.2	70.6 ± 23.9	32.9 ± 3.4	16.7 ± 4.7	37.6 ± 11.0	34.5 ± 20.7
1-Octen-3-ol	12.92		4.0	8.6 ± 2.6		8.6	11.2 ± 6.4
(*Z*)-3-hexenyl butanoate	13.07	19.6 ± 12.1	32.4 ± 13.4	38.3 ± 10.8	30.6 ± 16.0	45.2 ± 13.8	42.5 ± 21.5
(*Z*)-3-hexenyl 2-methylbutanoate	13.37	17.2 ± 6.5	15.1 ± 5.4	13.3 ± 4.7	11.0 ± 3.3	12.0 ± 2.9	11.1 ± 4.7
Decanal	13.96	30.7 ± 8.3	53.8 ± 23.2	15.4 ± 3.8	34.3 ± 10.4	64.7 ± 11.1	23.4 ± 17.1
β-Bourbonene	14.38	89.3 ± 21.1			49.0 ± 20.9	8.9	
Benzyl aldehyde	14.43	28.5	17.2 ± 6.6	3.5 ± 1.5	9.3 ± 0.9	22.9 ± 3.9	10.9 ± 5.2
Linalool	15.27	21.4 ± 9.0	12.9 ± 7.4	5.7 ± 2.1	28.8 ± 12.9	11.9 ± 1.9	11.3 ± 4.5
Unidentified 2	15.54	13.5 ± 0.8	30.8 ± 13.6	15.5 ± 2.3	7.6 ± 3.6	25.2 ± 6.0	14.8 ± 6.6
Unidentified 3	15.71	34.0 ± 5.5			22.5 ± 5.2		3.0 ± 1.7
Unidentified 4	16.11	28.4 ± 4.5			19.5 ± 5.0	8.2	
(*E*)-β-caryophyllene	16.22	70.8 ± 11.6	11.2 ± 6.6	5.1 ± 1.6	39.6 ± 9.2	9.7 ± 3.5	0.6
Hexyl hexanoate	16.67	10.1 ± 1.8	9.7 ± 5.4	103.0 ± 144.5	5.6 ± 2.0	10.9 ± 1.6	149.3 ± 209.2
Unidentified 5	17.26	8.7 ± 2.3			7.2 ± 2.1		
Unidentified 6	17.36	9.3 ± 2.1			4.8 ± 1.3		
(*Z*)-3-hexenyl hexanoate	17.72	88.6 ± 67.6	18.1	2.9 ± 3.7	15.1 ± 4.7	1.6 ± 0.4	2.6 ± 3.0
Unidentified 7	17.75	9.3 ± 1.1			5.5 ± 1.0		
(*E*)-β-farnesene	18.28	2.9 ± 1.3	10.3 ± 6.3	0.3	1.6 ± 0.5	7.3 ± 4.7	1.2 ± 1.1
Unidentified 8	18.40	3.0 ± 0.9	4.9	6.6	1.4 ± 0.4	2.5	
Germacrene D	18.74	214.9 ± 29.8	17.8	5.3 ± 1.1	149.4 ± 34.1	12.0 ± 2.6	14.2 ± 5.5
Unidentified 9	19.17	2.7 ± 0.1	13.1		1.1 ± 0.4	2.9	3.9 ± 3.8
(*E,E*)-α-farnesene	19.74	71.7 ± 22.6	73.5 ± 55.5	319.5 ± 257.7	59.2 ± 16.4	104.3 ± 70.1	618.9 ± 646.1
Unidentified 10	19.89	3.9 ± 0.5	6.3 ± 3.4	1.4 ± 0.2	2.6 ± 0.8	3.8 ± 0.8	1.6 ± 0.8
Methyl salicylate	20.21	16.2 ± 8.9	7.3 ± 2.9	5.1 ± 1.3	12.4 ± 3.9	16.7 ± 8.7	7.3 ± 1.9
(*Z*)-3-hexenyl benzoate	29.33	223.0 ± 169.6	6.7 ± 4.3	4.3 ± 2.3	22.2 ± 8.9	7.3 ± 3.4	1.5 ± 0.4
Total emission	—	3219.7 ± 841.7	4276.1 ± 2093.9	5377.9 ± 1359.7	2393.4 ± 597.7	3644.2 ± 746.1	4383.1 ± 1482.9

Compounds confirmed by comparison with synthetic compounds are bold.
[a] Mean percentages relative to the internal standard area (50 ng heptyl acetate).

Table 2. Amounts of volatile compounds detected in apple trees headspace that were significantly affected by the phenology

Compound	APF (%IS SE)[a]	IF (%IS SE)[a]	CFR (%IS SE)[a]
2-Hexanone		55.4 a	39.8 26.1 b
3-Carene[b]	13.2 2.2	31.1 38.8	
Limonene	22.4 8.1 ab	34.4 6.7 a	8.6 1.6 b
(*E*)-2-hexenal	11.3 3.0 b	33.8 8.5 a	22.0 7.6 ab
(*E*)--ocimene	21.4 4.2 a	1.8 1.9 b	2.1 0.7 b
Hexyl acetate	18.8 9.3 b	27.7 7.1 ab	239.6 148.0 a
Octanal	14.7 1.9 a	16.2 3.4 a	5.3 1.0 b
2-Methyl-6-methylene-1,7-octadien-3-one			35.3 13.8
Nonanal	31.1 4.9 b	69.0 11.3 a	14.8 2.1 c
UnidentiÞed 1	24.3 4.4 b	52.3 12.2 a	33.6 7.9 ab
1-Octen-3-ol		6.3 3.3	9.6 2.4
Decanal	32.5 6.0 b	60.3 10.1 a	18.1 4.5 b
-Bourbonene	69.1 15.1	8.9	
Benzyl aldehyde	15.7 7.9 a	20.3 3.4 a	6.7 2.5 b
Linalool	24.8 6.9 a	12.3 3.1 ab	7.8 2.1 b
UnidentiÞed 3	28.3 3.9 a		3.0 1.7 b
UnidentiÞed 4	24.3 3.4	8.2	
(*E*)--caryophyllene	56.6 8.6 a	10.6 3.5 b	3.6 2.0 c
UnidentiÞed 5	7.8 1.3		
UnidentiÞed 6	7.1 1.5		
(*Z*)-3-hexenyl hexanoate	51.8 32.8 a	5.7 4.8 b	2.7 1.6 b
UnidentiÞed 7	7.6 1.0		

(*E*)--farnesene	2.2 0.7 ab	8.8 3.2 a	0.9 0.6 b
Germacrene D[b]	185.2 22.9	12.9 2.3	9.7 3.0
UnidentiÞed 10	3.3 0.5 a	4.9 1.4 a	1.5 0.4 b
(*Z*)-3-hexenyl benzoate	131.7 91.1 a	7.0 2.4 b	3.1 1.3 b

Compounds conÞrmed by comparison with synthetic compounds are bold. Values in the same row with different letters differed signiÞcantly (0.05).

[a] Mean percentages of morning and dusk samples relative to the IS area (50 ng heptyl acetate). [b] Compounds with a signiÞcant time of the day by phenological period interaction.

Retention timesof15.54,15.71,16.11,17.26,17.37,17.75, 18.40, 19.17, and 19.89 min, respectively.

The most abundant compounds were (*Z*)-3-hexenyl acetate, a common green-leaf volatile, which was present in percentages ranging from 1,817 to 4,199% of the internal standard (IS), its associated alcohol, (*Z*)-3-hexenol, which was 71.7Ð253.9% IS, and (*E*,*E*)-farnesene, 71.7Ð618.9% IS (Table 1). Other compounds found in considerable amounts were hexyl acetate (9.1Ð304.5% IS), 4,8-dimethyl-1,3,7-nonatriene (26.7Ð200.9% IS), hexyl hexanoate (5.6Ð149.3% IS), and germacrene D (5.3Ð214.9% IS). Several compounds are reported for the Þrst time from apple plants, to our knowledge. These include 2-hexanone, 2-methyl-6methylene-1,7-octadien-3-one, and 1-octen-3-ol. One of them, 1-octen-3-ol, has been reported from pear volatile collections (Scutareanu et al. 1997).

No signiÞcant differences in total volatile release (sum of all peak areas) were found between phenological periods (df 2, *F* 1.74, *P* 0.20); however, a tendency to increase emission as the season advancedcanbeobserved(Table1).Mostoftheemitted compounds were detected in all the studied phenological periods (Table 1). Exceptions were 2-hexanone and 1-octen-3-ol absent on APF period; unidentiÞed3absentonIF;3-carene,-bourbonene,and unidentiÞed 4 absent on CFR; unidentiÞed 5 and 6 onlydetectedonAPF;and2-methyl-6-methylene-1,7octadien-3-one only present on IF. SigniÞcant differenceswerefoundamongthestudiedperiodsformany of the compounds detected in all the treatments (Table 2). Most of the compounds that showed signiÞcant differences between seasonal periods were emitted in greater amounts in APF and/or IF than in CFR periods. All saturated aldehydes appeared in smaller amounts in CFR than in IF (Table 2). This has been reported previously (Mattheis et al. 1991), and it is attributed to the reduction of aldehydes to alcohols before esteriÞcation during fruit ripening. Perhaps this process can also explain that several esters (hexyl acetate, hexyl butanoate, and hexyl hexanoate) tendedtobemoreabundantintheCFRperiod(Table 1), although signiÞcant differences were only found for hexyl acetate (Table 2). (*E*,*E*)--farnesene, which has been described as one of the most abundant compounds in apple fruit emissions (Bengtsson et al. 2001), and has been shown to modify female behavior (Wearing and Hutchins 1973, Hern and Dorn 1999), showed a clear tendency to be present in higher amounts in CFR period than in the other two periods, although no signiÞcant differences between periods were found (df 2, *F* 0.88, *P* 0.14; Table 1).

No signiÞcant variation between day and dusk periods was found in total emission of volatiles (df 1, *F* 0.82, *P* 0.37); however, there was a tendency for emissions to be higher in the morning than at dusk (Table 1). Although variation between morning and dusk was apparent in many compounds, it was significant only for six of them: (*E*)--caryophyllene (df 1, *F* 8.54, *P* 0.01) and unidentiÞed compound 7 (df 1, *F* 7.84, *P* 0.03) were emitted in greater amountsinthemorning,hexyl2-methylbutanoatewas found in some of the morning collections, but never at dusk, and 2-hexanone (df 1, *F* 788.6, *P* 0.001),

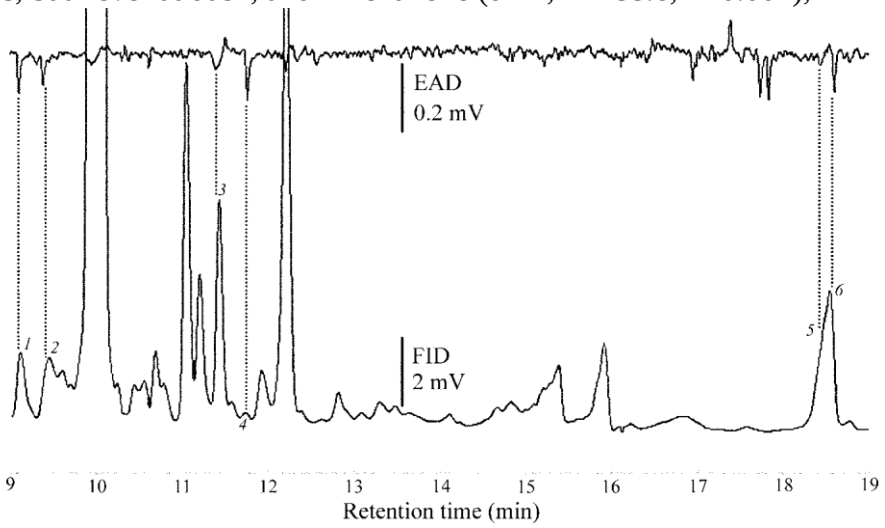

Fig. 1. GC-FID (bottom) and GC-EAD (top) traces of a volatile collection from an apple tree (variety Golden Smothee) done in the CFR period at dusk and using the antenna of a male *C. pomonella*. Peaks of compounds that produced discernible EAD responses are labeled. (1) hexyl acetate, (2) 4,8-dimethyl-1,3,7-nonatriene, (3) (*Z*)-3-hexenol nonanal, (4) hexyl butanoate, (5) (*E*,*E*)--farnesene, and (6) 2-cyclopentyl cyclopentanone. MS analysis revealed that (5) and (6) were two different compounds.

octanal(df1,*F*4.37,*P*0.05),and(*Z*)-3-hexenol (df 1, *F* 5.69, *P* 0.03) were more abundant at dusk.VariationintheemissionproÞleofplantvolatiles between light and dark periods has been reported in other species (Nielsen et al. 1995, Staudt et al. 1997, 2000, Huber et al. 2005).

Our results disagree with those of Ba¨ckman et al. (2001),who identified lower amounts of all volatiles at dusk than in the photophase with (*E*)--caryophyllene, (*E*)--farnesene, and (*E*,*E*)--farneseneÑ theonlyvolatilesdetectedduringthescotophase.The disagreement could result from differences in temperature during collections between the two studies. Ba¨ckman et al. (2001) registered temperatures from 12.2 to 18C, whereas we registered higher temperatures overall. Temperature is known to affect volatile emission by plants. For example *Betula pendula* Roth and *Sambucus nigra* L. increase the emission of both total volatiles and most individual compounds after a saturation curve between 16 and 40C, under constant humidity and light intensity (Zhang et al. 1999a). Similarly emission of herbivore-induced plant volatiles by *Zea mays* L. seedlings also varies depending on the environmental temperature (Gouinguene´ and Turlings 2002).

GC-EAD Analysis of Volatile Collections. EAG responses were very weak and were only consistently detected for hexyl acetate, (*Z*)-3-hexenol nonanal, 4,8-dimethyl-1,3,7-nonatriene,hexylbutanoate,(*E*,*E*)-farnesene, and 2-cyclopentylcyclopentanone (Fig. 1).

These compounds gave average EAG responses of 0.021, 0.034, 0.047, 0.021, 0.027, and 0.026 mV, respectively. EAG responses to 2-cyclopentylcyclopentanonewereinterestinginthatthiscompoundisemitted by the oven bags used for volatile collection (Gramshaw and Soto-Valdez 1998) and is used in fragrance industry because of its fruity aroma (ZEON Corp. 2005). No responses were detected to compoundsthatwerepresentinoursamplesandhadbeen described previously as GC-EADÐresponsive (Ba¨ckmanetal.2001,Bengtssonetal.2001),suchaslinalool, (*E*)--caryophyllene, or (*E*)--farnesene. The lack of responsiveness to these compounds could be caused bytheirsmallconcentrationinoursamples.Totestthe sensitivity of our GC-EAD setup, we injected synthetic standards of several plant volatiles (i.e., linalool and pear ester) and obtained clear responses to amounts 10 ng (data not shown).

Another reason for lack of responsiveness to some compounds might be differences among populations. Differences in host preference among populations of *C. pomonella* have been previously reported. Phillips andBarnes(1975)foundthatwildpopulationscoming from apple strongly preferred apple for oviposition, whereas those coming from walnut and plum showed a preference for ovipositing in walnut. More recently, *C.pomonella*wildpopulationsfrompear(France)and walnut (Italy) showed a response to walnut stimuli by increasing egg laying, whereas a wild population from apple (Sweden) did not (Witzgall et al. 2005). This couldalsoexplaindifferencesinÞeldtrappingefÞcacy of pear ester between American and European apple orchards, as well as between tree species.

Among the compounds we detected as GC-EAD active, (*E*,*E*)--farnesene is known to have a behavioral effect both on females and larvae of *C. pomonella* (Wearing and Hutchins 1973, Yan et al. 1999), hexyl acetatehasbeenreportedasarepellenttothefemales in olfactometer assays but ineffective in wind tunnel (HernandDorn2004),(*Z*)-3-hexenolhasbeenshown to act as a synergist of the sex pheromone in wind tunnel (Yang et al. 2004), and 4,8-dimethyl-1,3,7-non-

Table 3.Antennal responses of males and females of *C. pomonella* L. to synthetic compounds

Males			Females		
Compounds	Percent STD SE[a]	Duncan groups[b]	Compounds	Percent STD SE[a]	Duncan groups[b]
hexyl butanoate	277.9 ± 52.6		decanal	185.2 ± 18.2	
(*Z*)-3-hexenyl butanoate[c]	261.6 ± 49.0		(*Z*)-3-hexenyl hexanoate	203.5 ± 42.1	
(*Z*)-3-hexenyl hexanoate	303.1 ± 101.7		linalool	201.2 ± 49.5	
decanal	255.7 ± 44.3		ethyl (*E*,*Z*)-2,4-decadienoate	212.9 ± 62.3	
butyl hexanoate[c]	267.8 ± 71.7		hexyl hexanoate	198.7 ± 56.8	
nonanal	219.2 ± 44.1		nonanal	176.9 ± 23.3	
hexyl hexanoate	181.6 ± 35.1		hexyl butanoate	155.3 ± 25.8	
2-cyclopentyl cyclopentanone	171.8 ± 38.7		2-cyclopentyl cyclopentanone	146.4 ± 20.0	
octanal[c]	202.2 ± 55.1		(*Z*)-3-hexenyl benzoate	123.0 ± 17.6	
ethyl (*E*,*Z*)-2,4-decadienoate	171.3 ± 31.2		(*Z*)-3-hexenyl butanoate[c]	133.3 ± 27.2	
linalool	160.0 ± 45.4		hexyl acetate	129.9 ± 21.9	
hexyl acetate	137.5 ± 20.4		butyl hexanoate[c]	120.0 ± 20.2	
(*Z*)-3-hexenyl benzoate	123.8 ± 22.9		farnesene (racemic)	115.0 ± 18.0	
limonene[c]	159.6 ± 49.5		(*Z*)-3-hexenyl acetate	110.6 ± 17.6	
6-methyl-5-hepten-2-one	146.4 ± 36.8		farnesol (racemic)	107.8 ± 21.6	
methyl salicylate	86.8 ± 25.4		(*E*)-β-farnesene	100.8 ± 14.1	
farnesol (racemic)	81.4 ± 13.9		6-methyl-5-hepten-2-one	96.0 ± 14.1	
(*Z*)-3-hexenyl acetate	98.5 ± 18.0		octanal[c]	89.1 ± 16.0	
(*Z*)-3-hexenol[c]	66.7 ± 18.4		methyl salicylate	58.1 ± 16.6	
farnesene (racemic)	73.3 ± 10.9		(*E*)-2-hexenal	54.5 ± 4.9	
(*E*)-2-hexenal	49.7 ± 15.9		β-myrcene[c]	57.2 ± 10.1	
(*E*)-β-farnesene	72.0 ± 12.1		limonene[c]	52.7 ± 11.8	
benzyl aldehyde	50.6 ± 6.9		benzyl aldehyde	45.8 ± 12.3	
(*E*)-β-caryophyllene	32.1 ± 4.5		(*Z*)-3-hexenol[c]	44.2 ± 13.1	
β-pinene	17.8 ± 7.7		β-pinene	23.3 ± 16.5	
β-myrcene[c]	34.3 ±1 4.5		(*E*)-β-caryophyllene	29.5 ± 6.2	

[a] Averaged relative response to the standard stimulus, 50 g hexyl acetate.

[b] DuncanÕs multiple range separation of means of the transformed log(x 1) variable, 0.05. Differences among compounds within each sex. [c] Compounds with signiÞcant differences in response between sexes, 0.05.

[c]

atriene is frequently found in volatile emissions from insect-attackedplants(Scutareanuetal.1997,DeBoer et al. 2004).

EAGRecordingswithSyntheticCompounds. Mean EAG responses after hexane response subtraction rangedfrom0.3to5.6mV,dependingoninsectsexand compound. Mean overall response of the experiment was 2.55 0.07 mV. Compounds that generated responses 4 mV were nonanal and decanal in both sexes and (*Z*)-3-hexenyl butanoate only in males. Over one half of the females (63.4%) were mated but, as expected, mating status (virgin versus

mated) had no effect on the EAG response to the different compounds (df 1, *F* 0.31, *P* 0.58).

A significant interaction between sex and compound on the EAG response was found (df 25, *F* 2.44, *P* 0.001). Pairwise comparison of least square means of the sex-by-compound interaction revealed differences in EAG response between sexes for six compounds: (*Z*)-3-hexenol (*P* 0.006), octanal (*P* 0.03), limonene (*P* 0.002), (*Z*)-3-hexenyl butanoate (*P*0.02),butylhexanoate(*P*0.03),and-myrcene (*P* 0.001) (Table 3). In all cases except -myrcene, the response of the male antenna was larger than the response of the female antenna. The responses to -myrcene were small compared with the othersÑ 57.2% and 34.3% of the standard in females and males, respectively. Consequently, this compound would be difÞcult to detect in GC-EAD analysis of plant volatile collections. Amounts of compounds in volatile collections are usually small, and detection of active compounds by GC-EAD can become difÞcult. For *C. pomonella,* we recommend the use of males in this kind of experiments, but we also think that, after identification of GC-EAD-active compounds, comparative assays between sexes with synthetics should be made.

Most of the compounds tested generated EAG responses that did not differ signiÞcantly from those of the pear ester [ethyl (*E,Z*)-2,4-decadienoate], the commercial *C. pomonella* attractant, regardless of the sex. Four compounds showed smaller responses than the pear ester in both sexes [benzyl aldehyde, (*E*)-caryophyllene, -pinene, and -myrcene], Þve compounds showed smaller responses only in females [octanal, methyl salicylate, (*E*)-2-hexenal, limonene, and (*Z*)-3-hexenol], and one compound produced smaller responses only in males [(*E*)--farnesene] (Table 3).

In both sexes, the maximum EAG responses were recorded to some of the aliphatic esters tested, two aldehydes (decanal and nonanal), linalool, and 2-cyclopentylcyclopentanone (Table 3). High EAG responses to linalool and some aliphatic esters such as (*Z*)-3-hexenyl hexanoate, butyl hexanoate, or ethyl (*E,Z*)-2,4-decadienoate have been previously reported (Ansebo et al. 2004). To our knowledge, this is the Þrst report of nonanal and decanal eliciting antennal responses in *C. pomonella.* These two compounds tend to be more abundant at dusk than in the morningthroughouttheentireseason(Table1).Nonanal has recently been found to act as a repellent to mated females in an olfactometer assay (Vallat and Dorn 2005); however, a different effect of the compound depending on the dose cannot be rejected, because it has been previously described for -farnesene, which acts as an attractant at low concentrations (634 and 63.4 ng loaded on Silicon/Teßon septum), but as a repellent at high concentration (12,688 ng loaded on Silicon/Teßon septum), to mated famales (Hern and Dorn 1999). A mixture of decanal and nonanal has been shown ineffective in catching adult codling moth both in walnut and apple orchards(LightandKnight2005).Recently,thesetwo compounds have been reported to be minor componentsinalarvalaggregationpheromone(Jumeanetal. 2005).

The responses that we recorded to (*Z*)-3-hexenol are slightly smaller compared with other compounds, especially in females. Mean relative antennal responses were 66.7 and 44.2% for males and females, respectively (Table 3). However, we found this compound to be emitted in signiÞcantly higher amounts at dusk than in the morning, and it is known to act as a synergistofpheromoneinthewindtunnel(Yangetal. 2004). Moreover, EAG responses to (*Z*)-3-hexenol as high as those of pear ester have been also reported (Ansebo et al. 2004). Despite the low EAG responses to (*Z*)-3-hexenol in our study, we think that this compound is an appropriate candidate for future behavioral assays.

We recorded EAG responses to (*E*)--caryophyllene of only 32.1 and 29.5% of the standard in males and females, respectively. This compound produced discernible antennal

responses in GC-EAD trials with plant volatile collections (Bengtsson et al. 2001) and synthetic compounds (Ansebo et al. 2004), and attracted mated females in olfactometer assays (Vallat and Dorn 2005). The relatively low responses to (*E*)--caryophyllene in this study might be caused by a different ratio of stereoisomers in the tested chemicals or to differences among populations. Population differences in response to host-plant volatiles have been reported in another apple pest, *Rhagoletis pomonella*(Walsh)(Diptera:Tephritidae)(Linnetal. 2003).

Three other compounds that have shown low EAG responses in our test have been found to act as attractants or repellents in behavioral tests. These compounds were (*E*)--farnesene (72% of the standard in males), benzyl aldehyde (50.6 and 45.8% of the standardinmalesandfemales,respectively),and-pinene (17.8 and 23.3% of the standard in males and females, respectively;Table3).(*E*)--farneseneisknowntobe attractive in wind tunnel when mixed with (*E*,*E*)-farnesene (Coracini et al. 2004) and by itself in the Þeld (Coracini et al. 2004, Yang et al. 2005). Recently, benzyl aldehyde and -pinene have been shown to act as repellents to mated females of *C. pomonella* in olfactometer assays (Vallat and Dorn 2005). To our knowledge, no previous references of EAG responses to these two compounds exist. The EAG technique is a valid method for determining antennal responsiveness to selected compounds, but the strength of EAGresponse does not necessarily correlate with behavioral response, so behavioral tests and Þeld trapping become always necessary for the identiÞcation of the behaviorally active compounds.

The antenna of *C. pomonella* responds to many apple volatile compounds that are emitted not only by apple but also by other host and nonhost plants. This suggests that *C. pomonella* attraction to host plants might be regulated by the ratios among common plant volatiles, rather than by the presence of species-speciÞc compounds. The use of ubiquitous volatiles has been recently suggested as the prevalent mechanism mediating host-plant recognition by phytophagous insects (Bruce et al. 2005). Apple, hawthorn (*Crataegus* spp.), and dogwood (*Cornus florida* L.) host races of *R. pomonella* respond to blends that are host-speciÞc but share some components (Zhang et al. 1999b, Nojima et al. 2003a, b). For example, the six-component dogwood blend contains 54.9% ethyl acetate and 27.5% 3-methylbutan-1-ol and the sixcomponent hawthorn blend contains 94.3 and 4.0% of these two compounds, respectively. Although blends are similar, dogwood-origin *R. pomonella* shows signiÞcantly greater upwind ßies to the dogwood blend than to the hawthorn blend (Nojima et al. 2003b).

Most of the compounds released by apple trees, some of which are reported in here for the Þrst time, are also emitted by many other plants that might be or not suitable hosts for *C. pomonella.* We conÞrm the presence in the apple tree blend of compounds that elicit behavioral responses in *C. pomonella* adults and larvae. However, it is the blend, more than individual compounds, that seems to be responsible for the attractionofphytophagousinsectstohostplants(Bruce et al. 2005). Because of weak GC-EAD responses and the unreliability of EAG alone to predict behavioral responses to host volatiles, further behavioral studies are required to determine the composition of an attractant apple volatile blend for *C. pomonella.* Nonanal, decanal, and (*Z*)-3-hexenol are some of the candidate compounds to be included in these tests, but several others will probably be involved. We have shown that apple tree volatile emission in situ differs between day and dusk. Therefore, the inßuence of environmental conditions, such as light intensity and temperature, on the plant volatile emissions should be taken into consideration when establishing the ratios of the different compounds to be tested in behavioral assays with *C. pomonella.*

Acknowledgments

This work was partially funded by project RTA01Ð079 from the Programa Nacional de Recursos y Tecnologõ´as Agroalimentarias (Spain) and by projects AGL2003Ð06599CO02Ð02andAGL2004Ð05812/AGRfromthePlanNacional de IDI (Spain). D. Casado has a fellowship from MECD (Spain). C. Gemeno is funded by the Programa Ramo´n y Cajal (MCYT, Spain).

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