

# Plant Odor Analysis of Apple: Antennal Response of Codling Moth Females to Apple Volatiles during Phenological Development

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Volatile compounds were collected from apple branches (*Malus domestica*) at different developmental stages, and the antennal response of codling moth females (*Cydia pomonella*) to these compounds was recorded by electroantennography coupled to gas chromatography. Presence of a range of terpenoid compounds, many of which had antennal activity, was characteristic for volatile collections from branches with leaves, and from small green apples. Nine compounds from branches with leaves and green fruit consistently elicited an antennal response: methyl salicylate, (*E*)- $\beta$ -farnesene,  $\beta$ -caryophyllene, 4,8-dimethyl-1,3(*E*),7-nonatriene, (*Z,E*)- $\alpha$ -farnesene, linalool, germa-crene D, and (*E,E*)- $\alpha$ -farnesene. The bouquet emitted from flowering branches contained in addition several benzenoid compounds which were not found after bloom. Small green apples, which are the main target of codling moth oviposition during the first seasonal flight period, released very few esters. In comparison, fully grown apples released a large number of esters, but fewer terpenoids. The study of apple volatiles eliciting an antennal response, together with a survey of the seasonal change in the release of these compounds, is the first step toward the identification of volatiles mediating host-finding and oviposition in codling moth females.

**Keywords:** Host plant volatiles; phenology; headspace collection; electroantennography; apple; *Malus domestica*; codling moth; *Cydia pomonella*; Tortricidae; Lepidoptera

## INTRODUCTION

Volatile compounds from apple, *Malus domestica*, have been widely studied, especially with respect to aromas from flowers or ripe fruit important for human use (1–4). Apple is, also, host for a wide range of herbivorous insects, and evidence is accumulating that host-finding in insects is largely guided by volatile phytochemicals.

Codling moth, *Cydia pomonella* L. (Lepidoptera, Tortricidae), is the economically most important insect on apple worldwide. Male attraction to female sex pheromone has been studied over the past three decades for the development of environmentally safe control methods. However, control by pheromones is not efficient at high population densities (5–7). One important drawback of the mating disruption technique is that only male behaviors are being affected. The efficacy of pheromonal methods will undoubtedly be greatly enhanced by compounds permitting, in addition, the manipulation of female behaviors.

The orientation of gravid codling moth females to oviposition sites has long been known to be guided by olfactory cues (8–10), but only one behaviorally active constituent of apple odor, (*E,E*)- $\alpha$ -farnesene, has been identified (11–14). This compound stimulates oviposition and short-range attraction of adults and larvae, but is also produced by a wide variety of nonhost plants and cannot account for long-range attraction of codling moth to pome fruit trees.

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We have reinvestigated the volatile compounds released from apple at various developmental stages, including branches with green fruit, during the peak of codling moth seasonal flight period. As a first step toward the identification of the behaviorally active compounds, we have screened headspace collections from apple with female codling moth antennae coupled to gas chromatography.

## MATERIALS AND METHODS

**Volatile Collections.** Volatile collections were made from the following portions of the cultivar Discovery: cut apple branches with flowers; branches with mature foliage, 1–2 weeks after bloom; branches with leaves and small fruit, 1–3 weeks after bloom; green fruit alone, 3 weeks after bloom; and ripe apples, 12–14 weeks after bloom. At least 3 samples per developmental stage were analyzed by mass spectrometry, and 5 samples were analyzed by electroantennography.

Freshly cut branches up to 30 cm long (with 4–10 apples), or 450–550 g apples, were confined in a 2-L glass jar which was closed with a ground-glass fitting. The cut end of the branch was held in a 10-mL vial of water. A charcoal-filtered airstream was pulled over the plant material from the bottom to the top of the jar, and over a 50-mg Super Q trap (80/100 mesh; Alltech, Deerfield, IL) which was held between plugs of glass-wool in a 4 × 40-mm glass tube. Before use, traps were rinsed sequentially with 3 mL of methanol, ether, and redistilled hexane, after 15-min treatments in ultrasonic baths in ether and hexane, respectively. The air flow was 150 mL/min, exchanging the headspace in the jar 4.5 times/h (15). Collections were done for ca. 12 h, at 20 to 22 °C and 10 to 30 Lux. The charcoal filter for incoming air and the Super Q trap for outgoing air were connected with glass fittings to the jar. All glassware was heated to 375 °C during 10 h before use.

After volatile collections, the traps were immediately extracted with 0.5 mL of hexane (redistilled; LabScan, Malmö,

Sweden), and 500 ng heptyl acetate (99.8% chemical purity) was added as an internal standard. Sample volumes were reduced to 50–60  $\mu$ L, at ambient temperature in Francke-vials with an elongated tip (5 cm  $\times$  2 mm i.d.). Samples were stored in sealed glass capillary tubes at –19 °C.

**Gas Chromatography and Mass Spectrometry (GC-MS).** Compounds in trap eluents were identified on a Hewlett-Packard 5970 B GC-MS instrument, with electron impact ionization at 70 eV, and interfaced to a Hewlett-Packard 5890 GC, using a 30 m  $\times$  0.25 mm DB-Wax column (J&W Scientific, Folsom, CA). The temperature program was from 50 °C (hold 5 min) at 8 °C/min to 230 °C (hold 10 min), and the carrier gas was helium.

Additional GC analyses were done on a Hewlett-Packard 5890 instrument with a flame ionization detector (FID), on a DB-Wax column and a nonpolar SE-54 column (25 m  $\times$  0.32 mm i.d., Kupper & Co., Bonaduz, Switzerland), using the same temperature program. Column linear gas flow was 2.5 mL/min, injector temperature was 220 °C, detector (FID) temperature was 250 °C, and gas flows for FID air and hydrogen were 400 and 30 mL/min, respectively.

**Gas Chromatography – Electroantennographic Detection (GC-EAD).** The response of codling moth antennae to apple volatiles was studied by coupled GC-EAD (16). A Hewlett-Packard 6890 GC with a HP-INNOWax column (30 m  $\times$  0.25 mm i.d.), which was programmed from 50 °C (hold 5 min) at 8 °C/min to 230 °C (hold 10 min), was interfaced to electroantennogram recording equipment (Syntech, Hilversum, The Netherlands).

Excised antennae of 2-d-old codling moth females were mounted individually in an antenna holder between two saline-filled wells. The column effluent was split between the FID and the antenna holder. One arm of the split column led into a glass tube (8 mm i.d.), with a charcoal-filtered and humidified air stream (0.5 L/min). Codling moth female antennae were at 0.5 cm from the end of this glass tube, 30 cm from the GC outlet. The GC was operated in splitless injection mode. EAD-outlet temperature was 220 °C; the split ratio between FID and EAD was 1:1. The antenna holder was connected to an amplifier (JoAc, Lund, Sweden), and FID and EAG signals were recorded simultaneously using GC-EAD software (Syntech).

**Chemicals.** The identity of GC-EAD-active compounds in volatile collections was verified by comparison to synthetic or authentic compounds. Germacrene D was isolated from thermomechanical pulp (TMP) turpentine of spruce heartwood (17). (Z,E)- $\alpha$ -farnesene was synthesized according to Ramaiah et al. (18); (E,E)- $\alpha$ -farnesene and (E)- $\beta$ -farnesene were purchased from Bedoukian Research Inc. (Danbury, CT);  $\beta$ -caryophyllene and a racemic mixture of linalool were from Firmenich (Geneva, Switzerland), and  $\beta$ -bourbonene was from Siber Hegner (Zurich, Switzerland). The ester compounds were synthesized from corresponding acid and alcohol precursors according to standard procedures.

**Behavioral Tests.** A laboratory wind tunnel with a flight section of 100  $\times$  100  $\times$  250 cm was lit diffusely from above at 5 Lux, wind speed was 25 cm/s, temperature ranged from 18 to 20 °C, and relative humidity was  $\geq$  60%. Contaminated air from the wind tunnel was aspirated by a fan and was led out of the building. Apple branches (up to 30 cm long, with up to 10 fruits; 2 to 4 weeks after bloom; cv. Discovery), or apples (ca. 500 g, 1 to 3 cm diameter), were enclosed in a 2-L glass jar used for volatile collections. Air from a tank passed over the plant material and left the jar at 25 cm/s, the outlet (4 mm i.d.) was 30 cm from the ground.

Codling moths were reared on a semi-artificial diet (19) and were sexed after eclosion. Three- to 4-d-old codling moth females, which had mated on the day after emergence, were kept in batches of 15 in 1-L plexiglass cylinders coated with disposable 0.025-mm polyethylene sheeting. Both cylinder openings were covered with plastic screen. Plexiglass cylinders with females were placed into the downwind section of the wind tunnel, after the screen had been removed, and their behaviors (activation, taking flight, flying upwind, landing at the outlet of the glass jar) were recorded for 30 min; 90 females

were tested with each source. Tests were done 0 to 3 h after onset of scotophase. The number of moths attracted were compared with an analysis of variance, followed by a Tukey multiple range test ( $P < 0.05$ ).

For behavioral observations under natural photoperiod, freshly emerged lab-reared codling moth females and males were released in a greenhouse. The walls consisted of a metal mesh (pore size 2.5 mm<sup>2</sup>) and the roof was covered by glass. Apple branches used in wind tunnel studies were placed at 1.5 m from the ground. Further observations were made in an adjacent apple orchard (cv. Aroma and Discovery), between ca. 21 and 23 h (0 to 2 h after onset of scotophase).

## RESULTS AND DISCUSSION

**Apple Volatiles Eliciting an Antennal Response.** Compounds identified from apple branches with flowers or fruit, and from fruit alone, are shown in Table 1. Antennae of codling moth females consistently responded to terpenoid and benzenoid compounds which were predominantly released from branches with flowers or green apples. They also responded to several esters, which were present in collections from mature fruit (Tables 1 and 2). These compounds were identified according to GC retention times and mass spectra, in comparison with synthetic or authentic compounds. Female antennae detected even small amounts of (E)- $\beta$ -farnesene, (Z,E)- $\alpha$ -farnesene, and methyl salicylate, while other, more abundant compounds, such as (Z)-3-hexenyl acetate and (E)- $\beta$ -ocimene, gave no significant antennal response (Tables 1 and 2; Figure 1).

Among the most abundant compounds, found in all samples, was (E,E)- $\alpha$ -farnesene. It elicited a strong antennal response, and is an attractant for codling moth larvae and adults (11, 12). The (Z,E)-isomer, which has similar behavioral activity (13, 14), co-occurred at smaller amounts.

Other compounds with strong antennal activity were 4,8-dimethyl-1,3(E),7-nonatriene and  $\beta$ -caryophyllene. Germacrene D is considered an important biogenetic precursor of many sesquiterpenes (20), and has been identified from apple and other Rosaceae (21, 22). GC retention times and mass spectrum were identical to those of germacrene D isolated from TMP turpentine and cubebe oil. An antennal receptor neuron tuned to germacrene D has recently been found in the tobacco budworm moth, *Heliothis virescens* (17).

Headspace collections used in this study were made in the laboratory from cut apple branches and picked apples (Table 1). It has been shown that the release rates of the compounds eliciting antennal activity increased after apple branches had been cut from the tree (23). The release of (E,E)- $\alpha$ -farnesene, germacrene D, 4,8-dimethyl-1,3(E),7-nonatriene, and (E)- $\beta$ -ocimene from apple leaves and fruits is known to increase in response to insect feeding (21, 24).

**Flowering Branches.** Benzenoid compounds were characteristic for flowering branches. The main compound was benzyl alcohol, which gave a comparatively weak antennal response in relation to its abundance (Tables 1 and 2). In contrast, female antennae responded strongly to methyl salicylate, which was a minor constituent of headspace collections from branches with flowers and branches with green fruit.

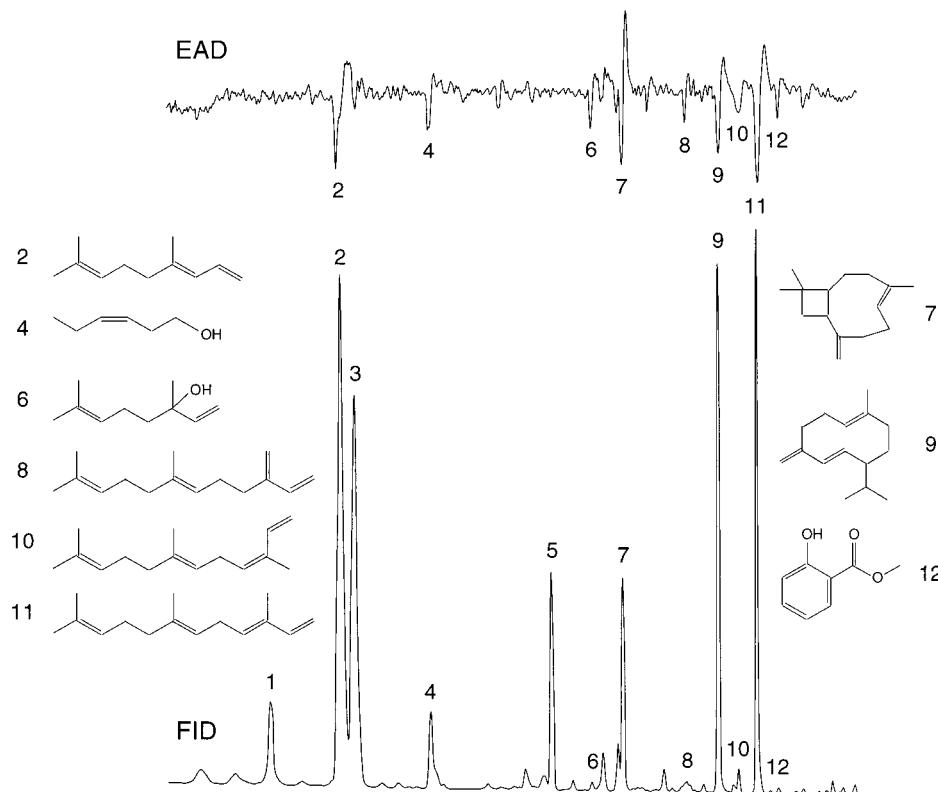
The seasonal flight period of codling moth starts toward the end of the flowering period. Females oviposit on or close to green fruit, but they are not known to be attracted to apple flowers (25). Terpenoid compounds found in collections from flowering branches were prob-

**Table 1. Volatile Compounds in Headspace Collections from Apple (cv. Discovery) at Different Phenological Stages and Their Antennal Activity in Codling Moth Females**

compound <sup>a</sup>	leaves, flowers <sup>b</sup>	leaves <sup>c</sup>	leaves, fruit <sup>d</sup>	fruit <sup>e</sup>	full-grown fruit <sup>f</sup>
acids					
acetic acid	<1				
alkanes					
dodecane			<1	2	
tridecane			<1	2	
tetradecane				1	4
pentadecane	1			2	
heptadecane	<1				
heneicosane	4				
alcohols					
2-hexanol			1		
3-hexanol		1			
(Z)-3-hexenol*	4	71	8	11	
aldehydes					
heptanal*			2	2	
octanal*			5	3	
nonanal*	<1		9	22	29
decanal*			1	1	
ketones					
2-heptanone			1		
2-octanone			1		
esters					
2-methyl-butyl acetate*					186
propyl propanoate*					18
butyl propanoate*					21
pentyl acetate*					14
2-methyl-butyl propanoate*					4
2-methyl-propyl butanoate*					4
<b>butyl butanoate*</b>					<b>36</b>
2-methyl-butyl butanoate*					25
butyl 2-methyl-butanoate*					4
pentyl butanoate*					7
hexyl acetate*		4	1	1	357
2-methyl-butyl 2-methyl-butanoate*					29
(E)-2-hexenyl acetate*					4
(Z)-3-hexenyl acetate*	23	417	114	27	27
butyl pentanoate					7
pentyl 2-methyl-butanoate					12
<b>propyl hexanoate*</b>					<b>4</b>
<b>hexyl propanoate*</b>					<b>7</b>
2-methyl-hexyl propanoate*					2
<b>butyl hexanoate*</b>					<b>29</b>
<b>hexyl butanoate*</b>					<b>21</b>
<b>hexyl 2-methyl-butanoate*</b>					<b>112</b>
2-methyl-butyl hexanoate*					17
(Z)-3-hexenyl butanoate*	3		1		
(Z)-3-hexenyl 2-methyl-butanoate*	<1		2	1	
<b>hexyl hexanoate*</b>					<b>25</b>
butyl octanoate*					4
(Z)-3-hexenyl hexanoate*	<1		<1		4
2-methyl-butyl octanoate*					7
benzenoids					
benzyl acetate*	14				
benzaldehyde*	7				
<b>methyl 2-hydroxy benzoate (methyl salicylate)*</b>	3		3	1	
(Z)-3-hexenyl benzoate*	3				
<b>benzyl alcohol*</b>	127				
1-methoxy-4-propenyl benzene (anethole)					4
irregular terpenes					
<b>4,8-dimethyl-1,3,(E)7-nonatriene*</b>	24	13	26	21	34
4,8,12-trimethyl-1,3,(E)7,(E)11-tridecatetraene*	3		2		
(E)6,10-dimethyl-5,9-undecadien-2-one*	1				
monoterpene					
δ-3-carene*				3	
(E)-β-ocimene*	19	13	9	1	27
<b>3,7-dimethyl-1,6-octadien-3-ol (β-linalool)*</b>	18	2	2	2	
sesquiterpenes					
β-bourbonene*	<1		2	<1	
β-caryophyllene*	8	29	13	7	
(E)-β-farnesene*	5	79	1	3	
germacrene D*	39	21	27	7	
(Z,E)-α-farnesene*	1	4	2	2	7
(E,E)-α-farnesene*	100	100	100	100	100
δ-cadinene*			1	1	

<sup>a</sup> Amounts relative to (E,E)-α-farnesene. Bold-faced compounds consistently elicited antennal activity in codling moth females as determined by GC-EAD (see Table 2). The average amount of (E,E)-α-farnesene collected from a branch with small fruit was  $3.3 \pm 2.1 \mu\text{g/L}$  ( $N = 5$ ). Asterisks indicate compounds for which synthetic or authentic standards were available. <sup>b</sup> Flowering branches ( $N = 4$ ).

<sup>c</sup> Mature foliage, 1–2 weeks after bloom ( $N = 3$ ). <sup>d</sup> Branches with green fruit, 1–3 weeks after bloom ( $N = 8$ ). <sup>e</sup> Green apples, 3 weeks after bloom ( $N = 5$ ). <sup>f</sup> Ripe apples, 12–14 weeks after bloom ( $N = 6$ ).



**Figure 1.** Response of a codling moth female antenna to volatile compounds from an apple branch with green fruit (cv. Discovery, 3 weeks after bloom), as analyzed by GC-EAD. Top, antennal signal (EAD); bottom, GC trace (FID). The active compounds are 4,8-dimethyl-1,3(*E*),7-nonatriene (**2**), (*Z*)-3-hexenol (**4**), linalool (**6**),  $\beta$ -caryophyllene (**7**), (*E*)- $\beta$ -farnesene (**8**), germacrene D (**9**), (*Z,E*)- and (*E,E*)- $\alpha$ -farnesene (**10, 11**), and methyl salicylate (**12**). Antennae did not respond to (*E*)- $\beta$ -ocimene (**1**), (*Z*)-3-hexenyl acetate (**3**), or  $\beta$ -bourbonene (**5**).

ably released from leaves; they have not been reported from apple flowers (4, 26).

**Branches with Green Apples.** The headspace of apple branches with green fruit, collected at the seasonal peak of codling moth oviposition in the field, contained nine compounds which were detected by codling moth female antennae (Table 1; Figure 1). These

compounds, except methyl salicylate, were also found in collections from foliage alone. The most obvious difference between leaves and green fruit was an almost inverse ratio of (*Z*)-3-hexenyl acetate and (*E,E*)- $\alpha$ -farnesene (Table 1).

Three terpenoids released from green fruit,  $\delta$ -3-carene,  $\beta$ -bourbonene, and  $\alpha$ -cadinene, were not present

**Table 2. Antennal Response of Codling Moth Females to Volatile Compounds from Apple as Determined by GC-EAD**

compound	mean response ± SD (mV)	relative response ± SD (mV*100/ng) <sup>a</sup>
alcohols		
( <i>Z</i> )-3-hexenol <sup>b</sup>	0.058 ± 0.014	1.64 ± 0.65
esters		
butyl butanoate <sup>c</sup>	0.037 ± 0.012	0.09 ± 0.02
propyl hexanoate <sup>c</sup>	0.088 ± 0.014	0.33 ± 0.15
hexyl propanoate <sup>c</sup>	0.104 ± 0.018	0.24 ± 0.05
butyl hexanoate <sup>c</sup>	0.058 ± 0.008	0.11 ± 0.02
hexyl butanoate <sup>c</sup>	0.066 ± 0.010	0.13 ± 0.03
hexyl 2-methyl-butanoate <sup>c</sup>	0.192 ± 0.018	0.20 ± 0.19
hexyl hexanoate <sup>c</sup>	0.075 ± 0.030	0.72 ± 0.37
benzenoids		
methyl 2-hydroxy benzoate (methyl salicylate) <sup>d</sup>	0.086 ± 0.029	13.79 ± 4.14
benzyl alcohol <sup>d</sup>	0.098 ± 0.018	0.04 ± 0.04
irregular terpenes		
4,8-dimethyl-1,3( <i>E</i> )-7-nonatriene <sup>b</sup>	0.078 ± 0.011	2.51 ± 2.45
monoterpene		
3,7-dimethyl-1,6-octadien-3-ol ( $\beta$ -linalool) <sup>d</sup>	0.100 ± 0.032	1.09 ± 0.27
sesquiterpenes		
$\beta$ -caryophyllene <sup>b</sup>	0.105 ± 0.020	4.40 ± 4.05
( <i>E</i> )- $\beta$ -farnesene <sup>b</sup>	0.056 ± 0.012	5.03 ± 2.41
germacrene D <sup>b</sup>	0.068 ± 0.014	0.60 ± 0.32
( <i>Z,E</i> )- $\alpha$ -farnesene <sup>b</sup>	0.069 ± 0.013	1.36 ± 0.71
( <i>E,E</i> )- $\alpha$ -farnesene <sup>b</sup>	0.114 ± 0.016	0.47 ± 0.32

<sup>a</sup> Mean antennal response in relation to stimulus amount. <sup>b</sup> Volatile collections from branches with green fruit (*N* = 5). <sup>c</sup> Volatile collections from ripe apples (*N* = 5). <sup>d</sup> Volatile collections from flowering branches (*N* = 5).

in collections from leaves alone. These compounds were not detected by the female antenna. Longer sampling intervals, or collection from larger amounts of foliage, would be required to determine whether these compounds are released also from foliage. Presence of  $\beta$ -bourbonene and  $\alpha$ -cadinene may even be an artifact, as both compounds have been reported as isomerization products of germacrene D (27, 28);  $\delta$ -3-carene has previously been identified from apple (29).

**Full-Grown Apples.** The volatile blend collected from apples 6–8 weeks after bloom (apples up to 5 cm in diameter, cv. Discovery; data not shown) contained fewer and smaller amounts of the terpenoid compounds, whereas several esters were seen to emerge. The relative abundance and the number of esters further increased in collections from mature apples, 12–14 weeks after bloom (Table 1). This increase in ester production associated with apple ripening is well documented (30–32). Female antennae responded to some of these esters; the most active one, in relation to abundance, was hexyl hexanoate (Table 2).

**Behavioral Response of Codling Moth Females.** In the orchard, codling moth females were regularly observed to fly upwind over several meters toward branches with green apples for oviposition. Cut branches with green apples elicited upwind orientation flights from mated females also in a greenhouse. For the laboratory wind tunnel tests done with plant material in glass jars used for volatile collections an air stream was passed through the jar. Branches with green apples attracted 18% of the females tested to the outlet of this jar; but branches with leaves alone attracted significantly fewer females (4%;  $N = 90$ ). Blank air and a branch with green cherries (*Prunus avium*) were not attractive at all.

Visual observations in the field and the wind tunnel showed that the upwind flight behavior of females toward apple was quite similar to the upwind flight behavior of males toward sources of sex pheromone (33). However, there is seemingly an important difference with respect to the chemical stimulus eliciting orientation flights in males and females. The females release a pheromone blend which is quite constant in composition and proportion (34, 35). In comparison, ovipositing females are attracted to both green and ripening apples, during the first and second seasonal flight period, and to various apple cultivars, which greatly differ with respect to the blend of compounds released (Table 1; 25, 30, 36).

The compounds that account for long-range orientation of gravid females to apple have not been identified, but a strong antennal response to several terpenoid compounds, including (*E,E*)- $\alpha$ -farnesene, indicates that at least some of these compounds may provide important cues. Compounds which do not elicit an antennal response are less likely to induce a behavioral response.

The role of the esters released from full-grown fruit, which elicited an antennal response, is quite unclear. These compounds are not released from immature apples during the first seasonal flight period, thus they are not required for female attraction. They may synergize female attraction to (*E,E*)- $\alpha$ -farnesene released from full-grown fruit, but large amounts of these compounds might even have an opposite effect. It has been observed that females lay fewer eggs on apples toward the end of the summer (37). Apple maggot, *Rhagoletis pomonella*, which oviposits only on full-grown

fruit, is attracted to a blend of apple esters (38). However, extracts from apples that are too ripe for optimal larval development and survival had a deterrent effect on female attraction (39). Ongoing studies aim at the identification of the role of apple terpenoids and esters in codling moth reproductive behavior.

#### ACKNOWLEDGMENT

We thank G. Birgersson (Göteborg), W. Francke (Hamburg), and R. U. Unelius (Kalmar) for generously providing us with samples of synthetic compounds.

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Received for review January 12, 2001. Revised manuscript received May 10, 2001. Accepted May 14, 2001. This study was supported by the Foundation for Strategic Environmental Research (MISTRA), the Swedish Natural Science Research Council (NFR), and the Swedish Council for Forestry and Agricultural Research (SJFR) which is gratefully acknowledged.

JF0100548