Universitat de Lleida

Departament de Química

PhD Thesis

Cydia pomonella (L.) behavior and responses to host volatiles

Daniel Casado González

Lleida 2007

A mi familia,
por su apoyo incondicional.

Agradecimientos

Estos cinco últimos años han sido de una gran importancia para mi crecimiento tanto a nivel profesional como personal. Mirando atrás veo cómo ha cambiado mi vida y me doy cuenta de que tengo mucho que agradecer a las personas que me han acompañado a lo largo de todo este tiempo.

En primer lugar tengo mucho que agradecer a los Drs. Magí Riba, César Gemeno y Jesús Avilla, quienes han supervisado y dirigido mi trabajo. Siempre estaré en deuda con ellos por sus consejos, enseñanzas y paciencia.

A los Drs. Peter Witzgall y Marie Bengtsson, que me acogieron en su grupo de investigación durante mis estancias en Suecia, y que enriquecieron mi formación ampliando mi visión científica.

A todos los estudiantes de doctorado, profesores y técnicos de laboratorio de los departamentos de Química de la Universitat de Lleida, y de Protección de Cultivos del Centro UdL-IRTA de R+D, que siempre me han ayudado cuando lo he necesitado, y con quienes he pasado tantos ratos agradables. Especialmente agradecido estoy con Montse, que tanto me ayudó en mis inicios en el mundo de la cromatografía, y con Mire, por tantas horas que hemos compartido.

A todos los amigos que tanto se han preocupado por la marcha de esta tesis y por mi futuro. Especialmente a los otros dos mosqueteros, Santi y Jorge, y mis otras dos dimensiones, David y Dani, que me ayudaron tanto cuando no podía salir del hoyo y lo veía todo de color negro. También me siento especialmente agradecido con Bàrbara, por este último año de compañía maravillosa, con Jordi, mi intrépido compañero de aventuras, y con Meritxell, por las fantásticas horas de trabajo que compartimos este último verano.

A mis padres, José y Dolores, así como a mi hermana, Eva, por toda una vida de cariño, preocupación y apoyo. Sin ellos nada tendría sentido.

A todos aquellos que me haya dejado en el tintero, porque aunque aquí no aparezcan sus nombres, sí que los llevo en el corazón.

Abstract

Volatile compounds from apple and walnut trees were collected in the field from attached branches, bearing leaves and fruits, enclosed in plastic bags in the morning and at dusk, in different periods of the season. Collections were analyzed by gas chromatography-mass spectrometry (GC-MS), and gas chromatography-electroantennodetection (GC-EAD) using antennae of male *Cydia pomonella*. Forty four compounds in apple and 90 in walnut were detected by GC-MS. Emissions of both plant species widely differed both qualitatively and quantitatively. Apple emissions were dominated by aliphatic compounds, whereas walnut ones by terpenes. Diel and seasonal variations were found in emissions of both plant species. GC-EAD revealed activity for 5 compounds in apple collections and 10 in walnut ones. Further electroantennographic (EAG) analyses with males and females revealed important EAG-activity for many other volatiles emitted by apple. In these analyses male responses were equal to or higher than those of female for all compounds, except for β-myrcene. Amongst the EAD-active compounds in the GC-EAD analyses, hexyl butanoate was apple-specific, 3 compounds were walnut-specific (alloocimene, pinocarrvone, and caryophyllene oxide), and the remaining were shared by both plant species. 2-Cyclopentylcyclopentanone, a compound emitted by the plastic bags, also elicited strong EAG responses in antennae of both sexes.

Ethyl (*E*,*Z*)-2,4-decadienoate (pear ester), and (*E*)-β-farnesene were fully attractive for *C. pomonella* in field trapping. However, they did not elicit source contacts in wind tunnel, suggesting that other sensory cues are involved in their field attractiveness. Pre-exposure to the sex pheromone, (*E*,*E*)-8,10-dodecadien-1-ol (codlemone), decreased male upwind flight to itself in wind tunnel, but increased pear ester attractiveness; and had no effect on females. Similarly, trap captures with pear ester were found to increase under mating disruption. Pear ester acted as a codlemone antagonist when blended at large amounts. However, this effect disappeared when both compounds were loaded onto different septa; and males were unable to discriminate amongst codlemone and the antagonistic blend when offered side-by-side.

Oviposition and mating assays showed that *C. pomonella* diel oviposition and mating timings are modulated by temperature. In the field, oviposition activity was advanced by lower temperatures; and was maximum in the 3rd and 4th days of life. In the laboratory, oviposition was also advanced by lower temperatures, but for most of the assayed temperatures it peaked in the first hour of the scotophase. Oviposition did not take place at 12ºC, and fecundity was maximum at 22 and 27ºC. Mating activity occurred before than oviposition activity in a given day.

Keywords: *Cydia pomonella*, host-plant volatiles, behavior, EAG, GC-EAD, GC-MS, Lepidoptera, Tortricidae, pheromone pre-exposure, pear ester, (*E*)-β-farnesene, upwind flight, sex pheromone, walnut, apple, diel variation, temperature, diel activity, light intensity.

Resumen

Se recogieron colecciones de volátiles de manzano y de nogal emitidos por ramas intactas en campo, con hojas y frutos, rodeadas por bolsas de plástico, por la mañana y en el crepúsculo en diferentes momentos de la campaña. Estas muestras se analizaron mediante cromatografía de gases-espectrometría de masas (GC-MS) y cromatografía de gases-electroantenodetección (GC-EAD) usando antenas de machos de *Cydia pomonella*. Se detectaron 44 compuestos en manzano y 90 en nogal por GC-MS. Las emisiones de ambas especies variaron ampliamente, tanto cuantitativa como cualitativamente. Los compuestos alifáticos fueron mayoritarios en las emisiones de manzano, mientras que los terpenos lo fueron en las de nogal. Se observaron variaciones entre periodos tanto para la campaña como para el día. Se encontró actividad EAD para 5 compuestos en las muestras de manzano y 10 en las de nogal. Posteriores pruebas de electroantenografía (EAG) con machos y hembras revelaron una fuerte actividad EAG para múltiples volátiles emitidos por manzano. En estos ensayos la respuesta de los machos fue siempre igual o superior a la de las hembras, con la excepción del β-mirceno. Entre los compuestos activos en los análisis de GC-EAD, el butanoato de hexilo fue específico de manzano, 3 compuestos fueron específicos de nogal (aloocimeno, pinocarvona, y óxido de cariofileno) y los restantes eran compartidos por ambas especies de planta. 2-Ciclopentilciclopentanona, un compuesto emitido por las bolsas de plástico, también provocó intensas respuestas EAG en antenas de ambos sexos.

(*E*,*Z*)-2,4-Decadienoato de etilo (éster de pera), y (*E*)-β-farneseno fueron completamente atrayentes en campo, pero no provocaron contactos con la fuente en túnel de viento, sugiriendo que otros estímulos sensoriales están relacionados con su funcionamiento en campo. La preexposición a la feromona sexual, (*E*,*E*)-8,10-dodecadien-1-ol, disminuyó la respuesta a ella de machos en túnel de viento, pero incrementó la respuesta al éster de pera; y no tuvo efecto alguno sobre las hembras. De forma similar, las capturas en campo con éster de pera fueron superiores en confusión sexual. El éster de pera actuó como antagonista de la feromona sexual al mezclarlo con ésta en cantidades elevadas. No obstante, el antagonismo desapareció cuando ambos compuestos se presentaban en septos separados; y los machos no discriminaron entre la feromona sexual sola y la mezcla antagonista cuando ambas se ofrecieron una al lado de la otra.

Los ensayos de oviposición y apareamiento demostraron que la temperatura modula el momento del día en que estos comportamientos tienen lugar. En campo, la oviposición se avanzó a temperaturas más bajas; y fue máxima en el 3º y 4º días de vida. En laboratorio, la oviposición también se avanzó con temperaturas más bajas, pero para la mayoría de temperaturas ensayadas, el pico de oviposición tuvo lugar durante la primera hora de la escotofase. A 12ºC no hubo oviposición, y la fecundidad fue máxima a 22 y 27ºC. Para un día determinado. La actividad diaria de apareamiento tenía lugar antes que la de oviposición.

Palabras clave: *Cydia pomonella*, volátiles de planta huésped, comportamiento, EAG, GC-EAD, GC-MS, Lepidoptera, Tortricidae, preexposición a la feromona, éster de pera, (*E*)-β-farneseno, vuelo orientado, feromona sexual, nogal, manzano, variación diaria, temperatura, actividad diaria, intensidad lumínica.

Resum

Es van mostrejar volàtils de pomera i noguer emesos per branques intactes en camp, rodejades per bosses de plàstic, al matí i al crepuscle en diferents moments de la campanya. Aquestes mostres es van analitzar per cromatografia de gasos-espectrometria de masses (GC-MS) i cromatografia de gasos-electroantenodetecció (GC-EAD) fent servir antenes de mascles de *Cydia pomonella*. Es van detectar 44 compostos en pomera i 90 en noguer per mitjà de la GC-MS. Les emissions d'ambdues espècies van variar clarament, tant quantitativament com qualitativa. Els compostos alifàtics eren majoritaris en les emissions de pomera, mentre les terpens ho eren a les de noguer. Es va trobar activitat EAD per 5 compostos a les mostres de pomera i 10 a les de noguer. Posteriors proves d'electroantenografia (EAG) amb mascles i femelles van revelar una forta activitat EAG per nombrosos volàtils emesos per pomera. En aquests assajos la resposta dels mascles va ser sempre igual o superior a la de les femelles, excepte pel β-mircè. Entre els compostos actius en les anàlisis de GC-EAD, el butanoat d'hexil era específic de pomera, 3 compostos eren específics de noguer (al·loocimè, pinocarvona, i òxid de cariofil·lè) i la resta eren comuns entre ambdues espècies de planta. La 2-ciclopentilciclopentanona, un compost emès per les bosses de plàstic, també va provocar intenses respostes EAG en antenes d'ambdós sexes.

El (*E*,*Z*)-2,4-decadienoat d'etil (èster de pera), i el (*E*)-β-farnesè van ser completament atraients en camp, però no van estimular contactes amb la font en túnel de vent, suggerint que d'altres estímuls sensitius estan relacionats amb el seu funcionament en camp. La preexposició a la feromona sexual, (*E*,*E*)-8,10-dodecadien-1-ol, va disminuir la resposta dels mascles a aquesta en túnel de vent, però va incrementar la resposta a l'èster de pera; i no va tenir cap efecte sobre les femelles. De forma semblant, les captures en camp amb èster de pera van ser superiors sota confusió sexual. L'èster de pera va actuar com antagonista de la feromona sexual en barrejar-se a altes quantitats. No obstant, l'antagonisme va desaparèixer en presentar ambdós compostos en septes separats; i els mascles van ser incapaços de discriminar entre la feromona sexual sola i la barreja antagonista quan ambdues es van oferir una al costat de l'altra.

Assajos d'oviposició i aparellament van demostrar que la temperatura modula el moment del día en que aquests comportaments tenen lloc. En camp, l'oviposició es va avançar a temperatures baixes; i va ser màxima al 3r i 4t dies de vida. En laboratori, l'oviposició també es va avançar a temperatures més baixes, però per la majoria de temperatures assajades, el pic d'oviposició va tenir lloc durant la primera hora de l'escotofase. A 12ºC no hi va haver oviposició, i la fecunditat va ser màxima a 22 i 27ºC. Per un dia determinat. L'activitat diària d'aparellament tenia lloc abans que la d'oviposició.

Paraules clau: *Cydia pomonella*, volàtils de planta hoste, comportament, EAG, GC-EAD, GC-MS, Lepidoptera, Tortricidae, preexposició a la feromona, èster de pera, (*E*)-β-farnesè, vol orientat, feromona sexual, noguer, pomera, variació diària, temperatura, activitat diària, intensitat lumínica.

Table of contents

List of tables………… **x**
List of figures………… **xi**
List of abbreviations………… **xiii**

Chapter I. Introduction………… **1**
1. Apple, pear and walnut growing in Spain………… 3
2. Volatile emission by plants………… 3
3. The role of odors in insect behavior………… 5
3.1. Semiochemicals………… 5
3.2. Sex pheromones………… 6
3.3. Plant semiochemicals………… 8
3.4. Semiochemicals in pest control………… 11
3.5. Detection of odors by insects………… 12
4. *Cydia pomonella* (L.)………… 14
4.1. Taxonomy………… 14
4.2. Description………… 14
4.3. Life history………… 15
4.4. *Cydia pomonella* as a pest………… 16
4.5. Management………… 18
5. General methods………… 20
5.1. Insects………… 20
5.2. Volatile collection………… 20
5.3. Electroantennographic recordings………… 22
5.4. Wind tunnel assays………… 23
5.5. Oviposition assays………… 23
5.6. Field Trapping………… 25
6. References………… 26

Chapter II. General objectives………… **41**

Chapter III. Day-night and phenological variation of apple tree volatiles and electroantennogram responses in *Cydia pomonella* (Lepidoptera: Tortricidae)………… **45**

Chapter IV. Diel variation of walnut tree volatiles and electrophysiological responses in *Cydia pomonella* (Lepidoptera: Tortricidae)………… **57**

Chapter V. Pheromone pre-exposure and mating modulate codling moth (Lepidoptera: Tortricidae) response to host plant volatiles………… **85**

Chapter VI. Sex pheromone and pear ester upwind attraction of *Cydia pomonella* (L.) males: blend effect and compound discrimination………… **93**

Chapter VII. Effect of temperature and light intensity on *Cydia pomonella* (L.) oviposition and mating diel activity………… **113**

Chapter VIII. Main results and global discussion………… **133**
1. Diel and seasonal variations of volatile emissions by *Cydia pomonella* (L.) host plants and electroantennogram responses………… 135
2. Behavioral responses to pear ester, (*E*)-β-farnesene, and codlemone………… 139
3. Influence of temperature and light intensity on oviposition and mating behaviors.. 141
4. Final conclusions………… 143
5. References………… 145

List of tables

Chapter I

Table 1. Some examples of semiochemicals responsible of attraction to hosts or of oviposition stimulation in insects of different taxa…………………………………… 9

Chapter III

Table 1. Volatile compounds detected in apple tree headspace at three phenological stages in the morning and at dusk………………………………………………….. 50
Table 2. Amounts of volatile compounds detected in apple trees headspace that were significantly affected by the phenology……………………………………… 51
Table 3. Antennal responses of males and females of *C. pomonella* L. to synthetic compounds……………………………………………………………………. 53

Chapter IV

Table 1. Mean amounts of volatile compounds detected in walnut trees headspace at two seasonal periods in the morning and at dusk…………………………………….. 68
Table 2. Mean percentage contribution of the major compounds to the total emission of walnut tree volatiles…………………………………………………... 72
Table 3. *F*- and *P*-values of the ANOVA for compounds and chemical groups that showed significant variations……………………………………………………… 73

Chapter VI

Table 1. Summary of composition of lures formulated and used in the study………. 98

List of figures

Chapter I

Figure 1. The codling moth, *Cydia pomonella* (L.), in different stages…………….... 15
Figure 2. *Cydia pomonella* (L.) damage in different hosts………………………….... 17
Figure 3. Scheme of the volatile collection system………………………………….. 21
Figure 4. Mating box for rearing and bioassays…………………………………….... 24

Chapter III

Figure 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*………………………………………………….. 52

Chapter IV

Figure 1. Percentage contribution of the different groups of compounds to the total emission of walnut tree volatiles…………………………………………………... 66
Figure 2. GC-FID (bottom) and GC-EAD (top) traces of a volatile collection from a walnut tree *in situ*, made during the morning in mid-Summer, using the antenna of a male *C. pomonella*……………………………………………………………………. 74

Chapter V

Figure 1. Wind tunnel response of codling moth Cydia pomonella males and females to 10 mg of plant volatiles pear ester ethyl (E,Z)-2,4-decadienoate, or (E)-β-farnesene, and to 100 mg of sex pheromone codlemone (E,E)-8,10-dodecadienol….. 90
Figure 2. Wind tunnel response of codling moth Cydia pomonella males to 1 mg of codlemone (E,E)-8,10-dodecadienol and a 1 : 10 000 mg-blend of codlemone and pear ester ethyl (E,Z)-2,4-decadienoate………………………………………………. 90

Chapter VI

Figure 1. Mean percentage of *Cydia pomonella* males responding to single lures loaded with codlemone, pear ester, or both compounds, in the wind tunnel………… 100
Figure 2. Mean percentage of *Cydia pomonella* male attraction to different lures of codlemone and pear ester in the wind tunnel……………………………………… 102
Figure 3. Percentage of upwind flying *Cydia pomonella* males that changed at least once of plume in a two-source wind tunnel assay of attraction to codlemone and pear ester……………………………………………………………………………… 103
Figure 4. Mean time spent to contact a source by *Cydia pomonella* males in the wind tunnel when flown to one or two sources containing codlemone, pear ester, or a blend of both……………………………………………………………………... 105

Chapter VII

Figure 1. Oviposition and mating by codling moth females under natural conditions of light and temperature in two different European locations……………………….. 120
Figure 2. Oviposition pattern of *Cydia pomonella* females within the first five days of life, under natural conditions of light and temperature in two different locations, Lleida (41º 37' N, 0º 38' E) and Alnarp (55º 55' N, 13º 37' E)……………………… 121
Figure 3. Daily mean oviposition of *Cydia pomonella* females within the first five days of life, under natural conditions of light and temperature in two different locations, Lleida (Spain, 41º 37' N, 0º 38' E) in mid-September, and Alnarp (Sweden, 55º 55' N, 13º 37' E) in late-Sweden……………………………………… 122
Figure 4. Mean female fecundity of *Cydia pomonella* under laboratory conditions, at five different constant temperatures, and a light intensity of ca. 2500 lux………… 123
Figure 5. Mean percentage of oviposition each hour from 4 hours before to 4 hours after scotophase onset, at 4 different constant temperatures…………………………. 124
Figure 6. Mean cumulated percentage of oviposition each hour from 4 hours before to 4 hours after scotophase onset, at 4 different constant temperatures……………… 125

List of abbreviations

Act	Activation
AL	Antennal lobe
APF	After petal fall
CFR	Close-to-full ripening
CpGV	*Cydia pomonella* granulovirus
dLS	Late-spring at dusk
dMS	Mid-summer at dusk
EAG	Electroantennography
EE12OH	(*E*,*E*)-8,10-dodecadien-1-ol
EtDD	Ethyl (*E*,*Z*)-2,4-decadienoate
F1	Flying upwind over 50 cm
F2	Flying upwind over 100 cm
F3	Flying upwind over 150 cm
FID	Flame ionization detector
GC	Gas chromatography
GC-EAD	Gas chromatography-electroantennodetection
GC-MS	Gas chromatography-mass spectrometry
IF	Immature fruit
IGR	Insect growth regulator
IS	Internal standard
LRI	Linear retention index
LS	Late-spring
MGC	Macroglomerular complex
mLS	Late-spring in the morning
mMS	Mid-summer in the morning
MS	Mid-summer
OBP	Odorant-binding protein
ODE	Odorant degrading enzymes
OR	Olfactory receptor
ORN	Olfactory receptor neuron
RT	Retention time
TCA	Total chromatographic area
Tch	Source contact
TF	Taking flight
VOC	Volatile organic compound
WF	Wing-fanning
Wlk	Walking and wing-fanning on the source

CHAPTER I

INTRODUCTION

1. Apple, pear and walnut growing in Spain

Apple is an important crop in Spain. More than 888,000 tones of apples were produced in 2003, although importations were higher than exportations. Apple is grown for cider production in about 8,700 ha, whereas a surface of approximately 37,200 ha is cultivated for fresh fruit. Fresh apple production is especially important in Catalonia, where apple is grown in almost 14,000 ha, and apple production raised the 370,000 tones in 2003 (MAPA 2004).

Pear production is similar in importance to fresh apple production in Spain. In 2003, pear growing covered a surface of approximately 38,100 ha in Spain, and pear production exceeded 728,000 tones. Spanish pear production is very concentrated in the Valley of the Ebro River. In Catalonia the surface dedicated to pear growing exceeded the 16,500 ha in 2003. Inside Catalonia, Lleida is the most important area of pear production with almost 15,500 ha cultivated in 2003. Spain is a net exporter of pear; in 2003 importations were of about 39,000 tones, whereas exportations amounted to about 130,000 tones (MAPA 2004).

Walnut production in Spain is more marginal than these of apple or pear. Walnut growing was carried out in about 5,500 ha in 2003. Walnut production in 2003 in Spain was about 9,500 tones. Despite productive surface has been almost duplicated in the last 15 years, the increase in production has been much more moderated. The highest concentration of walnut in Spain takes place in the Community of Valence (MAPA 2004).

2. Volatile emission by plants

Plants are constantly emitting substantial amounts of biogenic volatile organic compounds (VOCs) into the atmosphere. The emissions of plants are rather complex, and any plant species emits several dozens of chemical compounds. Emission inventories show isoprene and monoterpenes as the most prominent compounds, but alkanes, alkenes, alcohols, carbonyls, organic acids, esters, and ethers are also emitted (Kesselmeier and Staudt 1999). These volatiles are secondary metabolites, which are produced by plants but are not directly essential for basic photosynthetic or respiratory metabolism (Theis and Lerdau 2003). Secondary metabolites were considered for long

time as simple by-products of primary metabolism that served as waste products for plants, which lack an excretory system. However nowadays, they are considered from an ecological and evolutionary perspective, as was first pointed out by Fraenkel in 1959.

Volatile emissions by plants are known to vary depending on multiple factors. Besides the genetically predetermined diversity, for a given plant species temporal and spatial variations occur. These variations result from the interaction between plant and environment (Kesselmeier and Staudt 1999). Factors affecting emission include, amongst others, temperature and light (Schuh *et al.* 1997, Staudt *et al.* 1997, Staudt and Bertin 1998, Tarvainen *et al.* 2005), attack of herbivores (Hopke *et al.* 1994, Paré and Tumlinson 1997a,b, Llusià and Peñuelas 2001, Hern and Dorn 2001, Scutareanu *et al.* 2003, Gouinguené *et al.* 2003), mechanical damage (Paré and Tumlinson 1997a, Agelopoulus *et al.* 1999), plant phenology (Staudt *et al.* 1997, Bengtsson *et al.* 2001, Rapparini *et al.* 2001), drought stress (Ebel *et al.* 1995), rainfall and relative humidity (Vallat *et al.* 2005), and stomatal closure and physicochemical properties of the different volatile compounds (Niinemets and Reichstein 2003, Niinemets *et al.* 2004).

Multiple functions have been quoted for plant volatiles. It is widely assumed that floral scents serve to attract and guide pollinators (Reinhard *et al.* 2004). However, many floral volatiles have anti-microbial activity (Friedman *et al.* 2002, Hammer *et al.* 2003), and so they could also be involved in the protection of the reproductive parts of the plant, which are highly valuable (Dudareva *et al.* 2004).

Non-floral volatiles have also several functions. Volatile emissions increase the tolerance of the photosynthectic apparatus to several adverse conditions, such as heat (Sharkey and Singsaas 1995, Sharkey *et al.* 2001, Peñuelas and Llusià 2003), pollution (Loreto and Velikova 2001, Affek and Yakir 2002, Loreto *et al.* 2004), or water stress (Peñuelas and Llusià 2002). Emissions from foliage are also thought to protect plants against some herbivores (Pichersky and Gershenzon 2002), and herbivore-induced volatiles seem to be also a defense system against herbivory. This defense can be either indirect by attraction of predators and/or parasitoids (Paré and Tumlinson 1999, Dicke and Van Loon 2000), or direct by repelling (De Moraes *et al.* 2001, Kessler and Baldwin 2001) or intoxicating (Vancanneyt *et al.* 2001) herbivores.

Recent studies propose that one of the most abundant VOCs, isoprene, has a protection role, acting as a metabolic 'safety valve' (Rosenstiel *et al.* 2004), and that plant emissions of VOCs are largely determined by the physicochemical characteristics of the emitted compounds (Niinemets *et al.* 2004). By combining these new approaches,

Peñuelas and Llusià (2004) have hypothesized that VOC emissions by plants, and their functions, are the result of natural selection acting on pre-existing volatile heritable materials, that has led plants to take advantage of these unavoidable emissions, rising up functions of compounds that were originally simply nonfunctional metabolic byproducts. This theory also highlights that there is not necessarily a specific role for every VOC emitted by plants.

3. The role of odors in insect behavior

3.1. Semiochemicals

Semiochemicals are chemicals that mediate interactions between different organisms (Law and Regnier 1971), and they are classified in two main groups: pheromones and allelochemicals. Pheromones mediate relations between conspecific individuals. The term pheromone was first proposed in 1959 (Karlson and Butenandt 1959, Karlson and Luscher 1959) to chemicals that affected conspecific animals, but later the definition was modified to include also plant chemicals (Nordlund and Lewis 1976). Pheromones are usually classified depending on the behavior that they evoke, and the most common categories are sex, aggregation, alarm, epideictic and trail pheromones. Sex pheromones are the most well documented pheromones, and are involved in mate location or courtship. Aggregation pheromones evoke an increase in density of conspecifics in the vicinity. Alarm pheromones lead to an effect of escaping and dispersion as a defensive response. Epideictic pheromones stimulate spacing between conspecifics, and result in a reduction of intraspecific competition. Trail pheromones are used to recruit other insects in a colony to new food sources or to facilitate migration in a colony to a new site (Jutsum and Gordon 1989).

On the other hand, allelochemicals are chemicals that mediate relationships between individuals of different species (Whittaker 1970a,b). Allelochemicals are divided in four types: allomones, kairomones, synomones, and apneumones. Allomones are chemicals which induce a behavioral or physiological response in the receiver that adaptively benefits the emitter (Brown 1968, Nordlund and Lewis 1976). On the contrary, a kairomone is a substance that, when it is perceived, generates a behavioral or physiological response in the receiver that represents an adaptive benefit to itself

(Brown *et al.* 1970, Nordlund and Lewis 1976). Synomones are chemical substances that mediate mutualistic interaction, and they stimulate a behavioral or physiological response in the receiver that adaptively benefits both, receiver and emitter (Nordlund and Lewis 1976). Finally, apneumones are chemicals emitted by a nonliving source that evoke a behavioral or physiological response in the receiver, that is adaptively favorable to itself, but detrimental to an organism of another species that may be found in or on the nonliving source (Nordlund and Lewis 1976).

Recently, Ruther *et al.* (2002a) proposed a classification of kairomones similar to that existing for pheromones, depending on their ecological function for the benefiting organism. They proposed the terms foraging, enemy-avoidance, sexual, and aggregation kairomones. Foraging kairomones are those used for food location for the organism itself or its offsprings, enemy-avoidance kairomones are used to reduce the impact of natural enemies, sexual kairomones are used for sexual purposes, and aggregation kairomones attract and/or arrest both sexes of conspecific individuals. The authors also suggested that this classification should be easily extended to other semiochemicals such as allomones.

3.2. Sex pheromones

As mentioned above, sex pheromones are the most widely known and documented semiochemicals. They are intraspecific semiochemicals that organisms use in their mating process (Roelofs 1981). Sex pheromones mediate both long-range attraction and close-range interactions in a wide variety of taxa (Cardé and Baker 1984). Despite this wide concept of sex pheromone, in many cases, components responsible for the long-range behavioral phases of mate location are different from those of close courtship (Jutsum and Gordon 1989).

A sex pheromone was identified and reported by first time in 1959, when Butenandt *et al.* identified the sex pheromone of *Bombix mori* L. They extracted the abdomen apex of 250,000 female moths, and found (*E*,*Z*)-10,12-hexadecadien-1-ol, named bombykol, to be the active compound. The identification of bombykol was soon followed by that of the sex pheromones of other insects: *Apis mellifera* L. (Butler and Fairey 1964), *Trichoplusia ni* (Hübner) (Berger 1966), and *Lymantria dispar* (L.) (Bierl *et al.* 1970).

Typically an insect pheromone comprises a few compounds, whose composition is well defined and generally very consistent amongst individuals (Gordon and Jutsum 1989). Insects synthesize the compounds of the pheromones to a high degree of purity; precisely control the geometrical and optical isomerism of the molecules, and the ratio of the blend (Löfstedt and Odham 1984, Löfstedt *et al.* 1982, 1985a,b). Different isomers can evoke opposite behaviors in a given responsive insect (Gordon and Jutsum 1989), and the blend ratio is species-specific and a powerful tool for sympatric reproductive isolation of close related species (Tumlinson *et al.* 1974, Roelofs and Brown 1982, Löfstedt *et al.* 1991).

Sex pheromones of female moth are relatively simple structures. These consist on straight hydrocarbon chains that contain an oxygenated functional group, and usually have some degree of unsaturation. The functional group can be an ester linkage, an alcohol, an aldehyde, or an epoxide (Howse *et al.* 1998, Jurenka 2003).

The main compound of the sex pheromone of *Cydia pomonella* (L.) is the (*E*,*E*)-8,10-dodecadienol (codlemone), and was identified by electroantennography in 1971 by Roelofs *et al.*. In later bioassays, it has been shown that female gland extracts are more attractive than codlemone alone; this makes evident that *C. pomonella* sex pheromone is multicomponent (Bartell and Bellas 1981, El-Sayed *et al.* 1999). Several authors have studied composition of *C. pomonella* female gland extracts and effluvia (Einhorn *et al.* 1984, Arn *et al.* 1985, Bäckman *et al.* 1997, Witzgall *et al.* 2001), and identified the following secondary compounds: dodecan-1-ol, tetradecan-1-ol, hexadecan-1-ol, (*E*,*Z*)-8,10-dodecadien-1-ol, (*Z*,*E*)-8,10-dodecadien-1-ol, (*Z*,*Z*)-8,10-dodecadien-1-ol, (*E*)-9-dodecen-1-ol, (*E*,*E*)-8,10-dodecadienal, (*E*)-8-dodecen-1-ol, and (*E*,*E*)-8,10-dodecadien-1-yl acetate.

In wind tunnel tests, dodecan-1-ol has been shown a synergist of codlemone (Arn *et al.* 1985, Einhorn *et al.* 1986), but this compound together with tetradecan-1-ol and (*E*)-9-dodecen-1-ol have been shown not to be emitted in a constant ratio, suggesting the absence of a behavioral role to them (Bäckman *et al.* 1997). (*E*,*Z*)-8,10-dodecadien-1-ol and (*E*,*E*)-8,10-dodecadien-1-yl acetate are known pheromone antagonists (Roelofs *et al.* 1972, Hathaway *et al.* 1974, El-Sayed *et al.* 1998), but when they are released in the amounts found in female gland extracts they act as synergists (Witzgall *et al.* 2001). Amongst the codlemone isomers, as seen above the (*E*,*Z*)-isomer can act as both a synergist or an antagonist depending on the blend ratio; the (*Z*,*Z*)-isomer has antagonistic effects both in wind tunnel and field capture; and (*Z*,*E*)-isomer has been

found to act as a synergist in wind tunnel but not under field conditions (El-Sayed *et al.* 1998, Witzgall *et al.* 2001). Although it seems clear that *C. pomonella* sex pheromone is multicomponent, the actual blend has not been yet determined as the main component by it self is highly efficient at attracting males, and codlemone is the only compound in the monitoring lures.

3.3. Plant semiochemicals

The semiochemical environment of cultivars must be decisive in determining the plant-insect interrelationships of agroecosystems (Metcalf and Metcalf 1992). Insects can use plant semiochemicals in their own benefit. Herbivorous insects use host-volatiles as chemical cues to find desirable feeding and oviposition sites (Visser 1986). However, semiochemicals act not only in a bitrophic system, as they can also be used by predators to find their preys (Dicke 1999).

Plant volatiles that are attractive to insects (i.e., Table 1) are usually lipophilic substances. The terpenoids are the most numerous and structurally varied of these plant volatiles, and they are formed by isoprenoid units, including some times oxygenated groups (Metcalf and Metcalf 1992). Terpenoids are derived from the mevalonic acid and are typically found in all parts of higher plants. Terpenoids are classified depending on the number of isoprenoid units they contain as: hemiterpenoids (1 unit), monoterpenoids (2 units), sesquiterpenoids (3 units), and diterpenoids (4 units) (Banthorpe 1994). Other important groups of compounds attractive for insects are phenylpropanoids and green leaf volatiles (Metcalf and Metcalf 1992). Phenylpropanoids present a C_6-C_3 structure and are derived from shikimic acid (Harborne, 1994). Green leaf volatiles are six carbon alcohols, aldehydes, and related esters that are product of the degradation of the C_{18} linolenic and linoleic fatty acids (Hatanaka 1993).

Many advances on *C. pomonella*-host plant interactions have been made, especially in the last 8 years. Antennal responses of *C. pomonella* adults have been recorded to numerous host volatiles (Bäckman *et al.* 2001, Bengtsson *et al.* 2001, Ansebo *et al.* 2004). However, behavioral responses are reported to a narrower range of compounds.

Table 1. Some examples of semiochemicals responsible of attraction to hosts or of oviposition stimulation in insects of different taxa.

Insect species	Chemical compounds	Host	References
Coleoptera			
Melolontha hippocastani	(*Z*)-3-hexen-1-ol	several species	Ruther *et al.* 2002b
Melolontha melolontha	(*Z*)-3-hexen-1-ol, (*E*)-2-hexen-1-ol, hexan-1-ol	several species	Reinecke *et al.* 2002
Pachnoda marginata	methyl salicylate, methyl anthranilate, cinnamic aldehydes, isovaleric acid, anethole, methyl benzoate, methyl cinnamate, isoamyl acetate, butyl butanoate, eugenol, phenylacetaldehyde, linalool, phenethyl propionate, acetoin, linalool oxide, citronellol, geraniol	several species	Larsson *et al.* 2003
Diptera			
Anastrepha obliqua	ethyl butanoate, isopropyl butanoate, hexan-1-ol, propyl butanoate, isobutyl butanoate, ethyl hexanoate, isopentyl butanoate, ethyl benzoate, ethyl octanoate	*Spondias mombin*	Cruz-López *et al.* 2006
Rhagoletis pomonella	butyl butanoate, propyl hexanoate, butyl hexanoate, hexyl butanoate, pentyl hexanoate	*Malus domestica*	Zhang *et al*. 1999
	3-methylbutan-1-ol, butyl hexanoate, (*E*)-4,8-dimethyl-1,3,7-nonatriene, dihydro-β-ionone, 3-methylbutyl acetate, ethyl acetate	*Crataegus* spp.	Nojima *et al.* 2003a
	3-methylbutan-1-ol, 1-octen-3-ol, β-caryophyllene, ethyl acetate, isoamyl acetate, dimethyl trisulfide	*Cornus florida*	Nojima *et al.* 2003b
Lepidoptera			
Argyresthia conjugella	2-phenyl etanol, methyl salicylate, decanal, anethole	*Sorbus aucuparia* & *Malus domestica*	Bengtsson *et al.* 2006
Cydia molesta	butyl hexanoate	*Malus domestica*	Natale *et al.* 2004
Cydia pomonella	α-farnesene	*Malus domestica*	Wearing and Hutchins 1973
	α-farnesene	*Malus domestica*	Hern and Dorn 1999
	α-farnesene	*Malus domestica*	Yan *et al.* 1999
	ethyl (*E*,*Z*)-2,4-decadienoate	*Pyrus communis*	Light *et al.* 2001
	(*E*,*E*)-α-farnesene, (*E*)-β-farnesene	*Malus domestica*	Coracini *et al.* 2004, Ansebo *et al.* 2004
	butyl hexanoate	*Malus domestica*	Hern and Dorn, 2004
Ectomyelois ceratoniae	acetaldehyde, etanol, ethyl hexanoate	*Phoenix dactylifera*	Cossé *et al.* 1994
Ephestia cautella	ethyl vanillin, nonanal, phenylacetaldehyde	chocolate	Olsson *et al*. 2005
Helicoverpa armigera	α-pinene, pentan-1-ol, (+)-Δ-3-carene, myrcene	*Cicer arietinum*	Rembold *et al.* 1991
	β-caryophyllene, α-humulene, α-guajene, α-muurolene, γ-muurolene, α-bulnesene	*Cajanus cajan*	Hartlieb & Rembold 1996
	(*E*)-β-caryophyllene, α-humulene, α-pinene, β-bisabolol, β-pinene, myrcene	*Gossypium hirsutum*	Jallow *et al.* 1999

Table 1. (Continued)

Helicoverpa armigera	benzaldehyde, (S)-(-)-limonene, (R,S)-(±)-linalool, (*E*)-myroxide, (*Z*)-β-ocimene, phenylacetaldehyde, (R)-(-)-piperitone	*Tagetes erecta*	Bruce & Cork 2001
Lobesia botrana	*p*-cymene, thujyl alcohol, piperitone, terpinen-4-ol, *d*-limonene, (*Z*)-verbenol, thujone	*Tanacetum vulgare*	Gabel *et al.* 1992
	β-caryophyllene, (*E*)-β-farnesene, (*E*)-4,8-dimethyl-1,3,7-nonatriene, linalool, octenol, ethyl hexanol, α-farnesene, methyl salicylate, (*Z*)-furan linalool oxide, (*E*)-furan linalool oxide	*Vitis vinifera*	Tasin *et al.* 2007
Mamestra brassicae	allyl isothiocyanate, (*E*)-2-hexenal, (*Z*)-3-hexenyl acetate, 4-pentenyl isothiocyanate, 2-phenylethyl isothiocyanate, benzyl isothiocyanate, 3-butenyl isothiocyanate	Brassicae species	Rojas 1999
	(*Z*)-3-hexenyl acetate, 1,8-cineole, α-terpinene, chrysanthenone, camphor	*Chrysanthenum* spp.	Rojas 1999
Manduca sexta	α-terpinene, methyl salicylate, (*E*)-β-ocimene, benzyl alcohol, decanal, nonanal, phenylacetaldehyde, geranyl acetona	*Lycopersicon esculentum*	Fraser *et al.* 2003
Plodia interpunctella	ethyl vanillin, nonanal, phenylacetaldehyde	chocolate	Olsson *et al.* 2005
Trichoplusia ni	phenylacetaldehyde, benzaldehyde, 2-phenylethanol, benzyl alcohol	*Abelia grandiflora*	Haynes *et al.* 1991
	benzaldehyde, benzyl acetate, phenylacetaldehyde	*Cestrum nocturnum*	Heath *et al.* 1992

In the early 70s was reported the attraction of larvae and adults, as well as the stimulation of oviposition in females by farnesene (Sutherland and Hutchins 1973, Wearing and Hutchins 1973, Sutherland *et al.* 1974), one of the most abundant compounds of apple headspace. More recently, a clear sexual dimorphism in the response of *C. pomonella* to α-farnesene in olfactometry has been found. Mated females preferred α-farnesene in comparison to solvent at low doses (63.4 and 634 ng), but they avoided α-farnesene at high dose (12,688 ng). On the other hand, males did not show preference, except for the highest dose assayed (12,688 ng), for which they showed preference in front of solvent (Hern and Dorn 1999). (*E*,*E*)-α-Farnesene has also been found to attract males in wind tunnel and field assays when combined with other volatiles, such as, (*E*)-β-farnesene or ethyl (*E*,*Z*)-2,4-decadienoate (Ansebo *et al.* 2004, Coracini *et al.* 2004).

The best results in *C. pomonella* attraction to plant volatiles have been achieved with ethyl (*E*,*Z*)-2,4-decadienoate, the pear ester, which is the only commercial kairomone of *C. pomonella*. This compound was identified in collections from ripe Bartlett pears (Light *et al.* 2001), elicits strong electroantennographic responses (Light

et al. 2001, Ansebo *et al.* 2004), and attracts individuals of both sexes in the field (Light *et al.* 2001, Knight and Light 2005, Light and Knight 2005).

A part from pear ester and (*E*,*E*)-α-farnesene there are behavioral references to other compounds. (*E*)-β-Farnesene is attractive to males in wind tunnel when combined with (*E*,*E*)-α-farnesene, and by it self in the field (Coracini *et al.* 2004). This compound also acts as a pheromone synergist in the wind tunnel (Yang *et al.* 2004). Linalool and (*Z*)-3-hexenol also increase the number of male contacts with the pheromone source in wind tunnel (Yang *et al.* 2004).

Hern and Dorn (2004) reported butyl hexanoate as a female-specific apple-derived kairomone for *C. pomonella*. This compound attracted females, both in olfactometer and wind tunnel, while no effect was observed on male behavior. Unfortunately, no data on field trapping are available for this compound.

3.4. Semiochemicals in pest control

The use of semiochemicals in pest control has favored the rationalization of pest control, and the evolution of integrated pest management strategies. Semiochemicals have simplified pest monitoring and have allowed the design of more environmentally-safe control techniques. Main uses of semiochemicals in pest control are monitoring, mass trapping, attract and kill, and mating disruption, though there are others (Howse *et al.* 1998). The most commonly used semiochemicals are the insect pheromones, especially sex pheromones (Jutsum and Gordon 1989), but a number of volatile plant kairomones have practical use as lures to attract insects for population monitoring or control (Metcalf and Metcalf 1992). There are evidences that synthetic herbivore-induced volatiles can be used for the attraction of natural enemies (James 2003).

The aims of monitoring a pest are to determine if and when the pest is present, and to decide the need and timing of the control measures. Mass trapping consists in selectively capture adults of a pest species, and reduce its population to levels below the threshold of damage. Attract and kill technique is a specific case of mass trapping, in which the trapped individuals are killed or sterilized. Control by mating disruption is achieved by the widespread application of synthetic pheromone over the crop; thereby mating encounters become disrupted (Howse *et al.* 1998).

The use of semiochemicals in *C. pomonella* control is basically narrowed to the main compound of the sex pheromone, although pear ester has been recently introduced

under some conditions. Since its description, codlemone has been gradually introduced in the management programs. Initially codlemone was only used for monitoring the populations, but later it started being used for control the pest by mating disruption (Charmillot 1990, Howell *et al.* 1992, Pfeiffer *et al.* 1993) and attract and kill (Charmillot *et al.* 2000). Nowadays, mating disruption is the most successful alternative to traditional chemical control and it is used worldwide (Calkins and Faust 2003). However, mating disruption presents several limitations, one of the most important being the population monitoring because of the loss of effectiveness of pheromone traps at catching males under mating disruption (Gut and Brunner 1996). Pear ester has been introduced in the last years in some *C. pomonella* control programs as a monitoring tool (Light *et al.* 2001, Knight and Light 2004a,b, 2005).

3.5. Detection of odors by insects

The olfactory system plays a very important role in the behavior of the large majority of insects (Hildebrand 1995, Hansson and Anton 2000). Most of the olfactory structures in insects are located on the antennae, and are functionally adapted to perceive airborne volatiles (Visser *et al.* 1986). The olfactory structures are the sensilla, and they can present a variety of morphological forms, hair-like *sensilla trichodea*, cone-like *sensilla basiconica*, sensory pits *sensilla coeloconica*, pore-plates *sensilla placodea*, and other (Metcalf and Metcalf 1992). One to several olfactory receptor neurons (ORNs) send dendrites into the lumen of the cuticular part of the sensillum. In the lumen, the outer dendritic segments are surrounded by the sensillum lymph. The cell-bodies of the ORNs are located below the base of the sensilla, embedded in the epithelium, where they are surrounded by auxiliary cells (Hansson 1995, Todd and Baker 1997, Hansson and Anton 2000). Axons of ORNs enter into the ipsilateral antennal lobe (AL) through the antennal nerve. ALs are paired sphere-shaped structures located at the base of the antennae in the deutocerebrum (Hildebrand 1995, Todd and Baker 1997, Hansson and Anton 2000), that receive information from ORNs, and are the primary olfactory centers of the insect central nervous system (Hansson and Anton 2000). Axons from antennal mechanosensory neurons bypass the AL and project to another part in the deutocerebrum (Hildebrand 1995).

When arrive to the AL, axons of ORNs terminate in glomeruli (Kaissling 1996) that are spheroidal neuropilar structures housing synaptic contacts between ORN axons

and AL interneurons (Hansson and Anton 2000). Pheromone receptor cells project their axons to the macroglomerular complex (MGC) (Hansson *et al.* 1992), whilst general odor receptor axons terminate in the ordinary glomeruli (Kaissling 1996). The MGC is formed by enlarged glomeruli and it is only found in male individuals of species using sex-pheromone communication (Hansson and Anton 2000). The MGC receives information exclusively from sex-pheromone-sensitive antennal neurons, and it is divided into subcompartiments that usually receive specifically information by just one pheromone component (Hansson *et al.* 1992, Todd and Baker 1997).

Odor molecules enter the sensilla through wall pores, and once in the sensillum lymph they interact with the odorant-binding proteins (OBPs). These proteins are lipid carriers, whose functions are to solubilize and to transport hydrophobic odorants into and through the aqueous sensillar lymph (Vogt *et al.* 1991). OBPs are secreted by the auxiliary cells in the insect sensilla, and play an essential role in the protection of odorants from the odorant degrading enzymes (ODEs), which are a biochemical diverse array of enzymes that rapidly modify or degrade the odorants. ODEs have the double functionality of eliminating odorants after their presentation to the recognition system, and protect the olfactory structures from harmful chemicals (Rützler and Zwiebel 2005).

For some time, it has been thought that the complex OBP-odorant directly interacted with the olfactory receptors (ORs) (Hildebrand 1995, Kaissling 1996), but recently it has been shown that this interaction does not always take place. Instead, other OBP receptors have been found in the neuron membrane different from ORs. In some cases, these OBP receptors may act in concert with the conventional ORs by directing OBPs to the site of specific evaluation (Rützler and Zwiebel 2005).

The olfactory receptors are membrane-bound macromolecules that are complementary in size, shape, and stereochemical configuration to the stimulating chemical and to the position, number and nature of its functional groups (Mustaparta, 1990). When odor molecules interact with receptor proteins, it results in a conformational change of the receptor macromolecule, that activates the receptor by opening ion channels that induce a depolarization of cell membranes and a chain-reaction is started, finally leading information to the brain (Metcalf and Metcalf 1992, Todd and Baker 1997). The more specialized the interaction between semiochemical and receptor, the lower the noise-level of irrelevant stimulation, and the higher the receptor sensitivity (Metcalf and Metcalf 1992).

In the case of *C. pomonella* the antennae of both sexes is filiform. The flagellum comprises approximately 65 flagellomeres, and approximately two-thirds of the circumference of each is covered by scales. On the scale-less part, sensilla of all morphological types are present at high density; on the other hand, on the scaled part are also present sensilla of all types hidden by the scales, but at lower density (Ansebo *et al.* 2005).

4. *Cydia pomonella* (L.)

4.1. Taxonomy

C. pomonella is commonly known as codling moth (Spanish, 'carpocapsa'). It is a Lepidoptera, of the Tortricidae family, subfamily Olethreutinae. The species has a long and complex taxonomic history that includes cases of synonymy and homonymy (Wearing *et al.* 2001). Amongst these synonyms and homonyms are *Phalaena pomonella*, *Phalaena pomonana*, *Pyralis pomana*, *Phalaena aeneana*, *Carpocapsa putaminana*, *Carpocapsa simpsoni*, *Enarmonia pomonella*, *Laspeyresia pomonella*, and *Carpocapsa pomonella* (De Liñán 1998, Wearing *et al.* 2001).

The species was first described by Linnaeus (1758) as *Phalaena Tinea pomonella*, and it subsequently was described by other early European authors, i.e. *Phalaena aenana* Villers and *Carpocapsa putaminana* Staudinger. Throughout the literature, the species was mainly referred as *Carpocapsa pomonella* (L.) from about 1830 to 1960, and as *Laspeyresia pomonella* (L.) from about 1960 to around 1980. Nowadays, *C. pomonella* (L.) should be the correctly used species name (Wearing *et al.* 2001).

4.2. Description

The adults are small moth showing different designs of grey mottled in their wings, with coppery markings in the wing tips. Adult wingspan is approximately 12 to 19 mm. The eggs are about 1 mm-diameter, disk-shaped and flattened. Eggs are white when laid, but following development, later a red ring appears, and finally, close to hatching, a black head can be seen. Newly hatched larvae are pinkish white with a black head. Mature larvae are about 19 mm long, more pinkish than newly hatched ones, and

they have mottled brown head. Pupae are about 1 cm long, and brown, with 10 abdominal segments that present 2 rows of tinny prickles each (Alfaro 1954, Bonnemaison 1964, García de Otazo *et al.* 1992, SIPMP 1999) (Figure 1).

4.3. Life history

Voltinism in a given area depends on the summer length and climatology. Time needed to complete a generation varies amongst authors, but is close to 600 degree-days (Pitcairn *et al.* 1991, García de Otazo *et al.* 1992, Ferreira *et al.* 1994), with lower and upper development thresholds of 10 and 31 ºC, respectively (Pitcairn *et al.* 1991, SIPMP 1999). In the coldest areas only one generation is present; whilst in the hotter ones four or five generations may occur (Chapman 1973, Audemard 1991). In the Ebro Valley Area there are three generations, partial the last one, and they peak in mid-May, Mid-July, and the end of August, respectively (Alfaro 1954, García de Otazo *et al.* 1992).

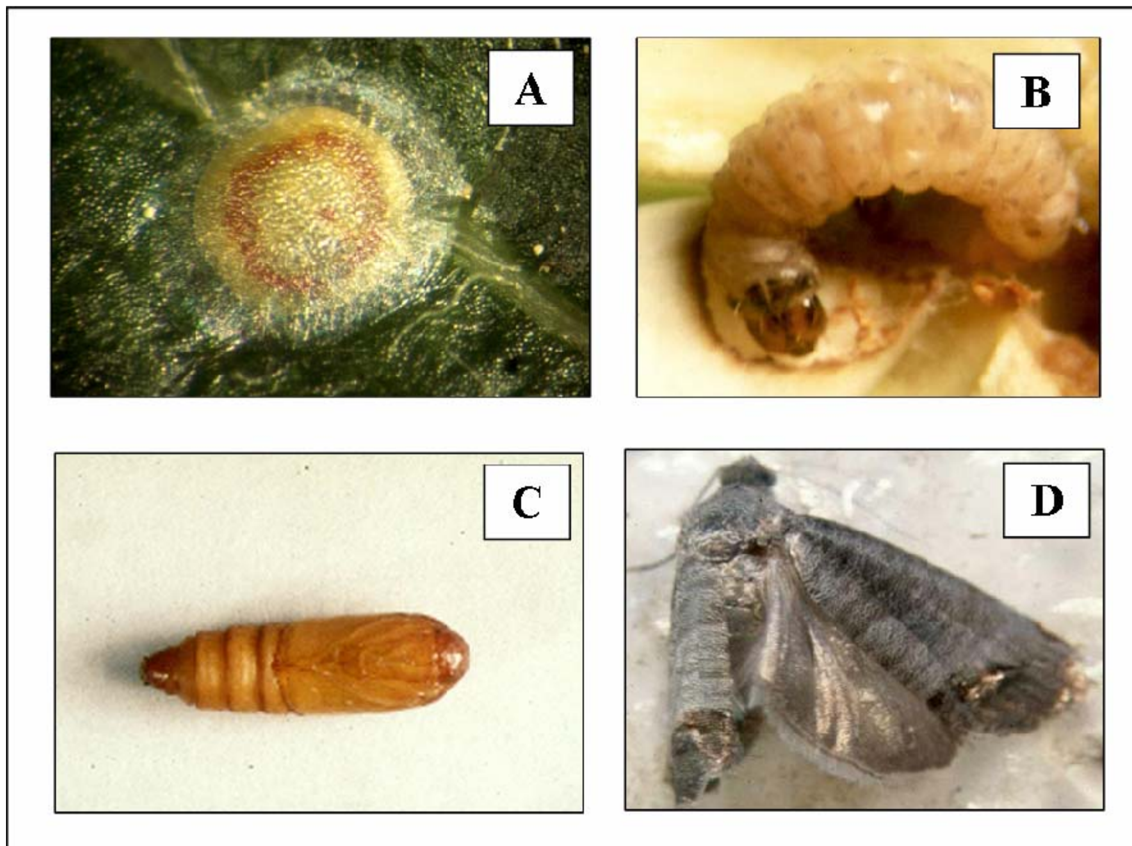

Figure 1. The codling moth, *Cydia pomonella* (L.), in different stages. A, red ringed egg; B, 5th instar larva; C, pupa; D, adult.

C. pomonella individuals overwinter as diapausing full-grown larvae, inside thick and silken cocoons, mainly located in bark clefts and apertures (Alfaro 1954, Bonnemaison 1964, SIPMP 1999). Larvae pupate in the cocoons in early spring, and shortly thereafter adults emerge, around apple flowering. That is mid-April in the Ebro Valley Area. Males live 8 to 15 and females 10 to 20 days after emergence (García de Otazo 1992). The species shows certain proterandry, and males emerge some days earlier than females (Alfaro 1954).

Adults are active for a few hours before and after twilight (Alfaro 1954, Riedl and Loher 1980, SIPMP 1999, Keil *et al.* 2001). Mating takes place at dusk when temperatures are above 15-17 ºC (Alfaro 1954, Bonnemaison 1964, García de Otazo *et al.* 1992, SIPMP 1999). Rainfall and strong wind can inhibit flight activity (Domínguez, 1989). Females lay eggs singly, or in small groups, close to fruits (Geier 1963, Blomefield *et al.* 1997), and preferably avoid pubescent surfaces (Geier 1963, Putman 1963, Jackson 1979, Hagley *et al.* 1980, Martí 2000); references to average female fecundity vary depending on the author, e.g. 20-40 eggs (Alfaro, 1954), 30-50 eggs (Bonnemaison 1964), and 30-70 eggs (SIPMP 1999). Larvae bore into the fruit within the first 24 hours after hatching, where they complete all the development (SIPMP 1999). Once larvae reach full growth they leave the fruit, either to pupate or to make the silk cocoon and overwinter on the tree bark (Alfaro 1954, SIPMP 1999). Diapause is induced primarily by a decreasing photoperiod (SIPMP 1999), and it can be induced at any larval instar, but incidence decreases as larval instar increases (Pons *et al.* 1994). Critical photoperiod for diapause induction at 25 ºC in laboratory is 15.25 h of light (Pons *et al.* 1994).

4.4. *Cydia pomonella* as a pest

C. pomonella is an oligophagous species that can attack an important number of fruit species, most of them belonging to Rosaceae family. Amongst its hosts are apple, pear, walnut, quince, apricot, plum, and some other *Prunus* species (Barnes 1991, De Liñán 1998, Wearing *et al.* 2001).

The species is thought to be of Eurasian origin, from where it widely spread around the world, along with the cultivation of apple and pears, principally in the 18th and 19th centuries (Barnes 1991). Nowadays, it occurs in most of the production areas of apple worldwide; however there are some exceptions such as Korea and Japan (Barnes

1991). The first references to the presence of *C. pomonella* in Spain are from the beginning of the 20th century (Benlloch et al. 1927).

It is a key pest in the management of pome fruits not only in Spain (Alfaro 1954, Domínguez 1989), but also worldwide (Chapman 1973, Barnes 1991, Falcon and Huber 1991, SIPMP 1999). Damage of *C. pomonella* is a consequence of larval feeding. Although the larvae can feed on leaves and bore into twigs, the most important damage is produce when they bore into fruits, making stings and deep entries (Alfaro 1954, SIPMP 1999) (Figure 2).

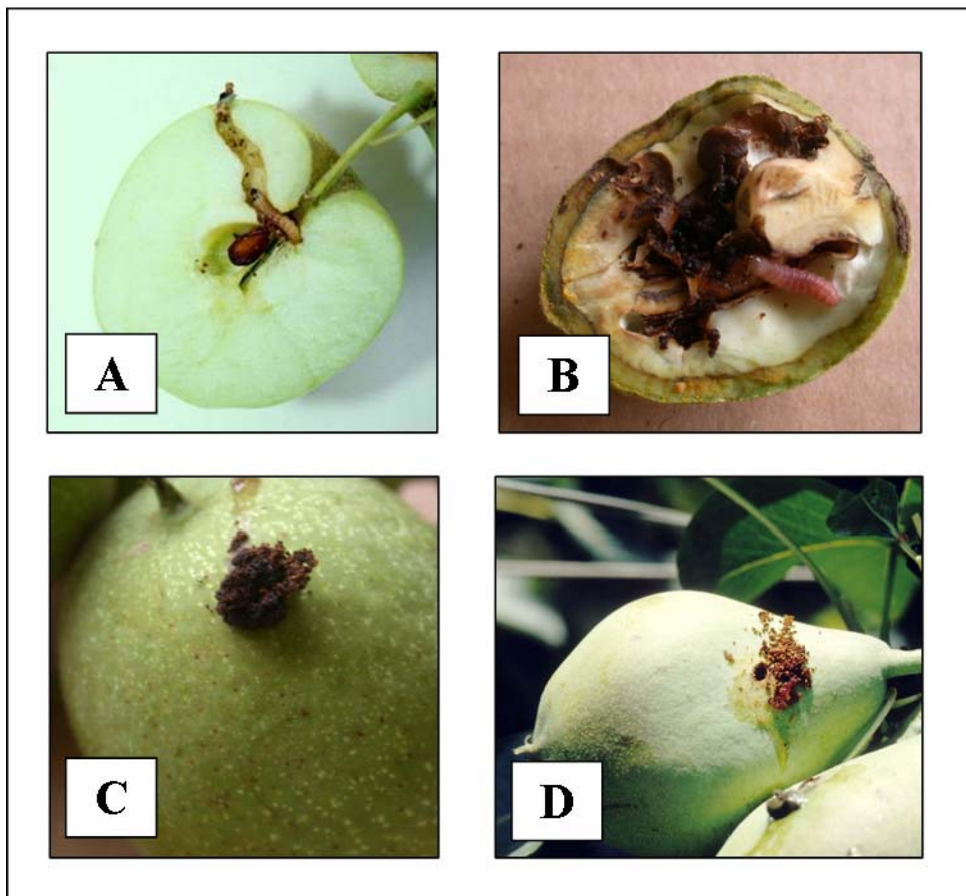

Figure 2. *Cydia pomonella* (L.) damage in different hosts. A, apple attacked fruit; B, walnut attacked fruit; C, larval entry in a walnut fruit; D, larval entry in a pear fruit.

Neonate larvae rapidly enter into a fruit (SIPMP 1999), and thereafter they are well protected against natural enemies and insecticides. The endophytic behavior of the larvae has led to a classic control based on numerous insecticide treatments, and this intensive chemical control has generated the appearance of resistant population of *C. pomonella* to several widely used insecticides, such as azinphosmethyl (Croft and Riedl

1991, Knight *et al.* 1994, Reuveny and Cohen 2004), or diflubenzuron (Charmillot *et al.* 1999). Moreover, cross resistance of populations to insecticides to which they had not been exposed have been found in some cases (Sauphanor *et al.* 1995, Dunley and Welter 2000, Reuveny and Cohen 2004).

Larvae usually feed in a single fruit, but can do it on several when they are small. Early attacked fruits fall from the tree before ripening. On the other hand, a proportion of the lately attacked fruits can stay on the tree, but they become unmarketable (Alfaro 1954, García de Otazo *et al.* 1992, Wearing *et al.* 2001). Larvae can enter the fruit through the sides, the stem end, or the calyx end (SIPMP 1999).

Although differences in susceptibility to infestation exist amongst cultivars, *C. pomonella* can cause severe damage in the absence of an appropriate management. In untreated apple orchards in limit univoltine areas, damage at harvest can be close to 15 % of fruit attacked, in areas with a second small generation it can reach around 35 %, and wherever two or more generations are present, damage level can range from 65 to 100 %. In a bivoltine area, damage may exceed 50 % in pear and walnut orchards (Barnes 1991).

4.5. Management

Classically *C. pomonella* control has been made by means of an intensive use of broad-spectrum insecticides. This kind of control leads to environmental and health problems, the appearance of resistant populations, and outbreaks of secondary pests. Nowadays, pheromone traps for monitoring are widely used, and they allow an easy pest monitoring to determine treatment need and timing. The use of more selective insecticides, such as insect growth regulators (IGR), is recommended, but organophosphates still are the most used. Control strategies must be especially careful to avoid the appearance of resistant populations to insecticides, as it has been already reported (e.g. Knight *et al.* 1994, Sauphanor and Bouvier 1995, Charmillot *et al.* 1999). In this sense it is necessary to combine insecticides with different action points, or to combine insecticide and parasite applications (Avilla *et al.* 1996, SIPMP 1999).

The most used tolerance thresholds are based on weekly male captures in traps baited with sex pheromone. These thresholds depend on the fruit species, geographical situation, and time of the season. Threshold in apple in Catalonia is 3 captures/trap/week, from petal falling to mid-June, and 2 captures/trap/week, from mid-

June to harvest. In pear in Catalonia, threshold is 5 captures/trap/week, from petal falling to the mid-June, and 3 captures/trap/week, from mid-June to harvest (Torà *et al.* 1995). When pheromone traps are not used, monitoring can be done on fruit. In this case threshold is 0.5 or 1 % of fruits presenting stings (García de Otazo *et al.* 1992, SIPMP 1999).

The most satisfactory alternative to chemical control is mating disruption. This technique is already widely used around the world (Calkins and Faust 2003), and should be the central issue in IPM programs of *C. pomonella* hosts. Despite mating disruption usually works well, it presents some limitations (Charmillot 1990), and it may need to be supplemented with insecticide sprays or parasite releases (SIPMP 1999). One of the most important limitations of mating disruption is how to carry out the population monitoring, as pheromone traps lose efficacy at trapping males (Gut and Brunner 1996).

Under an IPM context, a part from mating disruption and selective chemical insecticides, *Cydia pomonella* granulovirus (CpGV) can also be useful (Falcon and Huber 1991). CpGV is a larval entomopathogen, which was isolated in 1963 (Tanada 1964). This virus can be produce effectively under laboratory conditions, applied with the same equipment as chemical insecticides, and do not directly affects natural enemies (Falcon and Huber 1991).

A high number of natural enemies of *C. pomonella* have been reported, being the most important the parasitoid *Ascogaster quadridentata* Wesmael (Falcon and Huber 1991). However, natural enemies alone are unable to keep *C. pomonella* densities bellow the economic damage threshold (SIPMP 1999).

Few cultural techniques can help in *C. pomonella* control. Corrugated cardboard strips can be placed around tree trunk and large branches. Some of the diapausing larvae look for refuge and make their cocoon inside these strips, and removing them can help to reduce the overwintering population (Alfaro 1954). Removing attacked fruits at thinning can slightly reduce the damage level.

5. General methods

5.1. Insects

Most of the insects used in the different assays came from a *C. pomonella* colony started in 1992 from insects collected in an abandoned apple orchard in Lleida (Spain). The colony has been maintained in the Laboratory of Entomology of the Centre R+D UdL-IRTA in Lleida (Spain). The insects were reared on a semi-synthetic diet (Pons *et al.* 1994) under a 16:8 h (L:D) photoperiod at 25 ± 5 ºC.

In some specific assays, insects from another *C. pomonella* colony were used. This alternative colony was from the Chemical Ecology Department of the Swedish University of Agricultural Sciences, Alnarp, Sweden. This laboratory colony was interbred each summer with field adults from Scania (Sweden), and reared on semi-synthetic diet (Mani *et al.* 1978) under a 18:6 h (L:D) photoperiod at approximately 22-24 ºC.

5.2. Volatile collection

A dynamic headspace system similar to that described by Bäckman *et al.* (2001) was used for volatile collection (Figure 3). A 46 x 61 cm plastic oven bag (Pansaver®, M&Q Plastic Products Inc., Schuykill, USA) was placed over a tree branch and closed with a plastic clamp. A vacuum pump (NMP830 KNDC-12V, KNF Neuberger GmbH, Freiburg, Germany) pushed air through a stainless steel tube containing 1.3 g of activated charcoal (20/40 mesh, SKC Limited, Dorset, United Kingdom), into the bag at 0.5 l/min. A second vacuum pump simultaneously extracted air from the bag at 0.45 l/min through a glass trap containing 50 mg of Super-Q (80/100 mesh, Alltech associates Inc., Deerfield, USA) hold between two layers of glass wool. The temperature inside the bag was measured every 30 to 45 min by an electronic thermometer. Plastic bags were used only once to avoid contamination between samples.

Volatile collections were made in the spring and summer of 2004 over apple and walnut trees, located in Gimenells (Lleida, Spain, 41º 37' N). Apple trees belonged to a 1.1 ha Golden Smoothe orchard, and walnut trees to a 0.7 ha multivarietal orchard.

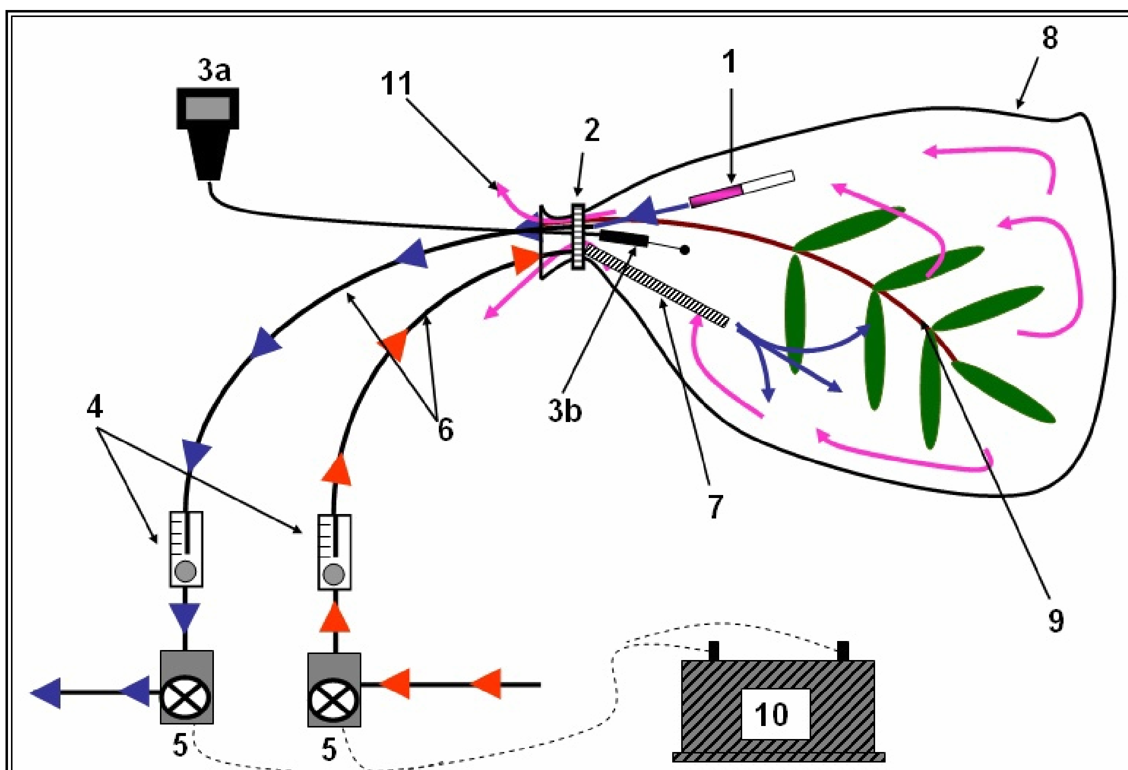

Figure 3. Scheme of the volatile collection system. (1) Super-Q glass trap, (2) plastic clamp, (3a) thermometer body and (3b) probe, (4) rotameters, (5) vacuum pumps, (6) teflon tubes, (7) activated charcoal stainless-steal fliter, (8) oven bag, (9) tree branch, (10) 12V battery, and (11) passive air flow.

Collections were performed always at 2 different times of the day over the same branch: morning (starting between 9:00 and 10:00, local time GMT+2), and dusk (beginning ca. 30 min before dusk). A minimum of two blank samples were always taken per diel 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 4 times with 100 µl of hexane to extract samples into conical-bottom vials. Fifty ng of heptyl acetate in 10 µl hexane were added as an internal standard and the vials were kept at -20 ºC until analysis. Before being reutilized traps were rinsed with approximately 2 ml of each hexane, diethyl ether and methanol. Immediately before analysis, samples were reduced under a soft stream of nitrogen to a few µl.

5.3. Electroantennographic recordings

Electroantennography (EAG) is a neurophysiological technique that allows monitoring the perception of a semiochemical by an insect (Jones and Olham 1999). In this technique the change of potential that occurs over the whole antenna following a chemical stimulus is measured, and it is thought to be the sum of all the receptor potentials elicited in all sensilla present on the antenna (Birch 1971). To carry out EAG recordings the antennal base and the antennal tip are connected to ground and a high impedance amplifier respectively (Hansson 1995). This technique was first developed by Schneider (1957a,b) to measure electrophysiological responses from antennae of male *Bombyx mori* L. to volatile compounds from its conspecific female sex pheromone and, since its invention, it has been widely used as a standard method in investigations of insect olfaction (Hansson 1995). Later the power of the EAG technique was highly increased by its combination with Gas Chromatography (GC), and Gas Chromatography-Electroantennodetection (GC-EAD) was born (Moorhouse *et al.* 1969). The use of EAG as a detector of GC effluents is a powerful analytical tool in the identification of behaviorally active compounds in complex blends (Roelofs 1977, 1984, Jones and Oldham 1999). Amongst the applications of GC-EAD are included the characterization of responses to pheromones and host odors (Bjostad 1998, Jones and Oldham 1999).

In this study, GC-EAD analyses were made on an Agilent Technologies 6890N gas chromatograph (Agilent Technologies Inc., Palo Alto, USA) coupled to an electroantennogram (Syntech, Hilversum, Holland). A column flow splitter (SGE Europe Ltd., Milton Keynes, United Kingdom) split GC effluent in two 0.32 mm ID methyl-deactivated capillary columns (SGE Europe Ltd., Milton Keynes, United Kingdom). Deactivated-columns were equal in length (ca. 30 cm), one of them led to the flame ionization detector (FID) and the other to the EAD preparation through a GC-EAD/SSR effluent interface (Syntech, Hilversum, Holland). GC-EAD interface temperature was held at 230 ºC by means of a TC-02 interface temperature controller (Syntech, Hilversum, Holland). Make-up nitrogen gas was added just before the split point to create a 30 ml/min flow into each branch. Excised antennae of 2- to 3-day-old insects were suspended between two glass capillary tubes containing 0.2 M KCl solution and gold electrodes. The electrodes were connected to a PR-05 probe (Syntech, Hilversum, Holland) which sent the signal to a computer for recording by GC-EAD

software (Syntech, Hilversum, Holland). A CS-05 stimulus controller (Syntech, Hilversum, Holland) continuously passed humidified air over the antenna at 1 l/min.

EAGs were conducted with compounds available as synthetics from reliable sources. A given test stimulus was loaded onto a piece of filter paper (20 x 5 mm), which was subsequently inserted into a Pasteur pipette. Stimuli were applied as 0.1 s air puffs which passed through the pipette and then were released into the 1 l/min humidified air stream which passed over the antenna. Puffs were generated by a CS-05 stimuli controller (Syntech, Hilversum, Holland). The quantity of each compound loaded onto filter paper amounted to 0.2 µmol, and hexyl acetate (50 µg, 0.35 µmol) was used as a standard. The pipettes were prepared a few minutes before recording. Excised antennae of 2- to 3-day-old individuals were used for EAG recordings.

5.4. Wind tunnel assays

Wind tunnel is used to assay upwind flight of insects to olfactory and visual attractants. The wind tunnel used had a flight section of 63 x 90 x 200 cm, and was diffusely illuminated from above and one of the two lateral walls white light, at approximately 20 lux. Wind speed was 30 cm/s, and air temperature ranged from 20 to 24 ºC (Witzgall *et al.* 2001).

Tests chemicals were loaded on red rubber septa (ABS, Dietikon, Switzerland) at the appropriate dosages. *C. pomonella* individuals were flown to single and combined sources, in the first 2 hours of the scotophase. Batches of 15 individuals were assayed. Individuals were flown one by one, and they were given up to 3 min to behave. The following behaviors were recorded: Activation (walking and wing-fanning), taking flight, flying upwind for 50, 100, and 150 cm towards the source, touching the source, and landing at the source.

5.5. Oviposition assays

Two different methodologies were used in oviposition assays. In the first type of oviposition assay, insects were sexed every day, and a couple (male and female) were placed inside 15 cm length x 2.5 cm diameter glass tubes. The number of eggs laid by every female at different intervals of the day was counted. This methodology was used

under semi-field conditions in two different locations, Alnarp (Southern Sweden, 55º 55' N and 13º 37' E) and Lleida (North-Eastern Spain, 41º 37' N and 0º 38' E).

In the second methodology groups of between 10 and 12 females, and 12 and 15 males were placed in mating boxes (Figure 4) on the day of their emergence. These mating boxes were cylindrical (31 cm length x 16 cm diameter), and lined with wax paper (Cut-Rite®, Reynolds®, Richmond, USA), which is an oviposition substrate suitable for *C. pomonella*. The ends of the cylinder were polyester covers lined with a pubescent adhesive non-woven fabric (Fixomull ® stretch, BSN medical GmbH & Co. KG, Hamburg, Germany), which is unsuitable for oviposition. Insects were thereafter kept in a climatic chamber at 22 ± 1 ºC under a 16:8 h (L:D) photo regime for 2 days to allow them to mate. Light intensity was of ca. 2500 lux.

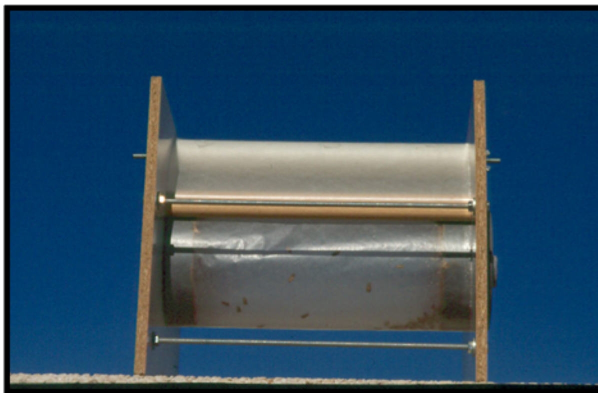

Figure 4. Mating box for rearing and bioassays.

At the onset of the third photophase the mating boxes were moved to other climatic chambers under the same photo regime, but different constant temperatures. On this third day the wax paper of the mating boxes was removed every hour from 4 hours before to 4 hours after the onset of the scotophase. The number of eggs laid during each hour was recorded. One hour before the first control, the wax paper had been removed to eliminate any eggs laid during the earlier days. After the end of the assay females were dissected to determine their mating status.

5.6. Field trapping

Field tests were conducted with Tetra traps (Arn *et al.* 1979), baited with different compounds loaded onto rubber septa. Traps were placed around 10 m apart each other, at random in a line along tree rows, and they were hung around 2 m from the ground.

Two fields were used: a 20-ha conventional managed orchard and a 6-ha pheromone-treated and insecticide-free orchard. Both were located close to Lleida (Spain). The pheromone-treated orchard was under mating disruption with 300 Checkmate CM WS dispensers/ha (Trécé, Adair, USA), containing 270 mg codlemone per dispenser.

6. References

Affek, H. P. and D. Yakir. 2002. Protection by isoprene against singlet oxygen in leaves. Plant Physiol. 129:269-277.

Agelopoulos, N. G., A. M. Hooper, S. P. Maniar, J. A. Pickett, and L. J. Wadhams. 1999. A novel approach for isolation of volatile chemicals released by individual leaves of a plant in situ. J. Chem. Ecol. 25:1411-1425.

Alfaro, A. 1954. Investigaciones Sobre la Biología de *Cydia pomonella* L. en las Condiciones de las Vegas Aragonesas y Ensayos de Tratamiento en Ella Basados. Publicaciones del Ministerio de Agricultura, Dirección General de Coordinación, Crédito y Capacitación Agraria, Madrid (Spain).

Ansebo, L., M. D. A. Coracini, M. Bengtsson, I. Liblikas, M. Ramírez, A. K. Borg-Karlson, M. Tasin, and P. Witzgall. 2004. Antennal and behavioural response of codling moth *Cydia pomonella* to plant volatiles. J. Appl. Entomol. 128:488-493.

Ansebo, L., R. Ignell, J. Löfqvist, and B. S. Hansson. 2005. Responses to sex pheromone and plant odours by olfactory receptor neurons housed in *sensilla auricillica* of the codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae). J. Insect Physiol. 51:1066-1074.

Arn, H., S. Rauscher, and A. Schmid. 1979. Sex attractant formulations and traps for the grape moth *Eupoecilia ambiguella* Hb. Mitt. Schweiz. Entomol. Ges. 52:49-55.

Arn, H., P. M. Guerin, H. R. Buser, S. Rauscher, and E. Mani. 1985. Sex pheromone blend of the codling moth, *Cydia pomonella*: evidence for a behavioral role of dodecan-1-ol. Experientia 41:1482-1484.

Audemard, H. 1991. Population dynamics of the codling moth, pp. 329-338 In L. P. S. Van Der Geest and H. H. Evenhuis [eds.], Tortricid Pests: Their Biology, Natural Enemies and Control. World Crop Pests. Elsevier, Amsterdam (Holland).

Avilla, J., M. J. Sarasúa, J. García de Otazo, R. Torà, J. Vall, and J. J. Roca. 1996. Control integrado de plagas y enfermedades en huertos de peral en Lleida. Frut. Prof. 78:69-89.

Banthorpe, D. V. 1994. Terpenoids, pp. 289-359 In J. Mann, R. S. Davidson, J. B. Hobbs, D. V. Banthorpe, and J. B. Harborne [eds.], Natural Products. Their Chemistry and Biological Significance. Addison Weskley Longman, Harlow (United Kingdom).

Barnes, M. M. 1991. Codling moth occurrence, host race formation, and damage, pp. 313-327 In L. P. S. Van Der Geest and H. H. Evenhuis [eds.], Tortricid Pests: Their Biology, Natural Enemies and Control. World Crop Pests. Elsevier, Amsterdam (Holland).

Bartell, R. J. and T. E. Bellas. 1981. Evidence for naturally occurring, secondary compounds of codling moth female sex pheromone. J. Aust. Entomol. Soc. 20:197-199.

Bäckman, A. C., M. Bengtsson, and P. Witzgall. 1997. Pheromone release by individual females of codling moth, *Cydia pomonella*. J. Chem. Ecol. 23:807-815.

Bäckman, A. C., M. Bengtsson, A. K. Borg Karlsson, I. Liblikas, and P. Witzgall. 2001. Volatiles from apple (*Malus domestica*) eliciting antennal responses in female codling moth *Cydia pomonella* (L.) (Lepidoptera: Tortricidae): effect of plant injury and sampling technique. Z. Naturforsch. C 56:262-268.

Bengtsson, M., A. C. Bäckman, I. Liblikas, M. I. Ramirez, A. K. Borg Karlson, L. Ansebo, P. Anderson, J. Löfqvist, and P. Witzgall. 2001. Plant odor analysis of apple: antennal response of codling moth females to apple volatiles during phenological development. J. Agric. Food Chem. 49:3736-3741.

Bengtsson, M., G. Jaastad, G. Knudsen, S. Kobro, A. C. Bäckman, E. Pettersson, and P. Witzgall. 2006. Plant volatiles mediate attraction to host and non-host plant in apple fruit moth, *Argyresthia conjugella*. Entomol. Exp. Appl. 118:77-85.

Benlloch, M., J. Nonell, J. Berro, and F. Gómez Clemente. 1927. Trabajos de las estaciones de fitopatología agrícola. Bol. Patol. Veget. Entomol. Agric. 2:48-60.

Berger, R. S. 1966. Isolation, identification, and synthesis of the sex attractant of the cabbage looper, *Trichoplusia ni*. Ann. Entomol. Soc. Am. 59:767-771.

Bierl, B. A., M. Beroza, and C. W. Collier. 1970. Potent sex attractant of the gypsy moth: its isolation, identification, and synthesis. Science 170:87-88.

Birch, M. C. 1971. Intrinsic limitations in the use of electroantennograms to bioassay male pheromones in Lepidoptera. Nature 233:57-58.

Bjostad, L. B. 1998. Electrophysiological methods, pp. 339-375 In J. G. Millar and K. F. Haynes [eds.], Methods in Chemical Ecology. Chapman and Hall, Norwall (USA).

Blomefield, T. L., K. L. Pringle, and A. Sadie. 1997. Field observations on oviposition of codling moth, *Cydia pomonella* (Linnaeus) (Lepidoptera: Olethreutidae), in an unsprayed apple orchard in South Africa. Afr. Entomol. 5:319-336.

Bonnemaison, L. 1964. Enemigos Naturales de las Plantas Cultivadas y Forestales. Occidente S.A., Barcelona (Spain).

Brown, W. L., Jr. 1968. An hypothesis concerning the function of the metapleural glands in ants. Am. Nat. 102:188-191.

Brown, W. L., Jr., T. Eisner, and R. H. Whittaker. 1970. Allomones and kairomones: transspecific chemical messengers. BioScience 20:21-22.

Bruce, T. J. and A. Cork. 2001. Electrophysiological and behavioral responses of female *Helicoverpa armigera* to compounds identified in flowers of african marigold, *Tagetes erecta*. J. Chem. Ecol. 27:1119-1131.

Butenandt, A., R. Beckmann, D. Stamm, and E. Hecker. 1959. Uber den sexualockstoff des seidenspinners *Bombyx mori*: reindarstelling und konstitution. Z. Naturforsch. 14b:283-284.

Butler, C. G. and E. M. Fairey. 1964. Pheromones of the honeybee: biological studies of true mandibular gland secretion of the queen. J. Apic. Res. 3:65-76.

Calkins, C. O. and R. J. Faust. 2003. Overview of areawide programs and the program for suppression of codling moth in the western USA directed by the United States Department of Agriculture - Agricultural Research Service. Pest Manag. Sci. 59:601-604.

Cardé, R. T. and T. C. Baker. 1984. Sexual communication with pheromones, pp. 355-383 In W. J. Bell and R. T. Cardé [eds.], Chemical Ecology of Insects. Chapman and Hall, London (United Kingdom).

Chapman, P. J. 1973. Bionomics of the apple-feeding Tortricidae. Annu. Rev. Entomol. 18:73-96.

Charmillot, P. J. 1990. Mating disruption technique to control codling moth in Western Switzerland, pp. 165-182 In R. L. Ridgway, R. M. Silverstein, and M. N. Inscoe [eds.], Behavior-Modifying Chemicals for Insect Management. Marcel Dekker Inc., New York (USA).

Charmillot, P. J., D. Pasquier, B. Sauphanor, J. C. Bouvier, and R. Olivier. 1999. Carpocapse des pommes: premier cas de résistance au diflubenzuron en Suisse. Rev. Suisse Vit. Arbor. Hort. 31:129-132.

Charmillot, P. J., D. Hofer, and D. Pasquier. 2000. Attract and kill: a new method for control of the codling moth *Cydia pomonella*. Entomol. Exp. Appl. 94:211-216.

Coracini, M. D. A., M. Bengtsson, and P. Witzgall. 2004. Attraction of codling moth males *Cydia pomonella* to apple volatiles. Entomol. Exp. Appl. 100:1-10.

Cossé, A. A., J. J. Endris, J. G. Millar, and T. C. Baker. 1994. Identification of volatile compounds from fungus-infected date fruit that stimulate upwind flight in female *Ectomyelois ceratoniae*. Entomol. Exp. Appl. 72:233-238.

Croft, B. A. and H. Riedl. 1991. Chemical control and resistance to insecticides of the codling moth, pp. 371-387 In L. P. S. Van Der Geest and H. H. Evenhuis [eds.], Tortricid Pests: Their Biology, Natural Enemies and Control. World Crop Pests. Elsevier, Amsterdam (Holland).

Cruz-López, L., E. A. Malo, J. Toledo, A. Virgen, A. Del Mazo, and J. C. Rojas. 2006. A new potential attractant for *Anastrepha obliqua* from *Spondias mombin* fruits. J. Chem. Ecol. 32:351-365.

De Liñán, C. 1998. Entomología Agroforestal. Agrotécnicas S.L., Madrid (Spain).

De Moraes, C. M., M. C. Mescher, and J. H. Tumlinson. 2001. Carterpillar-induced nocturnal plant volatiles repel conspecific females. Nature 410:577-580.

Dicke, M. 1999. Are herbivore-induced plant volatiles reliable indicators of herbivore identity to foraging carnivorous arthropods? Entomol. Exp. Appl. 91:131-142.

Dicke, M. and J. J. A. Van Loon. 2000. Multitrophic effects of herbivore-induced plant volatiles in an evolutionary context. Entomol. Exp. Appl. 97:237-249.

Domínguez, F. 1989. Plagas y Enfermedades de las Plantas Cultivadas. Mundi-Prensa, Madrid (Spain).

Dudareva, N., E. Pichersky, and J. Gershenzon. 2004. Biochemistry of plant volatiles. Plant Physiol. 135:1893-1902.

Dunley, J. E. and S. Welter. 2000. Correlated insecticide cross resistance in azinphosmethyl resistant codling moth (Lepidoptera: Tortricidae). J. Econ. Entomol. 93:955-962.

Ebel, R. C., J. P. Mattheis, and D. A. Buchanan. 1995. Drought stress of apple trees alters leaf emissions of volatile compounds. Physiol. Plant. 93:709-712.

Einhorn, J., F. Beauvais, M. Gallois, C. Descoins, and R. Causse. 1984. Constituants secondaires de la phéromone sexuelle du Carpocapse des Pommes, *Cydia pomonella* L. (Lepidoptera, Tortricidae). Compt. Rend. Acad. Sci. Paris 299:773-778.

Einhorn, J., P. Witzgall, H. Audemard, B. Boniface, and R. Causse. 1986. Constituants secondaires de la phéromone sexuelle du carpocapse des pommes, *Cydia pomonella* L. (Lepidoptera, Tortricidae). II. Première approche des effets comportamentaux. Compt. Rend. Acad. Sci. Paris 302:263-266.

El-Sayed, A., R. C. Unelius, I. Liblikas, J. Löfqvist, M. Bengtsson, and P. Witzgall. 1998. Effect of codlemone isomers on codling moth (Lepidoptera: Tortricidae) male attraction. Environ. Entomol. 27:1250-1254.

El-Sayed, A., M. Bengtsson, S. Rauscher, J. Löfqvist, and P. Witzgall. 1999. Multicomponent sex pheromone in codling moth (Lepidoptera: Tortricidae). Physiol. Chem. Ecol. 28:775-779.

Falcon, L. A. and J. Huber. 1991. Biological control of the codling moth, pp. 355-369 In L. P. S. Van Der Geest and H. H. Evenhuis [eds.], Tortricid Pests: Their Biology, Natural Enemies and Control. World Crop Pests. World crop pests. Elsevier, Amsterdam (Holland).

Ferreira, V. S., H. Riedl, M. J. Sarasúa, and J. Avilla. 1994. Modelización del desarrollo y mortalidad de *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) a temperatura constante. Invest. Agr. 2:79-86.

Fraser, A. M., W. L. Mechaber, and J. G. Hildebrand. 2003. Electroantennographic and behavioral responses of the sphinx moth *Manduca sexta* to host plant volatiles. J. Chem. Ecol. 29:1813-1833.

Friedman, M., P. R. Henika, and R. E. Mandrell. 2002. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. J. Food Prot. 65:1545-1560.

Gabel, B., D. Thiéry, V. Suchy, F. Marion-Poll, P. Hradsky, and P. Farkas. 1992. Floral volatiles of *Tanacetum vulgare* L. attractive to *Lobesia botrana* Den. et Schiff. females. J. Chem. Ecol. 18:693-701.

García de Otazo, J., J. Sió, R. Torà, and M. Torà. 1992. Peral. Control Integrado de Plagas y Enfermedades. Agrolatino, Barcelona (Spain).

Geier, P. W. 1963. The life history of codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), in the Australian capital territory. Aust. J. Zool. 11:323-367.

Gouinguené, S., H. Alborn, and T. C. J. Turlings. 2003. Induction of volatile emissions in maize by different larval instars of *Spodoptera littoralis*. J. Chem. Ecol. 29:145-162.

Gut, L. and J. F. Brunner. 1996. Implementing Codling Moth Mating Disruption in Washington Pome Fruit Orchards. Tree Fruit Research Extension Center Information Series [1]. Washington State University, Wenatchee (USA).

Hagley, E. A. C., J. F. Bronskill, and E. J. Ford. 1980. Effect of the physical nature of leaf and fruit surfaces on oviposition by the codling moth, Cydia pomonella (Lepidoptera: Tortricidae). Can. Entomol. 112:503-510.

Hammer, K. A., C. F. Carson, and T. V. Riley. 2003. Antifungal activity of the components of *Melaleuca alternifolia* (tea tree) oil. J. Appl. Microbiol. 95:853-860.

Hansson, B. S., H. Ljunberg, E. Hallberg, and C. Löfstedt. 1992. Functional specialization of olfactory glomeruli in a moth. Science 256:1313-1315.

Hansson, B. S. 1995. Olfaction in Lepidoptera. Experientia 51:1003-1027.

Hansson, B. S. and S. Anton. 2000. Function and morphology of the antennal lobe: new developments. Annu. Rev. Entomol. 45:203-231.

Harborne, J. B. 1994. Phenolics, pp. 361-388 In J. Mann, R. S. Davidson, J. B. Hobbs, D. V. Banthorpe, and J. B. Harborne [eds.], Natural Products. Their Chemistry and Biological Significance. Addison Weskley Longman, Harlow (United Kingdom).

Hartlieb, E. and H. Rembold. 1996. Behavioral response of female *Helicoverpa* (*Heliothis*) *armigera* HB. (Lepidoptera: Noctuidae) moths to synthetic pigeonpea (*Cajanus cajan* L.) kairomone. J. Chem. Ecol. 22:821-837.

Hatanaka, A. 1993. The biogeneration of green odour by green leaves. Phytochemistry 34:1201-1218.

Hathaway, D. O., T. P. McGovern, M. Beroza, H. R. Moffitt, L. M. McDonough, and B. A. Butt. 1974. An inhibitor of sexual attraction of male codling moths to a synthetic sex pheromone and virgin females in traps. Environ. Entomol. 3:522-524.

Haynes, K. F., J. Z. Zhao, and A. Latif. 1991. Identification of floral compounds from *Abelia grandiflora* that stimulate upwind flight in cabbage looper moths. J. Chem. Ecol. 17:637-646.

Heath, R. R., P. J. Landolt, B. Dueben, and B. Lenczewski. 1992. Identification of floral compounds of night-blooming jessamine attractive to cabbage looper moths. Environ. Entomol. 21:854-859.

Hern, A. and S. Dorn. 1999. Sexual dimorphism in the olfactory orientation of adult *Cydia pomonella* in response to α-farnesene. Entomol. Exp. Appl. 92:63-72.

Hern, A. and S. Dorn. 2001. Induced emissions of apple fruit volatiles by the codling moth: changing patterns with different time periods after infestation and different larval instars. Phytochemistry 57:409-416.

Hern, A. and S. Dorn. 2004. A female-specific attractant for the codling moth, *Cydia pomonella* (L.), from apple fruit volatiles. Naturwissenschaften 91:77-80.

Hopke, J., J. Donath, S. Blechert, and W. Boland. 1994. Herbivore-induced volatiles: the emission of acyclic homoterpenes from leaves of *Phaseolus lunatus* and *Zea mays* can be triggered by a β-glucosidase and jasmonic acid. Fed. Eur. Biochem. Soc. Lett. 352:146-150.

Howell, J. F., A. L. Knight, T. R. Unruh, D. F. Brown, J. L. Krysan, C. R. Sell, and P. A. Kirsch. 1992. Control of codling moth in apple and pear with sex pheromone-mediated mating disruption. J. Econ. Entomol. 85:918-912.

Howse, P., I. Stevens, and O. Jones. 1998. Insect Pheromones and Their Use in Pest Management. Chapman and Hall, London (United Kingdom).

Jackson, D. M. 1979. Codling moth egg distribution on unmanaged apple trees. Ann. Entomol. Soc. Am. 72:361-368.

Jallow, M. F. A., M. P. Zalucki, and G. P. Fitt. 1999. Role of chemical cues from cotton in mediating host selection and oviposition in *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). Aust. J. Entomol. 38:359-366.

James, D. G. 2003. Field evaluation of herbivore-induced plant volatiles as attractants for benefical insects: methyl salicylate and the green lacewing, *Chrysopa nigricornis*. J. Chem. Ecol. 29:1601-1609.

Jones, G. R. and N. J. Oldham. 1999. Pheromone analysis using capillary gas chromatographic techniques. J. Chromatogr. A 843:199-236.

Jurenka, R. A. 2003. Biochemistry of female moth sex pheromones, pp. 53-80 In G. Blomquist and R. Vogt [eds.], Insect Pheromone Biochemistry and Molecular Biology. Elsevier Academic Press, London (United Kingdom).

Jutsum, A. R. and R. F. S. Gordon. 1989. Insect Pheromones in Plant Protection. Biddles Ltd., Guildford (United Kingdom).

Kaissling, K. E. 1996. Peripheral mechanisms of pheromone reception in moths. Chem. Senses 21:257-268.

Karlson, P. and A. Butenandt. 1959. Pheromones (Ectohormones) in insects. Annu. Rev. Entomol. 4:39-58.

Karlson, P. and M. Luscher. 1959. "Pheromones," a new term for a class of biologically active substances. Nature 183:155-176.

Keil, S., S. Dorn, and H. Gu. 2001. Diel patterns of locomotor activity in Cydia pomonella: age and sex related differences and effects of insect hormone mimics. Physiol. Entomol. 26:306-314.

Kesselmeier, J. and M. Staudt. 1999. Biogenic volatile organic compounds (VOC): An overview on emission, physiology and ecology. J. Atmos. Chem. 33:23-88.

Kessler, A. and I. T. Baldwin. 2001. Defensive function of herbivore-induced plant volatile emissions in nature. Science 291:2141-2144.

Knight, A. L., J. F. Brunner, and D. Alston. 1994. Survey of azinphosmethyl resistance in codling moth (Lepidoptera: Tortricidae) in Washington and Utah. J. Econ. Entomol. 87:285-292.

Knight, A. L. and D. M. Light. 2004a. Use of ethyl (E,Z)-2,4-decadienoate in codling moth management: stimulation of oviposition. J. Entomol. Soc. Brit. Columbia 101:53-60.

Knight, A. L. and D. M. Light. 2004b. Use of ethyl (E,Z)-2,4-decadienoate in codling moth management: kairomone species specificity. J. Entomol. Soc. Brit. Columbia 101:61-67.

Knight, A. L., R. Hilton, and D. M. Light. 2005. Monitoring codling moth (Lepidoptera: Tortricidae) in apple with blends of ethyl (*E*,*Z*)-2,4-decadienoate and codlemone. Environ. Entomol. 34:598-603.

Larsson, M. C., M. C. Stensmyr, S. B. Bice, and B. S. Hansson. 2003. Attractiveness of fruit and flower odorants detected by olfactory receptor neurons in the fruit chafer *Pachnoda marginata*. J. Chem. Ecol. 29:1253-1268.

Law, J. H. and F. E. Regnier. 1971. Pheromones. Annu. Rev. Biochem. 40:533-548.

Light, D. M., A. L. Knight, C. A. Henrick, D. Rajapaska, B. Lingren, J. C. Dickens, K. M. Reynolds, R. G. Buttery, G. Merrill, J. Roitman, and B. C. Campbell. 2001. A pear-derived kairomone with pheromonal potency that attracts male and female codling moth, *Cydia pomonella* (L.). Naturwissenschaften 88:333-338.

Light, D. M. and A. L. Knight. 2005. Specificity of codling moth (Lepidoptera: Tortricidae) for the host plant kairomone, ethyl (2E,4Z)-2,4-decadienoate: field bioassays with pome fruit volatiles, analogue, and isomeric compounds. J. Agric. Food Chem. 53:4046-4053.

Linnaeus, C. 1758. Systema Naturae. Laurentii Salvii, Stockholm (Sweden).

Llusià, J. and J. Peñuelas. 2001. Emission of volatile organic compounds by apple trees under spider mite attack and attraction of predatory mites. Exp. Appl. Acar. 25:65-77.

Loreto, F. and V. Velikova. 2001. Isoprene produced by leaves protects the photosynthetic apparatus against ozone damage, quenches ozone products, and reduces lipid peroxidation of cellular membranes. Plant Physiol. 127:1781-1787.

Loreto, F., P. Pinelli, F. Manes, and H. Kollist. 2004. Impact of ozone on monoterpene emissions and evidence for an isoprene-like antioxidant action of monoterpenes emitted by *Quercus ilex* leaves. Tree Physiol. 24:361-367.

Löfstedt, C. and G. Odham. 1984. Analysis of moth pheromone acetates by selected ion monitoring using electron impact and chemical ionization mass spectrometry. Biomed. Mass Spectrom. 11:106-113.

Löfstedt, C., B. S. Lanne, J. Löfqvist, M. Appelgren, and G. Bergstrom. 1985. Individual variation in the pheromone of the turnip moth *Agrotis segetum*. J. Chem. Ecol. 11:1181-1196.

Löfstedt, C., C. E. J. Linn, and J. Löfqvist. 1985. Behavioural response of turnip moth males *Agrotis segetum* to sex pheromone in a flight tunnel and in the field. J. Chem. Ecol. 11:1209-1222.

Löfstedt, C., W. M. Herrebout, and S. Menken. 1991. Sex pheromones and their potential role in the reproductive isolation in small ermine moths (Yponomeutidae). Chemoecology 2:20-28.

Mani, E., W. Riggenbach, and M. Mendik. 1978. Zucht des apfelwicklers (*Laspeyresia pomonella* L.) auf künstlichem nährboden, 1968-1978. Mitt. Schweiz. Entomol. Ges. 51:315-326.

MAPA (Ministerio de Agricultura, Pesca y Alimentación). 2004. Anuario de Estadísitica Agroalimentaria. http://www.mapa.es/es/estadistica/pags/anuario/Anu_04/indice.asp.

Martí, S. 2000. Host Selection, Reproductive Biology, Host-Specific Development and Mortality of the Codling Moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) in Apple and Pear. PhD Thesis. Oregon State University, Corvallis (USA).

Metcalf, R. L. and E. R. Metcalf. 1992. Plant Kairomones in Insect Ecology and Control. Chapman and Hall, New York (USA).

Moorhouse, J. E., R. Yeadon, P. S. Beevor, and B. F. Nesbitt. 1969. Method for use in studies of insect chemical communication. Nature 223:1174-1175.

Mustaparta, H. 1990. Chemical information processing in the olfactory system of insects. Physiol. Rev. 70:199-245.

Natale, D., L. Mattiacci, E. Pasqualini, and S. Dorn. 2004. Apple and peach fruit volatiles and the apple constituent butyl hexanoate attract female oriental fruit moth, *Cydia molesta*, in the laboratory. J. Appl. Entomol. 128:22-27.

Niinemets, U. and M. Reichstein. 2003. Controls on the emission of plant volatiles through stomata: Differential sensitivity of emission rates to stomatal closure explained. J. Geophys. Res. 108, 4208, DOI: 10.1029/2002JD002620.

Niinemets, U., F. Loreto, and M. Reichstein. 2004. Physiological and physicochemical controls on foliar volatile organic compound emissions. Trends Plant Sci. 9:180-186.

Nojima, S., C. Linn, Jr., and W. Roelofs. 2003. Identification of host fruit volatiles from flowering dogwood (*Cornus florida*) attractive to dogwood-origin *Rhagoletis pomonella* flies. J. Chem. Ecol. 29:2347-2357.

Nojima, S., C. Linn, Jr., B. Morris, A. Zhang, and W. Roelofs. 2003. Identification of host fruit volatiles from hawthorn (*Crataegus* spp.) attractive to hawthorn-origin *Rhagoletis pomonella* flies. J. Chem. Ecol. 29:321-336.

Nordlund, D. A. and W. J. Lewis. 1976. Terminology of chemical releasing stimuli in intraspecific and interspecific interactions. J. Chem. Ecol. 2:211-220.

Olsson, P. O. C., O. Anderbrant, C. Löfstedt, A. K. Borg-Karlson, and I. Liblikas. 2005. Electrophysiological and behavioral responses to chocolate volatiles in both sexes of the pyralid moths *Ephestia cautella* and *Plodia interpunctella*. J. Chem. Ecol. 31:2947-2961.

Paré, P. W. and J. H. Tumlinson. 1997a. De novo biosynthesis of volatiles induced by insect herbivory in cotton plants. Plant Physiol. 114:1161-1167.

Paré, P. W. and J. H. Tumlinson. 1997b. Induced synthesis of plant volatiles. Nature 385:30-31.

Paré, P. W. and J. H. Tumlinson. 1999. Plant volatiles as a defense against insect herbivores. Plant Physiol. 121:325-331.

Peñuelas, J. and J. Llusià. 2002. Linking photorespiration, monoterpenes and thermotolerance in *Quercus*. New Phytol. 155:227-237.

Peñuelas, J. and J. Llusià. 2003. BVOCs: plant defense against climatic warming? Trends Plant Sci. 8:105-109.

Peñuelas, J. and J. Llusià. 2004. Plant VOC emissions: making use of the unavoidable. Trends Ecol. Evol. 19:402-404.

Pfeiffer, D. G., W. Kaakeh, J. C. Killian, M. W. Lachance, and P. Kirsch. 1993. Mating disruption for control of damage by codling moth in Virginia apple orchards. Entomol. Exp. Appl. 67:57-64.

Pichersky, E. and J. Gershenzon. 2002. The formation and function of plant volatiles: perfumes for pollinator attraction and defense. Curr. Opin. Plant Biol. 5:237-243.

Pitcairn, M. J., C. Pickel, L. A. Falcon, and F. G. Zalom. 1991. Development and survivorship of *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) at ten constant temperatures. Pan-Pac. Entomol. 67:189-194.

Pons, S., M. Eizaguirre, M. J. Sarasúa, and J. Avilla. 1994. Influencia del fotoperiodo sobre la inducción de diapausa de *Cydia pomonella* (Lepidoptera: Tortricidae) en laboratorio y campo. Invest. Agr. Prod. Prot. Veg. 9:477-491.

Putman, W. L. 1963. The codling moth, *Carpocapsa pomonella* (L.) (Lepidoptera: Tortricidae): a review with special reference to Ontario. Proc. Entomol. Soc. Ontario 93:22-60.

Rapparini, F., R. Baraldi, and O. Facini. 2001. Seasonal variation of monoterpene emission from *Malus domestica* and *Prunus avium*. Phytochemistry 57:681-687.

Reinecke, A., J. Ruther, T. Tolasch, W. Francke, and M. Hilker. 2002. Alcoholism in cockchafers: orientation of male *Melolontha melolontha* towards green leaf alcohols. Naturwissenschaften 89:265-269.

Reinhard, J., M. V. Srivivasan, and S. Zhang. 2004. Scent-triggered navigation in honeybees. Nature 427:411.

Rembold, H., A. C. Köhne, and A. Schroth. 1991. Behavioral response of *Heliothis armigera* Hb. (Lep., Noctuidae) moths on a synthetic chickpea (*Cicer arietinum* L.) kairomone. J. Appl. Entomol. 112:254-262.

Reuveny, H. and E. Cohen. 2004. Resistance of the codling moth *Cydia pomonella* (L.) (Lep., Tortricidae) to pesticides in Israel. J. Appl. Entomol. 128:645-651.

Riedl, H. and W. Loher. 1980. Circadian control of oviposition in the codling moth, Laspeyresia pomonella, Lepidoptera: Olethreutidae. Entomol. Exp. Appl. 27:38-49.

Roelofs, W., A. Comeau, A. Hill, and G. Milicevic. 1971. Sex attractant of the codling moth: characterization with electroantennogram technique. Science 174:297-299.

Roelofs, W., R. J. Bartell, A. S. Hill, R. T. Cardé, and L. H. Waters. 1972. Codling moth sex attractant-field trials with geometrical isomers. J. Econ. Entomol. 65:1276-1277.

Roelofs, W. 1977. The scope and limitations of the electroantennogram thecnique in identifying pheromone components, pp. 147-165 In N. R. Mc Farlane [ed.], Crop Protection Agents. Academic Press Inc. Ltd., London (United Kingdom).

Roelofs, W. 1981. Attractive and aggregating pheromones, pp. 215-235 In D. A. Nordlund, R. L. Jones, and W. J. Lewis [eds.], Semiochemicals. Their Role in Pest Control. John Wiley & Sons Inc., New York (USA).

Roelofs, W. L. and R. L. Brown. 1982. Pheromones and evolutionary relationships of Tortricidae. Annu. Rev. Ecol. Evol. Syst. 13:395-422.

Roelofs, W. 1984. Electroantennogram assays: rapid and convenient screening procedures for pheromones, pp. 131-159 In H. E. Hummel and T. A. Miller [eds.], Techniques in Pheromone Research. Springer-Verlag, New York (USA).

Rojas, J. C. 1999. Electrophysiological and behavioral responses of the cabbage moth to plant volatiles. J. Chem. Ecol. 25:1867-1883.

Rosenstiel, T. N., A. L. Ebbets, W. C. Khatri, R. Fall, and R. K. Monson. 2004. Induction of poplar leaf reductase: a test of extrachloroplastic control of isoprene emission rate. Plant Biol. 6:12-21.

Ruther, J., T. Meiners, and J. L. M. Steidle. 2002. Rich in phenomena-lacking in terms. A classification of kairomones. Chemoecology 12:161-167.

Ruther, J., A. Reinecke, and M. Hilker. 2002. Plant volatiles in the sexual communication of *Melolontha hippocastani*: response towards time-dependent bouquets and novel function of (Z)-3-hexen-1-ol as a sexual kairomone. Ecol. Entomol. 27:76-83.

Sauphanor, B. and J. C. Bouvier. 1995. Cross-resistance between benzoylureas and benzoylhydrazines in the codling moth, *Cydia pomonella* L. Pest. Sci. 45:369-375.

Schneider, D. 1957a. Elektrophysiologische untersuchungen von chemo- und mechanorezeptoren der antenne des seidenspinners *Bombyx mori* L. Z. Vergl. Physiol. 40:8-41.

Schneider, D. 1957b. Electrophysiological investigation on the antennal receptors of the silk moth during chemical and mechanical stimulation. Experientia 13:89-91.

Schuh, G., A. C. Heiden, T. Hoffmann, J. Kahl, P. Rockel, J. Rudolph, and J. Wildt. 1997. Emissions of volatile organic compounds from sunflower and beech: Dependence on temperature and light intensity. J. Atmos. Chem. 27:291-318.

Scutareanu, P., J. Bruin, M. A. Posthumus, and B. Drukker. 2003. Constitutive and herbivore-induced volatiles in pear, alder and hawthorn trees. Chemoecology 13:63-74.

Sharkey, T. D. and E. L. Singsaas. 1995. Why plants emit isoprene. Nature 374:769.

Sharkey, T. D., X. Y. Chen, and S. Yeh. 2001. Isoprene increases thermotolerance of fosmidomycin-fed leaves. Plant Physiol. 125:2001-2006.

SIPMP (Statewide Integrated Pest Manahement Project). 1999. Integrated pest management for apples and pears. University of California, Oakland (USA).

Staudt, M., N. Bertin, U. Hansen, G. Seufert, P. Ciccioli, P. Foster, B. Frenzel, and L. Fugit. 1997. Seasonal and diurnal patterns of monoterpene emissions from *Pinus pinea* (L.) under field conditions. Atmos. Environ. 31:145-156.

Staudt, M. and N. Bertin. 1998. Light and temperature dependence of the emission of cyclic and acyclic monoterpenes from holm oak (*Quercus ilex* L.) leaves. Plant Cell Environ. 21:385-395.

Sutherland, O. R. W. and R. F. N. Hutchins. 1973. Attraction of newly hatched codling moth larvae (*Laspeyresia pomonella*) to synthetic stereo-isomers of farnesene. J. Insect Physiol. 19:723-727.

Sutherland, O. R. W., R. F. N. Hutchins, and C. H. Wearing. 1974. The role of the hydrocarbon α-farnesene in the behaviour of codling moth larvae and adults, pp. 249-263 In L. Barton Brown [ed.], Experimental Analysis of Insect Behaviour. Springer-Verlag, Berlin (Germany).

Tanada, Y. 1964. A granulosis virus of the codling moth *Carpocapsa pomonella* (L.). Insect Pathol. 6:378-380.

Tarvainen, V., H. Hakola, H. Hellen, J. Back, P. Hari, and M. Kulmala. 2005. Temperature and light dependence of the VOC emissions of Scots pine. Atmos. Chem. Phys. 5:989-998.

Tasin, M., A. C. Bäckman, M. Coracini, D. Casado, C. Ioratti, and P. Witzgall. 2007. Synergism and redundancy in a plant volatile blend attracting grapevine moth females. Phytochemistry 68:203-209.

Theis, N. and M. Lerdau. 2003. The evolution of function in plant secondary metabolites. Int. J. Plant Sci. 164:S93-S102.

Todd, J. L. and T. C. Baker. 1997. The cutting edge of insect olfaction. Am. Entomol. 43:174-182.

Torà, R., J. Sió, M. J. Sarasúa, and J. Avilla. 1995. Control integrado de plagas en huertos de manzano y de peral en Cataluña. Frut. Prof. 70:36-51.

Tumlinson, J. H., C. E. Yonce, R. E. Doolittle, R. R. Heath, C. R. Gentry, and E. R. Mitchell. 1974. Sex pheromones and reproductive isolation of the lesser peachtree borer and peachtree borer. Science 185:614-616.

Vallat, A., H. Gu, and S. Dorn. 2005. How rainfall, relative humidity and temperature influence volatile emissions from apple trees in situ. Phytochemistry 66:1540-1550.

Vancanneyt, G., C. Sanz, T. Farmaki, M. Peneque, F. Ortego, P. Castañera, and J. J. Sánchez-Serrano. 2001. Hydroperoxide lyase depletion in transgenic potato plants leads to an increase in aphid performance. Proc. Natl. Acad. Sci. USA 98:8139-8144.

Visser, J. H. 1986. Host odor perception in phytophagous insects. Annu. Rev. Entomol. 31:121-144.

Vogt, R. G., R. Rybczynski, and M. R. Lerner. 1991. Molecular cloning and sequencing of general odorant-binding proteins GOBP1 and GOBP2 from the tobacco hawk moth *Manduca sexta*: comparisons with other insect OBPs and their signal peptides. J. Neurosci. 11:2972-2984.

Wearing, C. H. and R. F. N. Hutchins. 1973. α-Farnesene, a naturally occurring oviposition stimulant for the codling moth, *Laspeyresia pomonella*. J. Insect Physiol. 19:1251-1256.

Wearing, C. H., J. D. Hansen, C. Whyte, C. E. Miller, and J. Brown. 2001. The potential for spread of codling moth (Lepidoptera: Tortricidae) via commercial sweet cherry fruit: a critical review and risk assessment. Crop Prot. 20:465-488.

Whittaker, R. H. 1970a. The biochemical ecology of higher plants, pp. 43-70 In E. Sondheimer and J. B. Simeone [eds.], Chemical Ecology. Academic Press, New York (USA).

Whittaker, R. H. 1970b. Communities and Ecosystems. Macmillan, New York (USA).

Witzgall, P., M. Bengtsson, S. Rauscher, I. Liblikas, A. C. Bäckman, M. Coracini, P. Anderson, and J. Löfqvist. 2001. Identification of further sex pheromone synergists in the codling moth, *Cydia pomonella*. Entomol. Exp. Appl. 101:131-141.

Yan, F., M. Bengtsson, and P. Witzgall. 1999. Behavioral response of female codling moths, *Cydia pomonella*, to apple volatiles. J. Chem. Ecol. 25:1343-1351.

Yang, Z., M. Bengtsson, and P. Witzgall. 2004. Host plant volatiles synergize response to sex pheromone in codling moth, *Cydia pomonella*. J. Chem. Ecol. 30:619-629.

Zhang, A., C. Linn, Jr., S. Wright, R. Prokopy, W. Reissig, and W. Roelofs. 1999. Identification of a new blend of apple volatiles attractive to the apple maggot, *Rhagoletis pomonella*. J. Chem. Ecol. 25:1221-1232.

CHAPTER II

GENERAL OBJECTIVES

The general objectives of the present PhD thesis were:

- To know the volatile emissions of organic compounds by two *Cydia pomonella* (L.) hosts, apple and walnut. To compare the emissions of these two hosts between them and between morning and dusk, and to establish which compounds have electroantennographic activity and may have behavioral effects on the pest.

- To know the behavioral effects of ethyl (*E*,*Z*)-2,4-decadienoate (pear ester), the only commercial kairomone for *C. pomonella*, and other host volatiles on the pest, in order to improve the understanding of how they interact with sex pheromone and environment, for a future better interpretation of field captures under different conditions.

- To increase the knowledge about how temperature and light intensity influence *C. pomonella* oviposition, and to improve the conditions for future bioassays in female response to plant volatiles.

CHAPTER III

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

D. CASADO,[1] C. GEMENO, J. AVILLA AND M. RIBA

Àrea de Protecció de Conreus, Centre R+D UdL-IRTA, Av. Alcalde Rovira Roure 191, E-25198 Lleida, Spain

Environ. Entomol. 35(2): 258–267 (2006)

ABSTRACT Volatile compounds from apple trees (variety Golden Smothee) were collected in the field from attached apple branches enclosed in plastic bags in the morning and at dusk and during three growth periods (after petal fall [APF], immature fruit [IF], and close-to-full ripening [CFR]). Collections were analyzed by gas chromatography–mass spectrometry (GC-MS) and gas chromatography–electroantennographic 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*)-α-farnesene, hexyl acetate, 4,8-dimethyl-1,3,7-nonatriene, hexyl hexanoate, and germacrene D. Most of the compounds that showed significant differences between periods were emitted in greater amounts in the APF and/or IF periods than in the CFR period. (*E*)-β-caryophyllene and an unidentified compound were significantly more abundant during the day, whereas 2-hexanone, octanal, and (*Z*)-3-hexenol were significantly more abundant at dusk. GC-EAD responses were very weak and significantly higher than background noise only to hexyl acetate, 4,8-dimethyl-1,3,7-nonatriene, nonanal, (*Z*)-3-hexenol, hexyl butanoate, and (*E*,*E*)-α-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 identified. Males showed a response equal to or higher than females to all compounds except β-myrcene.

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

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, first 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 Brunner 1996), 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-specific and bisexual attractant, which is the only commercial kairomone for *C. pomonella.* The pear ester was discovered by testing compounds emitted by ripe Bartlett pears (Light et al. 2001). The efficacy of the pear ester in the field 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

[1] Corresponding author, e-mail: daniel.casado@irta.es.

0046-225X/06/0258–0267$04.00/0 © 2006 Entomological Society of America

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 (Paré and Tumlinson 1997, 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 (Bäckman et al. 2001), but surprisingly, only (*E,E*)-α-farnesene, (*E*)-β-farnesene, and (*E*)-β-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 ± 5°C. 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) and supplied with water until used. Test males 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. β-farnesene (95%) was purchased from Chemos (Regenstauf, Germany). (*Z*)-3-hexenol (98%), methyl salicylate (≥99%), (−)-(*E*)-β-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 Química (Madrid, Spain). Octanal (99%), nonanal (95%), and decanal (95%) were purchased from Acros Organics (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 Trécé (Adair, OK). Farnesene (racemic) was bought from TCI (Tokyo, Japan). Hexyl acetate, butyl hexanoate, hexyl hexanoate, hexyl butanoate, (*Z*)-3-hexenyl hexanoate, and heptyl acetate were synthesized (yields >70% after distilling) following the method of Eras et al. (2002), and all had purities >95% after purification.

Volatile Collections. 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, 41°37′ N). A dynamic headspace system similar to that described by Bäckman 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, United Kingdom), into the bag at 0.5 ml/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 GMT+2), 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 μl of hexane to extract samples into conical-bottom vials. Fifty nanograms of heptyl acetate in 10 μl hexane were added as an internal standard, and the vials were kept at −20°C 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 μl.

The temperature inside the bag was measured every 30–45 min by an electronic thermometer. Average temperatures per sample ranged from 19.6 to 25.2°C (APF-morning), 15 to 21.8°C (APF-dusk), 23.8 to

30.4°C (IF-morning), 21 to 29°C (IF-dusk), 21.2 to 29°C (CFR-morning), and 20.2 to 27.3°C (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 μm) capillary column (J&W Scientific, Folsom, CA). The injector temperature was 250°C, and the split ratio was 1:5. The oven temperature started at 50°C and was maintained for 2 min, increasing at 5°C/min to 150°C, held for 5 min, increased at 10°C/min to 230°C, and finally was kept at 230°C for 10 min. The carrier gas was helium at a constant flow 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 230°C, respectively.

The samples were analyzed by GC-MS software (MSD-ChemStation version D.00.01; Agilent Technologies), spectra were compared with the available library (NIST library 75K), and identification was confirmed by injection of synthetic compounds when possible. Four to six volatile collections and at least one blank sample per day time and season period were 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 significant differences existed, a Duncan's multiple range means separation test was performed.

Gas Chromatographic–Electroantennographic Detection. Gas chromatographic–electroantennographic detection (GC-EAD) analyses were made on an Agilent Technologies 6890N gas chromatograph coupled to an electroantennogram (EAG; Syntech, Hilversum, Holland). A column flow splitter (SGE Europe, Milton Keynes, United Kingdom) split GC effluent 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 flame ionization detector (FID) and the other to the EAD preparation through a GC-EAD/single sensillum recording effluent interface (Syntech). GC-EAD interface temperature was held at 230°C 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 flow into each branch. Excised antennae of 2- to 3-d-old males were suspended between two glass capillary 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 humidified 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 after the end of the run, the antennae were challenged with 1-μg puffs of sex pheromone to check their responsiveness. Three to four volatile collections per diel and seasonal period were analyzed by GC-EAD.

EAG Recordings with Synthetic Compounds. EAGs were conducted with those compounds identified that 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 filter paper (20 by 5 mm), which was subsequently inserted into a Pasteur pipette. Stimuli were applied as 0.1-s air puffs that passed through the pipette and were released into a 1-liter/min humidified air stream that passed over the antenna. Puffs were generated by a CS-05 stimuli controller (Syntech). The quantity of each compound loaded onto filter paper amounted to 0.2 μmol (between 16.8 and 45.7 μg depending on the compound). Hexyl acetate (50 μg, 0.35 μmol) was used as a standard. In a previous study, we established a dose–response relationship to this compound between 0.1 and 1,000 μg with a saturation response of 3.4 mV (unpublished data). The pipettes were prepared a few minutes before recording. Excised antennae of 2- to 3-d-old males and virgin and mated females were stimulated with 12 puffs, 30–40 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 compound never had more than one replicate over the same antenna, and 10–12 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 $\log(x + 1)$ before ANOVA and Duncan's multiple range means 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 identified and therefore are listed as "unidentified 1–10." Unidentified compounds 2–10 are sesquiterpenes, with average

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*)-β-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
Unidentified 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
β-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
Unidentified 3	28.3 ± 3.9 a		3.0 ± 1.7 b
Unidentified 4	24.3 ± 3.4	8.2	
(*E*)-β-caryophyllene	56.6 ± 8.6 a	10.6 ± 3.5 b	3.6 ± 2.0 c
Unidentified 5	7.8 ± 1.3		
Unidentified 6	7.1 ± 1.5		
(*Z*)-3-hexenyl hexanoate	51.8 ± 32.8 a	5.7 ± 4.8 b	2.7 ± 1.6 b
Unidentified 7	7.6 ± 1.0		
(*E*)-β-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
Unidentified 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 confirmed by comparison with synthetic compounds are bold.
Values in the same row with different letters differed significantly ($\alpha = 0.05$).
[a] Mean percentages of morning and dusk samples relative to the IS area (50 ng heptyl acetate).
[b] Compounds with a significant time of the day by phenological period interaction.

retention times of 15.54, 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 71.7–253.9% IS, and (*E,E*)-α-farnesene, 71.7–618.9% IS (Table 1). Other compounds found in considerable amounts were hexyl acetate (9.1–304.5% IS), 4,8-dimethyl-1,3,7-nonatriene (26.7–200.9% IS), hexyl hexanoate (5.6–149.3% IS), and germacrene D (5.3–214.9% IS). Several compounds are reported for the first time from apple plants, to our knowledge. These include 2-hexanone, 2-methyl-6-methylene-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 significant 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 advanced can be observed (Table 1). Most of the emitted compounds were detected in all the studied phenological periods (Table 1). Exceptions were 2-hexanone and 1-octen-3-ol absent on APF period; unidentified 3 absent on IF; 3-carene, β-bourbonene, and unidentified 4 absent on CFR; unidentified 5 and 6 only detected on APF; and 2-methyl-6-methylene-1,7-octadien-3-one only present on IF. Significant differences were found among the studied periods for many of the compounds detected in all the treatments (Table 2). Most of the compounds that showed significant 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 esterification during fruit ripening. Perhaps this process can also explain that several esters (hexyl acetate, hexyl butanoate, and hexyl hexanoate) tended to be more abundant in the CFR period (Table 1), although significant differences were only found for hexyl acetate (Table 2). (*E,E*)-α-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 significant differences between periods were found (df = 2, $F = 0.88$, $P = 0.14$; Table 1).

No significant 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*)-β-caryophyllene (df = 1, $F = 8.54$, $P = 0.01$) and unidentified compound 7 (df = 1, $F = 7.84$, $P = 0.03$) were emitted in greater amounts in the morning, hexyl 2-methylbutanoate was found in some of the morning collections, but never at dusk, and 2-hexanone (df = 1, $F = 788.6$, $P = 0.001$),

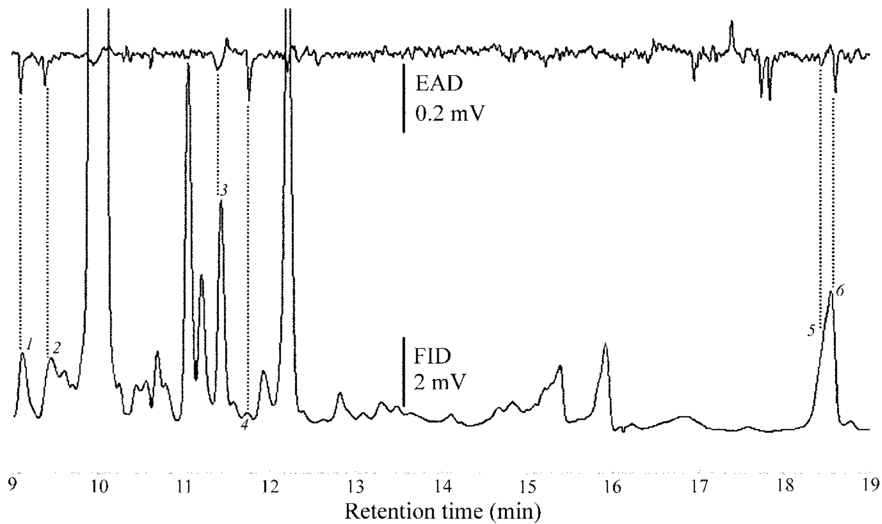

Fig. 1. GC-FID (bottom) and GC-EAD (top) traces of a volatile collection from an apple tree (variety Golden Smothee) done in the CFR period at dusk and using the antenna of a male *C. pomonella*. Peaks of compounds that produced discernible EAD responses are labeled. (1) hexyl acetate, (2) 4,8-dimethyl-1,3,7-nonatriene,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

octanal (df = 1, $F = 4.37$, $P = 0.05$), and (Z)-3-hexenol (df = 1, $F = 5.69$, $P = 0.03$) were more abundant at dusk. Variation in the emission profile of plant volatiles 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 Bäckman et al. (2001), who identified lower amounts of all volatiles at dusk than in the photophase with (*E*)-β-caryophyllene, (*E*)-β-farnesene, and (*E,E*)-α-farnesene—the only volatiles detected during the scotophase. The disagreement could result from differences in temperature during collections between the two studies. Bäckman et al. (2001) registered temperatures from 12.2 to 18°C, 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 40°C, 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 (Gouinguené 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, hexyl butanoate, (*E,E*)-α-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-cyclopentylcyclopentanone were interesting in that this compound is emitted 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 compounds that were present in our samples and had been described previously as GC-EAD–responsive (Bäckman et al. 2001, Bengtsson et al. 2001), such as linalool, (*E*)-β-caryophyllene, or (*E*)-β-farnesene. The lack of responsiveness to these compounds could be caused by their small concentration in our samples. To test the 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 and Barnes (1975) found that wild populations coming 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* wild populations from pear (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 could also explain differences in field trapping efficacy 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*)-α-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 acetate has been reported as a repellent to the females in olfactometer assays but ineffective in wind tunnel (Hern and Dorn 2004), (Z)-3-hexenol has been shown 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

Compounds	Males		Compounds	Females	
	Percent STD ± SE[a]	Duncan groups[b]		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 μg hexyl acetate.
[b] Duncan's multiple range separation of means of the transformed log(x + 1) variable, $\alpha = 0.05$. Differences among compounds within each sex.
[c] Compounds with significant differences in response between sexes, $\alpha = 0.05$.

atriene is frequently found in volatile emissions from insect-attacked plants (Scutareanu et al. 1997, De Boer et al. 2004).

EAG Recordings with Synthetic Compounds. Mean EAG responses after hexane response subtraction ranged from 0.3 to 5.6 mV, depending on insect sex and 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 ($P = 0.02$), butyl hexanoate ($P = 0.03$), and β-myrcene ($P < 0.001$) (Table 3). In all cases except β-myrcene, the response of the male antenna was larger than the response of the female antenna. The responses to β-myrcene were small compared with the others—57.2% and 34.3% of the standard in females and males, respectively. Consequently, this compound would be difficult 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 difficult. 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, com-

parative assays between sexes with synthetics should be made.

Most of the compounds tested generated EAG responses that did not differ significantly 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*)-β-caryophyllene, β-pinene, and β-myrcene], five 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*)-β-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 first report of nonanal and decanal eliciting antennal responses in *C. pomonella.* These two compounds tend to be more abundant at dusk than in the morning throughout the entire season (Table 1). 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 α-farnesene, which acts as an attractant at low concentrations (634 and 63.4 ng loaded on Silicon/Teflon septum), but as a repellent at high concentration (12,688 ng loaded on Silicon/Teflon 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 (Light and Knight 2005). Recently, these two compounds have been reported to be minor components in a larval aggregation pheromone (Jumean et al. 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 significantly higher amounts at dusk than in the morning, and it is known to act as a synergist of pheromone in the wind tunnel (Yang et al. 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*)-β-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*)-β-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) (Linn et al. 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*)-β-farnesene (72% of the standard in males), benzyl aldehyde (50.6 and 45.8% of the standard in males and females, respectively), and β-pinene (17.8 and 23.3% of the standard in males and females, respectively; Table 3). (*E*)-β-farnesene is known to be attractive in wind tunnel when mixed with (*E,E*)-α-farnesene (Coracini et al. 2004) and by itself in the field (Coracini et al. 2004, Yang et al. 2005). Recently, benzyl aldehyde and β-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 EAG-response does not necessarily correlate with behavioral response, so behavioral tests and field trapping become always necessary for the identification 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-specific 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-specific 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 six-component hawthorn blend contains 94.3 and 4.0% of these two compounds, respectively. Although blends are similar, dogwood-origin *R. pomonella* shows significantly greater upwind flies 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 first time, are also emitted by many other plants that might be or not suitable hosts for *C. pomonella.* We confirm 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 attraction of phytophagous insects to host plants (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 influence 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 RTA01–079 from the Programa Nacional de Recursos y Tecnologías Agroalimentarias (Spain) and by projects AGL2003–06599-CO02–02 and AGL2004–05812/AGR from the Plan Nacional de I+D+I (Spain). D. Casado has a fellowship from MECD (Spain). C. Gemeno is funded by the Programa Ramón y Cajal (MCYT, Spain).

References Cited

Agelopoulos, N. G., A. M. Hooper, S. P. Maniar, J. A. Pickett, and L. J. Wadhams. 1999. A novel approach for isolation of volatile chemicals released by individual leaves of a plant in situ. J. Chem. Ecol. 25: 1411–1425.

Ansebo, L., M.D.A. Coracini, M. Bengtsson, I. Liblikas, M. Ramírez, A. K. Borg-Karlson, M. Tasin, and P. Witzgall. 2004. Antennal and behavioral response of codling moth *Cydia pomonella* to plant volatiles. J. Appl. Entomol. 128: 488–493.

Bäckman, A. C., M. Bengtsson, A. K. Borg-Karlsson, I. Liblikas, and P. Witzgall. 2001. Volatiles from apple (*Malus domestica*) eliciting antennal responses in female codling moth *Cydia pomonella* (L.) (Lepidoptera: Tortricidae): effect of plant injury and sampling technique. Z. Naturforsch. C. 56: 262–268.

Bengtsson, M., A. C. Bäckman, I. Liblikas, M. I. Ramirez, A. K. Borg-Karlson, L. Ansebo, P. Anderson, J. Löfqvist, and P. Witzgall. 2001. Plant odor analysis of apple: antennal response of codling moth females to apple volatiles during phenological development. J. Agric. Food Chem. 49: 3736–3741.

Bosch, D., and J. Avilla. 2001. Evaluación de la eficacia en campo de (2E,4Z)-2,4-decadienoato de etilo, un atrayente de adultos de *Cydia pomonella* (L.), p. 74. *In* Proceedings, II Congreso Nacional de Entomología Aplicada. VIII Jornadas Científicas de la S.E.E.A., 12–16 November 2001, Pamplona, Spain.

Bouvier, J. C., A. Cuany, C. Monier, V. Brosse, and B. Sauphanor. 1998. Enzymatic diagnosis of resistance to deltamethrin in diapausing larvae of the codling moth, *Cydia pomonella* (L.). Arch. Insect. Biochem. 39: 55–64.

Bruce, T.J.A., L. J. Wadhams, and C. M. Woodcock. 2005. Insect host location: a volatile situation. Trends Plant Sci. 10: 269–274.

Calkins, C. O., and R. J. Faust. 2003. Overview of areawide programs and the program for suppression of codling moth in the western USA directed by the United States Department of Agriculture–Agricultural Research Service. Pest. Manag. Sci. 59: 601–604.

Charmillot, P. J., D. Hofer, and D. Pasquier. 2000. Attract and kill: a new method for control of the codling moth *Cydia pomonella.* Entomol. Exp. Appl. 94: 211–216.

Coracini, M.D.A., M. Bengtsson, and P. Witzgall. 2004. Attraction of codling moth males *Cydia pomonella* to apple volatiles. Entomol. Exp. Appl. 100: 1–10.

De Boer, J. G., M. A. Posthumus, and M. Dicke. 2004. Identification of volatiles that are used in discrimination between plants infested with prey or nonprey herbivores by a predatory mite. J. Chem. Ecol. 30: 2215–2230.

Eras, J., J. J. Méndez, M. Balcells, and R. Canela. 2002. Chlorotrimethylsilane: a suitable reagent for synthesis of chlorhydrin esters. J. Org. Chem. 67: 8631–8634.

Gouinguené, S., and T.C.J. Turlings. 2002. The effects of abiotic factors on induced volatile emissions in corn plants. Plant Physiol. 129: 1296–1307.

Gramshaw, J. W., and H. Soto-Valdez. 1998. Migration from polyamide 'microwave and roasting bags' into roast chicken. Food Addit. Contam. 15: 329–335.

Gut, L., and J. F. Brunner. 1996. Implementing codling moth mating disruption in Washington pome fruit orchards. Tree Fruit Research Extension Center Information Series, No. 1. Washington State University. Wenatchee, WA.

Hern, A., and S. Dorn. 1999. Sexual dimorphism in the olfactory orientation of adult *Cydia pomonella* in response to α-farnesene. Entomol. Exp. Appl. 92: 63–72.

Hern, A., and S. Dorn. 2002. Induction of volatile emissions from ripening apple fruits infested with *Cydia pomonella* and the attraction of adult females. Entomol. Exp. Appl. 102: 145–151.

Hern, A., and S. Dorn. 2004. A female-specific attractant for the codling moth, *Cydia pomonella* L., from apple fruit volatiles. Naturwissenschaften. 91: 77–80.

Howell, J. F., A. L. Knight, T. R. Unruh, D. F. Brown, J. L. Krysan, C. R. Sell, and P. A. Kirsch. 1992. Control of codling moth in apple and pear with sex pheromone-mediated mating disruption. J. Econ. Entomol. 85: 918–912.

Huber, F. K., R. Kaiser, F. Sauter, and P. Schiestl. 2005. Floral scent emission and pollinator attraction in two species of *Gymnadenia* (Orchidaceae). Oecologia (Berl.). 142: 564–575.

Jumean, Z., R. Gries, T. Unruh, E. Rowland, and G. Gries. 2005. Identification of the larval aggregation pheromone of codling moth, *Cydia pomonella.* J. Chem. Ecol. 31: 911–924.

Knight, A. L., and D. M. Light. 2005. Dose-response of codling moth (Lepidoptera: Tortricidae) to ethyl (*E*,*Z*)-2,4-decadienoate in apple orchards treated with sex pheromone dispensers. Environ. Entomol. 34: 604–609.

Knight, A. L., R. Hilton, and D. M. Light. 2005. Monitoring codling moth (Lepidoptera: Tortricidae) in apple with blends of ethyl (*E*,*Z*)-2,4-decadienoate and codlemone. Environ. Entomol. 34: 598–603.

Light, D. M., and A. L. Knight. 2005. Specificity of codling moth (Lepidoptera: Tortricidae) for the host plant kairomone, ethyl (2E,4Z)-2,4-decadienoate: field bioassays with pome fruit volatiles, analogue, and isomeric compounds. J. Agric. Food Chem. 53: 4046–4053.

Light, D. M., A. L. Knight, C. A. Henrick, D. Rajapaska, B. Lingren, J. C. Dickens, K. M. Reynolds, R. G. Buttery, G. Merrill, J. Roitman, and B. C. Campbell. 2001. A pear-derived kairomone with pheromonal potency that attracts male and female codling moth, *Cydia pomonella* (L.). Naturwissenschaften 88: 333–338.

Linn, C., Jr., J. L. Feder, S. Nojima, H. R. Dambroski, S. H. Berlocher, and W. Roelofs. 2003. Fruit odor discrimination and sympatric host race formation in *Rhagoletis.* Proc. Natl. Acad. Sci. U.S.A. 100: 11490–11493.

Mattheis, J. P., J. K. Fellman, P. M. Chen, and M. E. Patterson. 1991. Changes in headspace volatiles during physiological development of Bisbee Delicious apple fruit. J. Agric. Food Chem. 39: 1902–1906.

Nielsen, J. K., H. B. Jakobsen, P. Friis, K. Hansen, J. Møller, and C. E. Olsen. 1995. Asynchronous rhythms in the emission of volatiles from *Hesperis matronalis* flowers. Phytochemistry 38: 847–851.

Nojima, S., C. Linn Jr., B. Morris, A. Zhang, and W. Roelofs. 2003a. Identification of host fruit volatiles from hawthorn (*Crataegus* spp.) attractive to hawthorn-origin *Rhagoletis pomonella* flies. J. Chem. Ecol. 29: 321–336.

Nojima, S., C. Linn Jr., and W. Roelofs. 2003b. Identification of host fruit volatiles from flowering dogwood (*Cornus florida*) attractive to dogwood-origin *Rhagoletis pomonella* flies. J. Chem. Ecol. 29: 2347–2357.

Paré, P. W., and J. H. Tumlinson. 1997. De novo biosynthesis of volatiles induced by insect herbivory in cotton plants. Plant Physiol. 114: 1161–1167.

Phillips, P. A., and M. M. Barnes. 1975. Host race formation among sympatric apple, walnut, and plum populations of the codling moth, *Laspeyresia pomonella.* Ann. Entomol. Soc. Am. 68: 1053–1060.

Picone, J. M., H. S. MacTavish, and R. A. Clery. 2002. Emission of floral volatiles from *Mahonia japonica* (Berberidaceae). Phytochemistry 60: 611–617.

Pons, S., M. Eizaguirre, M. J. Sarasúa, and J. Avilla. 1994. Influencia del fotoperiodo sobre la inducción de diapausa de *Cydia pomonella* (Lepidoptera: Tortricidae) en laboratorio y campo. Invest. Agr. Produc. Protec. Veget. 9: 477–491.

Roelofs, W., A. Comeau, A. Hill, and G. Milicevic. 1971. Sex attractant of the codling moth: characterization with electroantennogram technique. Science 174: 297–299.

Scutareanu, P., B. Drukker, J. Bruin, M. A. Posthumus, and M. W. Sabelis. 1997. Volatiles from *Psylla*-infested pear trees and their possible involvement in attraction of anthocorid predators. J. Chem. Ecol. 23: 2241–2260.

Staudt, M., N. Bertin, U. Hansen, G. Seufert, P. Ciccioli, P. Foster, B. Frenzel, and L. Fugit. 1997. Seasonal and diurnal patterns of monoterpene emissions from *Pinus pinea* (L.) under field conditions. Atmos. Environ. 31: 145–156.

Staudt, M., N. Bertin, B. Frenzel, and G. Seufert. 2000. Seasonal variation in amount and composition of monoterpenes emitted by young *Pinus pinea* trees—implications for emission modeling. J. Atmos. Chem. 35: 77–99.

Trimble, R. M. 1998. Mating disruption for controlling the codling moth, *Cydia pomonella* (L.) (Lepidoptera:Tortricidae), in organic apple production in southwestern Ontario. Can. Entomol. 127: 493–505.

Vallat, A., and S. Dorn. 2005. Changes in volatile emissions from apple trees and associated response of adult female codling moths over the fruit-growing season. J. Agric. Food Chem. 53: 4083–4090.

Visser, J. H. 1986. Host odor perception in phytophagous insects. Annu. Rev. Entomol. 31: 121–144.

Vuorinen, T., A. M. Nerg, E. Vapaavuori, and J. K. Holopainen. 2005. Emission of volatile organic compounds from two silver birch (*Betula pendula* Roth) clones grown under ambient and elevated CO_2 and different O_3 concentrations. Atmos. Environ. 39: 1185–1197.

Wearing, C. H., and R.F.N. Hutchins. 1973. α-Farnesene, a naturally occurring oviposition stimulant for the codling moth, *Laspeyresia pomonella.* J. Insect Physiol. 19: 1251–1256.

Witzgall, P., L. Ansebo, Z. Yang, G. Angeli, B. Sauphanor, and M. Bengtsson. 2005. Plant volatiles affect oviposition by codling moths. Chemoecology 15: 77–83.

Yan, F., M. Bengtsson, and P. Witzgall. 1999. Behavioral response of female codling moths, *Cydia pomonella,* to apple volatiles. J. Chem. Ecol. 25: 1343–1351.

Yang, Z., M. Bengtsson, and P. Witzgall. 2004. Host plant volatiles synergize response to sex pheromone in codling moth, *Cydia pomonella.* J. Chem. Ecol. 30: 619–629.

Yang, Z., D. Casado, C. Ioratti, M. Bengtsson, and P. Witzgall. 2005. Effect of pheromone pre-exposure and mating on attraction of codling moth *Cydia pomonella* to host volatiles. Agric. Forest Entomol. 7: 1–6.

ZEON Corp. 2005. Specialty chemicals product list. (http://www.zeon.co.jp/business_e/enterprise/spechemi/index.html).

Zhang, A., C. Linn Jr., S. Wright, R. Prokopy, W. Reissig, and W. Roelofs. 1999b. Identification of a new blend of apple volatiles attractive to the apple maggot, *Rhagoletis pomonella.* J. Chem. Ecol. 25: 1221–1232.

Zhang, Q. H., G. Birgersson, J. Zhu, C. Löfstedt, J. Löfqvist, and F. Schlyter. 1999a. Leaf volatiles from nonhost deciduous trees: variation by tree species, season and temperature, and electrophysiological activity in *Ips typographus.* J. Chem. Ecol. 25: 1923–1943.

Received for publication 8 September 2005; accepted 5 January 2006.

CHAPTER IV

Diel Variation of Walnut Tree Volatiles and Electrophysiological Responses in *Cydia pomonella* (Lepidoptera: Tortricidae)

Daniel Casado*, César Gemeno, Jesús Avilla and Magí Riba

Àrea de Protecció de Conreus, Centre UdL-IRTA de R+D, Av. Alcalde Rovira Roure, 191, E-25198, Lleida, Spain

* Corresponding author, e-mail: danicasado@yahoo.es

ABSTRACT. Volatiles from walnut trees were collected in the field in the morning and at dusk at two different periods of the season. Headspace collections were analyzed by gas chromatography – mass spectrometry (GC-MS), and gas chromatography – electroantennodetection (GC-EAD). Ninety compounds were detected in walnut headspace collections, mainly oxygenated and hydrocarbon monoterpenes, and hydrocarbon sesquiterpenes. The most abundant compound was β-pinene, that together with (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, limonene, germacrene D, 1,8-cineole, sabinene, (*E*)-β-farnesene, (*E*)-β-caryophyllene, β-myrcene, and β-phellandrene constituted on average between 81.9 and 90.5% of the total chromatographic area. Differences between the two seasonal periods were significant for thirty-nine compounds. Significant differences between morning and dusk occurred for fourteen compounds, all but one were oxygenated compounds. Discernible and consistent GC-EAD responses were detected to eleven walnut-origin compounds in the collections, and confirmed with synthetics to seven of them. Except for alloocimene, pinocarvone and caryophyllene oxide, all the EAD-active compounds found in this study are also emitted by apple, another *Cydia pomonella* host.

KEYWORDS: *Cydia pomonella*; walnut; plant volatiles; GC-EAD; GC-MS; diel variation.

1. Introduction

Environmental concerns and the development of insecticide-resistant populations have promoted the use of more environmentally-safe techniques for pest control in agriculture and food production. Several of these techniques (i.e., mating disruption, attract and kill, mass trapping) depend on the availability of reliable attractants of insect adults. Pheromones of different types (sex, aggregation) are the most important class of attractants used in pest control.[1] and they are used in many crops all over the world since many years ago. However, host-plant volatiles are growing in importance in the control of phytophagous pests.[2-4]

The codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), is a serious pest in walnut (*Juglans regia* L.), apple (*Malus domestica* Borkh.), and pear (*Pyrus communis* L.) production. Its sex pheromone was first reported in 1971,[5] and at present it is widely used to monitor and control pest populations by means of mating disruption[6-7] and attract-and-kill.[8] Nowadays, mating disruption is the most successful alternative to traditional chemical control of *C. pomonella* and it is used worldwide.[9] However, pheromone traps are less effective to monitor adult populations under mating disruption,[10] and so new attractants are necessary as monitoring tools under mating disruption conditions. Furthermore, new attractants could eventually be used to develop new control strategies that affected both male and female adults.

In the last years many advances in *C. pomonella* response to host-volatiles have been made, with most of the effort focused on apple,[11-19] and pear.[20] Walnut belongs to a different family (Juglandaceae) than pear or apple (Rosaceae), however no studies have been conducted on *C. pomonella* response to walnut tree volatiles. In addition, most volatile collections made in the previous studies have been made by detaching plant parts[12,16] or during the photophase,[14,18] although mechanical damage can result in changes in volatile emission by plants[21-22] and *C. pomonella* is a species of crepuscular activity.

The aims of this study were to characterize and to compare volatile emissions of intact walnut trees in the field in the morning and at dusk, and to identify which compounds elicit electrophysiological responses in the antennae of *C. pomonella* adults. For this we collected volatiles from walnut trees *in situ* at dusk and in the morning and in two different phenological stages of the tree. Then we identified the volatiles by GC-

MS, and determined which of them elicited electrophysiological responses on the antennae of *C. pomonella* males by means of GC-EAD.

2. 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 semi-synthetic diet[23] under a 16:8 h (L:D) photoperiod at 25 ± 5 ºC. Newly emerged adults were sexed every day and males were kept apart from females in small groups (up to 10 individuals) in plastic boxes (15-cm diameter x 7-cm height). Insects were supplied with water until their use.

Solvents and chemicals

Solvents used were hexane, diethyl ether, and methanol, all of residue analysis quality (> 95%, > 99.8% and > 99.8%, respectively; Fluka Chemie GmbH, Buchs, Switzerland). Hexyl acetate, (*Z*)-3-hexenyl 2-methylbutanoate, butyl hexanoate and heptyl acetate were synthesized in good yields (> 70% after distilling) following the procedure described by Eras and coworkers[24] with GC purities of 95.3, 87.5, 97.9, and 96.6%, respectively. The impurities of heptyl acetate, which was used as internal standard (see bellow), were two ethyl-benzene isomers (0.9% both), xylene (1.5%), and dimethyl-benzene (0.6%). The remaining authentic samples were obtained from reliable commercial sources, and their purities ranged from 70 to 99.5%.

Volatile collections

Volatile collections were made in the spring and summer of 2004 in a 0.7 ha experimental multivarietal (mainly Chandler, Ferjean, Fernor, Howard, Lara, and Vina) walnut orchard, located in Gimenells (Lleida, Spain, 41º 37' N). A dynamic headspace setup was used for volatile collection.[19] A 46 x 61 cm plastic oven bag (Pansaver®, M&Q Plastic Products Inc., Schuykill, PA) was placed over a walnut tree branch and closed with a plastic clamp. A vacuum pump (NMP830 KNDC-12V, KNF Neuberger GmbH, Freiburg, Germany) pushed air through a stainless steel tube containing 1.3 g of activated charcoal (20/40 mesh, SKC Limited, Dorset, United Kingdom), into the bag at 0.5 l/min. A second vacuum pump simultaneously extracted air from the bag at 0.45 l/min through a glass trap containing 50 mg of Super-Q (80/100 mesh, Alltech

associates Inc., Deerfield, USA) hold between two layers of glass wool. Plastic bags were used only once to avoid contamination between samples, and only intact branches were sampled to avoid variations in emission due to herbivore damage.

Collections were made at 2 different periods in the season as follows: a) late-Spring (LS) between May 24 and June 7, over branches bearing leaves and 2 to 5 fruits about 1.5 cm of diameter; and b) mid-Summer (MS) between July 13 and 22, over branches bearing leaves and 2 to 3 fruit about 4 cm of diameter. In both periods, collections were always made at 2 different times of the day: morning (starting between 9:00 and 10:00, local time GMT+2) and dusk (beginning ≈30 min before dusk). Morning and dusk samples were taken over the same branch. Sample trees were chosen randomly regardless to varieties, this was done because our main objectives were to compare differences in emission between dusk and morning, and determine which walnut volatiles elicited EAD responses in *C. pomonella*. For this reason volatiles collections were made over the same branch in the morning and at dusk, thus minimizing the effect of differences amongst individuals and/or varieties. Nevertheless, we should inform that analyzed collections had been made over Chandler (45.5%), Lara (27.3%), Ferjean (13.6%), and other (13.6%). A minimum of two blank samples were taken per diel period and phenological stage from empty bags placed into the tree canopy.

Volatiles were collected for 2 h. Subsequently, Super-Q traps were taken to the laboratory and washed 4 times with 100 µl of hexane to extract samples into conical-bottom vials. Fifty nanograms of heptyl acetate in 10 µl hexane were added as an internal standard and the vials were kept at -20ºC until analysis. Before being reutilized traps were rinsed with approximately 2 ml of each hexane, diethyl ether and methanol. Immediately before analysis, samples were reduced under a soft stream of nitrogen to approximately 10 µl.

The temperature inside the bag was measured every 30 to 45 min by an electronic thermometer. Average temperatures inside the bags ranged from 26.8ºC to 32.6ºC (LS-morning), 20ºC to 25.4ºC (LS-dusk), 25.2ºC to 33.8ºC (MS-morning) and 24ºC to 28.5ºC (MS-dusk).

Gas chromatography – mass spectrometry (GC-MS)

GC-MS analyses were carried out on an Agilent Technologies 6890N GC interfaced to an Agilent Technologies 5973 Network quadrupole MS (Agilent

Technologies Inc., Palo Alto, USA). One microliter of the reduced sample was injected into the GC, and chromatographic separation was performed on a DB-Wax (30 m x 0.25 mm x 0.25 μm) capillary column (J&W Scientific, Folsom, USA). The injector temperature was 250ºC and the split ratio was 1:5. The oven temperature started at 50ºC and was maintained for 2 min, increased at 5ºC/min to 150ºC, held at this temperature for 5 min, then increased at 10ºC/min to 230ºC and finally kept at 230ºC for 10 min. The carrier gas was helium at a constant flow 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 ºC and 230 ºC, respectively.

Samples were analyzed by GC-MS software (MSD-ChemStation version D.00.01, Agilent Technologies Inc.). Spectra were compared to the available libraries for tentative identification (an own-made aroma library, NIST 75K, and Wiley 275). Identification was confirmed by injection of synthetic compounds when possible. When standards were not available, retention indexes and/or spectra were compared to those in the bibliography.[i.e. 25-28] Five volatile collections and at least one blank sample per diel and seasonal periods were analyzed by GC-MS. The amounts of all compounds that were not present in blanks (to avoid compounds emitted by bags, and the impurities of the internal standard) were estimated as a percentage of the internal standard peak. Linear retention indexes (LRI) were calculated using the following formula:

$$LRI = 100\left(\frac{t'_i - t'_Z}{t'_{Z+1} - t'_Z} + Z\right)$$

Where t'_i is the corrected retention time of the compound, t'_Z is the corrected retention time of the n-alkane eluting before the compound, t'_{Z+1} is the corrected retention time of the n-alkane eluting after the compound, and Z is the number of carbons of the n-alkane eluting before the compound. Retention times are corrected by subtracting the time of elution of a compound which is not retained by the capillary column, hexane in this case.

Gas chromatography – electroantennodetection (GC-EAD)

GC-EAD analyses were made on an Agilent Technologies 6890N gas chromatograph (Agilent Technologies Inc.) coupled to an electroantennogram (Syntech, Hilversum, Holland) as described by Casado and coworkers.[19] Excised antennae of 2- to

3-day-old males were suspended between two glass capillary 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 humidified air over the antenna at 1 l/min. Male antennae were used because of their better response to apple volatiles.[19]

Two microliters of the reduced samples were injected in the GC, and chromatographic conditions were the same as for GC-MS analyses except that the injector was set to splitless/split for 1 min after injection, and carrier flow rate was 1 ml/min. Between 2 and 3 min before the solvent peak and 1 min after the end of the run, the antennae were challenged with 1 μg puffs of sex pheromone to check their responsiveness (odor stimulus preparation and delivery followed the procedure described by Casado and coworkers).[19] Three to four volatile collections per diel and seasonal period were analyzed by GC-EAD, for a total of 13.

Extra GC-EAD were made with synthetics of those compounds that elicited EAD-responses in the above assay and were available, to confirm their activity. In this assay a mixture containing synthetic alloocimene, (*Z*)-3-hexenol, nonanal, (*E*)-β-caryophyllene, farnesene, and caryophyllene oxide was injected in the GC-EAD. The injected amount was 200 ng for each compound except farnesene. The available farnesene was a racemic mixture and the amount in the mixture was calculated to inject 200 ng of the main compound of the mixture. Although (*E*)-β-ocimene was present in an important amount as impurity in the available (*Z*)-β-ocimene, we decided not to include it in the synthetic blend. Ten replicates were conducted using all the other conditions mentioned above.

Data analysis

Comparison of the emission of volatiles between the different diel 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 significant differences occurred a Duncan's Multiple Range Test was performed. When the interaction diel-by-phenological period was significant, a pairwaise comparison of least square means was performed. Significance level was 0.05.

Identification of GC-EAD-active compounds was carried out by comparison of linear retention indexes of GC-MS and GC-EAD analyses. But no statistical analysis

was carried out amongst periods, as the objective of the GC-EAD was only to identify the EAD-active compounds.

3. Results

Emission of volatiles from walnut trees *in situ*

A total of 90 compounds were detected in the volatile collections from walnut branches *in situ* (Table 1). Seven compounds could not be identified; all of them were hydrocarbon sesquiterpenes and are named as 'unidentified 1 to 7'. Compounds were grouped in chemical categories as follows: 12 hydrocarbon monoterpenes, 20 oxygenated monoterpenes, 36 hydrocarbon sesquiterpenes, 5 oxygenated sesquiterpenes, 1 hydrocarbon homoterpene, 12 aliphatics, and 4 benzenoids (Table 1).

Hydrocarbon monoterpenes accounted for 51.7 to 63.0% on average of the Total Chromatographic Area (TCA) (Figure 1). Hydrocarbon sesquiterpenes represented between 13.3 and 29.4% of the TCA, and oxygenated monoterpenes and aliphatics were between 6.1 and 14.0% and between 7.9 and 10.9% TCA, respectively (Figure 1).

The predominant compound in all collection periods was the bicyclic hydrocarbon monoterpene β-pinene with an area that ranged on average from 8,075.3 to 12,728.6% of the Internal Standard area (IS) (Table 1), and between 28.6 and 41.2% TCA (Table 2). Sabinene (1,464.5 to 2,397.8% IS), β-myrcene (533.2 to 1,169.2% IS), limonene (1,514.6 to 2,624.2% IS), β-phellandrene (255.2 to 589.5% IS), and (*E*)-β-ocimene (717.1 to 4,241.9% IS) were other highly emitted hydrocarbon monoterpenes (Table 1). Amongst the rest of compounds 1,8-cineole (1,944.6 to 2,538.5% IS), (*E*)-β-caryophyllene (837.6 to 3,303.1% IS), (*E*)-β-farnesene (977.6 to 3,274.2% IS), germacrene D (679.5 to 5,293.8% IS), and (*Z*)-3-hexenyl acetate (1,286.9 to 3,645.4% IS) were also emitted in large amounts (Table 1). These 11 compounds constituted on average between 81.9 and 90.5% TCA, depending on the collection period (Table 2).

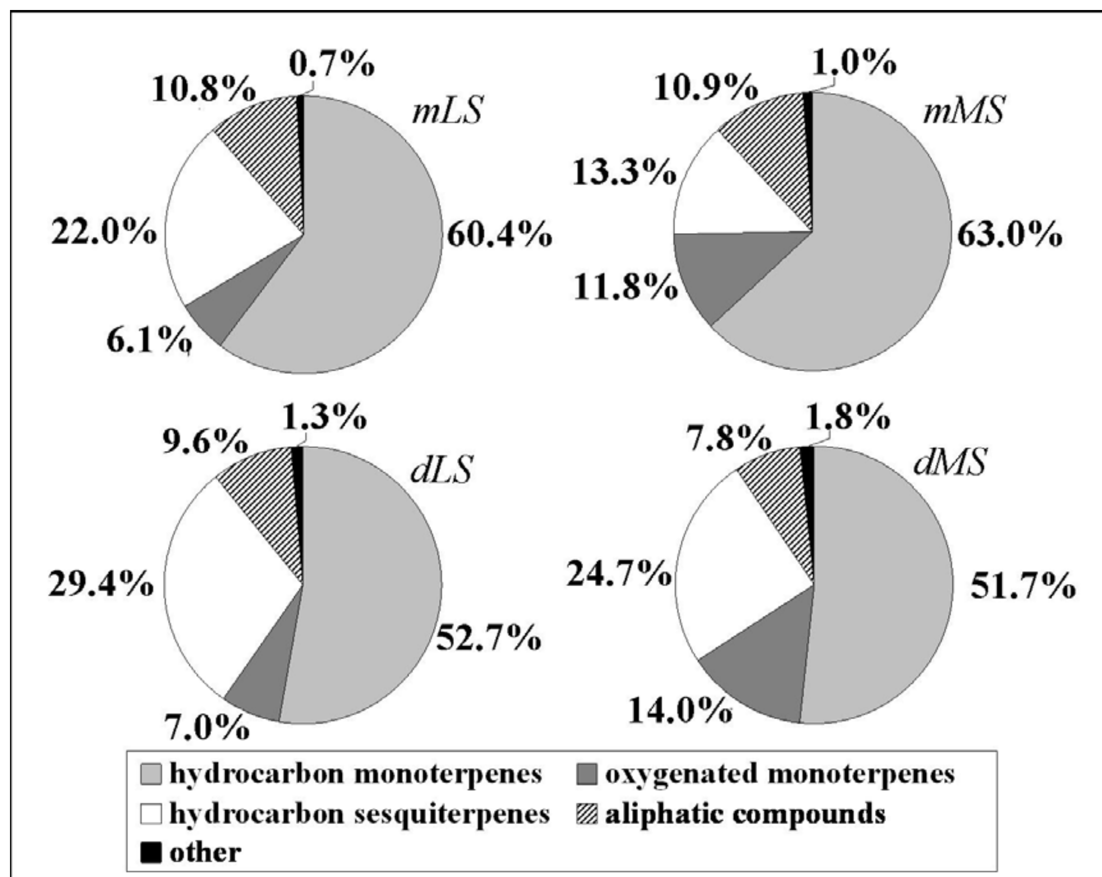

Figure 1. Percentage contribution of the different groups of compounds to the total emission of walnut tree volatiles. Percentages over total chromatographic area in the different collection periods: mLS, late-Spring period in the morning; mMS, mid-Summer period in the morning; dLS, late-Spring period at dusk; dMS, mid-Summer period at dusk.

Compounds that were undetected in one or more collection periods were pentyl acetate, (*Z*)-3-hexenyl 2-methylbutanoate, and (*Z*)-3-hexenyl benzoate at dusk in the MS period, δ-elemene in the morning in the MS period, nopinone in the LS period, and caryophyllene oxide isomer 1 in the morning collections in LS (Table 1).

No significant differences in total volatile release (sum of all peak areas) were found between LS and MS (df = 1, F = 4.33, P = 0.054), though a tendency for decreasing the emissions from LS to MS was observed (Table 1, bottom line). On the other hand, significant differences between the two periods were found for 4 groups of compounds. Three groups were more abundant in LS and one in MS (Table 3). For single compounds differences between the two seasonal periods were found in 38 cases. Twenty-two compounds were more abundant in LS and 16 in MS (Table 3).

No significant differences between morning and dusk for total volatile emissions (*df* = 1, *F* = 0.25, *P* = 0.626), or for any of the groups of compounds were found. Significant differences between the two diel periods were found for 12 single compounds. Eleven of them were more abundant at dusk and one in the morning (Table 3).

A significant seasonal by diel period interaction was found for 3 compounds: nonanal, decanal and (*Z*)-3-hexenyl 2-methylbutanoate. Further analysis of the data by means of a pairwise comparison of least square means of the interaction showed that for nonanal and decanal emissions were higher at dusk than in the morning in LS (*P* = 0.014, and *P* = 0.013, respectively), but no differences occurred in MS between diel periods (*P* = 0.987, and *P* = 0.665, respectively). As for (*Z*)-3-hexenyl 2-methylbutanoate, emissions at dusk were higher in LS than in MS (*P* = 0.001), whereas there were no significant differences between seasonal periods in the morning samples (*P* = 0.331).

GC-EAD analysis of volatile collections

EAD-Responses to 10 peaks were registered in a consistent and repetitive way. These responses matched with peaks corresponding to (*E*)-β-ocimene, alloocimene, (*Z*)-3-hexenol + nonanal, linalool, pinocarvone, (*E*)-β-caryophyllene, (*E*)-β-farnesene, germacrene D, (*E*,*E*)-α-farnesene + 2-cyclopentylcyclopentanone, and a caryophyllene oxide isomer (Figure 2). The average EAD responses, for all analyzed samples, generated by these compounds were of 0.030, 0.023, 0.026, 0.019, 0.012, 0.038, 0.028, 0.036, 0.024 and 0.014 mV, respectively. The compound 2-cyclopentylcyclopentanone is not produced by walnut; instead it is emitted by the plastic bags used in this experiment, as we found in a previous study using the same methodology.[19] However, in the same study we confirmed the electroantennographic activity of 2-cyclopentylcyclopentanone by EAGs with synthetic compound.

Table 1. Mean amounts of volatile compounds detected in walnut trees headspace at two seasonal periods in the morning and at dusk.

Compound	RT[c] (min)	LRI[b]	Morning LS % IS[a] ± SE	Morning MS % IS[a] ± SE	Dusk LS % IS[a] ± SE	Dusk MS % IS[a] ± SE
Hydrocarbon monoterpenes						
β-pinene	4.91	1123	11958.5 ± 2074.1	9703.9 ± 1363.4	12728.6 ± 1796.6	8075.3 ± 1906.2
sabinene	5.09	1132	2126.1 ± 648.7	1464.5 ± 230.9	2397.8 ± 653.2	1644.3 ± 747.1
β-myrcene	5.92	1172	1017.9 ± 209.7	533.2 ± 85.6	1169.2 ± 202.8	462.2 ± 157.5
α-terpinene	6.24	1187	33.2 ± 14.4	30.9 ± 6.7	26.3 ± 6.9	20.3 ± 6.1
limonene	6.68	1207	2291.3 ± 492.5	1756.3 ± 340.3	2624.2 ± 434.0	1514.6 ± 508.9
β-phellandrene	6.87	1215	589.5 ± 147.0	291.2 ± 38.0	518.7 ± 165.2	255.2 ± 76.0
(*Z*)-β-ocimene	7.51	1242	59.7 ± 13.9	28.2 ± 6.1	52.4 ± 14.9	25.0 ± 10.9
γ-terpinene	7.75	1252	56.9 ± 11.4	196.1 ± 157.1	58.1 ± 12.5	34.2 ± 8.7
(*E*)-β-ocimene	7.94	1260	4241.9 ± 659.8	717.1 ± 225.1	3926.5 ± 655.7	860.6 ± 283.0
p-cymene	8.31	1275	35.2 ± 7.9	142.9 ± 50.6	34.2 ± 5.0	113.6 ± 51.9
terpinolene	8.64	1289	120.7 ± 52.1	45.1 ± 10.0	94.5 ± 24.6	57.9 ± 16.1
alloocimene	10.85	1324	13.5 ± 5.5	23.3 ± 5.5	23.0 ± 3.6	21.6 ± 10.5
Oxygenated monoterpenes						
1,8-cineole	6.94	1218	1944.6 ± 719.2	2039.0 ± 917.9	2538.5 ± 779.7	2210.1 ± 1359.1
sabinene hydrate	13.26	1475	5.5 ± 3.5	27.5 ± 10.2	24.6 ± 9.3	56.2 ± 28.0
α-campholenal	13.70	1493	1.1 ± 1.1	11.2 ± 2.9	4.9 ± 2.3	34.0 ± 13.5
isopinocamphone	15.00	1547	106.2 ± 27.9	326.0 ± 106.0	78.0 ± 26.7	97.3 ± 49.1
linalool	15.28	1558	71.8 ± 16.1	29.1 ± 6.2	90.2 ± 22.8	109.4 ± 45.7
pinocarvone	15.55	1570	58.5 ± 15.8	181.2 ± 38.9	95.5 ± 15.1	431.9 ± 149.2
nopinone	15.78	1579	-	52.5 ± 21.6	-	35.3 ± 12.3
bornyl acetate	15.92	1585	96.3 ± 24.0	87.6 ± 15.8	174.4 ± 39.6	184.6 ± 65.0
terpinen-4-ol	16.49	1609	18.2 ± 3.6	27.2 ± 5.7	22.7 ± 3.3	42.7 ± 11.9
myrtenal	16.95	1629	20.8 ± 5.3	104.6 ± 26.1	55.5 ± 11.4	306.9 ± 104.0
sabina ketone	17.05	1634	0.4 ± 0.4	6.1 ± 2.6	6.0 ± 5.7	9.9 ± 2.6
3,6,6-trimethylnorpinan-2-one	17.11	1636	5.6 ± 2.4	29.7 ± 7.8	2.7 ± 2.7	7.9 ± 5.6
(*E*)-pinocarveol	17.71	1662	26.2 ± 7.7	128.3 ± 40.7	44.8 ± 15.4	282.3 ± 103.3
neral	18.30	1688	17.3 ± 4.8	57.4 ± 18.6	32.5 ± 5.6	102.1 ± 39.7
α-terpineol	18.71	1706	5.0 ± 3.2	9.5 ± 3.6	15.3 ± 5.8	19.6 ± 6.7

Table 1. (Continued)

Compound	RT[c] (min)	LRI[b]	Morning LS % IS[a] ± SE	Morning MS % IS[a] ± SE	Dusk LS % IS[a] ± SE	Dusk MS % IS[a] ± SE
neryl acetate	19.28	1732	10.6 ± 6.7	14.5 ± 5.4	18.0 ± 11.0	44.6 ± 12.3
carvone	19.36	1736	45.6 ± 35.1	27.5 ± 7.8	97.4 ± 48.1	49.7 ± 26.4
geranyl acetate	19.96	1763	35.5 ± 14.7	21.8 ± 4.2	103.0 ± 25.5	79.2 ± 23.7
myrtenol	20.79	1801	9.0 ± 3.8	55.9 ± 14.6	21.0 ± 6.7	91.5 ± 25.3
(*E*)-carveol	21.70	1844	3.7 ± 2.0	12.9 ± 3.9	2.0 ± 1.3	17.1 ± 5.0
Hydrocarbon sesquiterpenes						
α-cubebene	12.94	1462	47.1 ± 24.0	32.7 ± 11.5	63.5 ± 25.0	47.7 ± 23.0
α-longipinene	13.13	1470	11.0 ± 3.8	2.1 ± 2.1	13.2 ± 7.7	2.7 ± 2.7
cyclosativene	13.47	1483	13.3 ± 9.3	6.7 ± 4.9	19.0 ± 9.0	15.1 ± 9.8
δ-elemene	13.50	1485	30.9 ± 26.2	-	61.9 ± 39.2	18.5 ± 18.5
α-copaene	13.75	1495	63.6 ± 28.9	37.7 ± 13.4	67.1 ± 34.7	63.6 ± 33.1
α-bourbonene	14.22	1514	10.0 ± 2.9	18.7 ± 13.4	23.4 ± 8.8	36.2 ± 27.8
β-bourbonene	14.38	1521	224.0 ± 119.6	252.6 ± 122.8	310.0 ± 105.7	412.8 ± 275.4
(*Z*)-α-bergamotene	14.86	1541	33.8 ± 14.9	16.7 ± 5.2	61.5 ± 24.5	30.0 ± 14.4
β-cubebene	14.88	1542	32.2 ± 18.0	13.8 ± 6.7	45.4 ± 18.0	32.9 ± 23.2
(*E*)-β-bergamotene	15.37	1562	38.6 ± 20.2	19.3 ± 6.4	73.1 ± 27.8	46.8 ± 24.5
unidentified 1	15.73	1577	523.0 ± 303.1	118.5 ± 58.7	928.2 ± 438.2	417.0 ± 302.1
β-gurjunene	16.14	1594	523.2 ± 311.8	116.7 ± 55.1	935.1 ± 438.9	361.9 ± 248.8
(*E*)-β-caryophyllene	16.27	1600	2104.3 ± 982.9	837.6 ± 297.7	3303.1 ± 1283.0	1878.8 ± 1011.3
bicyclosesquiphellandrene	16.72	1619	9.3 ± 4.3	5.5 ± 2.4	14.4 ± 5.7	11.1 ± 5.0
unidentified 2	17.08	1635	8.1 ± 5.7	0.2 ± 0.2	3.4 ± 2.5	6.6 ± 5.2
alloaromadendrene	17.28	1644	151.4 ± 85.3	42.3 ± 17.8	267.3 ± 119.2	121.1 ± 90.8
unidentified 3	17.77	1665	183.6 ± 109.5	35.3 ± 17.3	343.3 ± 165.7	131.9 ± 89.5
α-humulene	17.88	1670	20.4 ± 13.4	26.1 ± 8.3	434.6 ± 246.9	372.1 ± 347.8
(*E*)-β-farnesene	17.97	1674	2689.1 ± 1493.1	977.6 ± 368.4	3274.2 ± 1576.9	2351.5 ± 1181.0
unidentified 4	18.04	1677	7.6 ± 2.4	9.3 ± 4.8	5.9 ± 2.8	6.7 ± 2.2
α-amorphene	18.40	1693	12.4 ± 4.4	15.2 ± 6.9	32.0 ± 16.0	37.0 ± 22.0
γ-curcumene	18.45	1695	81.0 ± 30.1	11.8 ± 4.0	427.1 ± 233.8	92.7 ± 55.2
germacrene D	18.82	1711	3335.0 ± 1677.3	679.5 ± 306.7	5293.8 ± 2164.2	2163.2 ± 1396.5
unidentified 5	18.93	1716	23.0 ± 14.4	6.0 ± 1.9	11.2 ± 3.7	6.8 ± 2.9

Table 1. (Continued)

Compound	RT[c] (min)	LRI[b]	Morning LS % IS[a] ± SE	Morning MS % IS[a] ± SE	Dusk LS % IS[a] ± SE	Dusk MS % IS[a] ± SE
α-zingiberene	19.12	1725	47.1 ± 37.6	7.1 ± 4.6	112.7 ± 67.3	29.0 ± 24.6
α-muurolene	19.19	1728	27.7 ± 18.8	13.4 ± 4.0	54.8 ± 21.9	37.3 ± 21.2
β-bisabolene + unidentified 6	19.27	1732	134.1 ± 79.4	25.6 ± 14.9	255.3 ± 114.0	32.0 ± 13.5
β-curcumene	19.55	1745	53.5 ± 28.1	9.5 ± 3.1	116.0 ± 47.1	35.1 ± 17.2
(*E*,*E*)-α-farnesene	19.76	1754	158.2 ± 66.5	23.3 ± 7.8	376.8 ± 234.1	63.0 ± 28.0
δ-cadinene	19.92	1762	73.6 ± 39.8	18.0 ± 9.1	94.7 ± 45.2	68.1 ± 45.8
β-sesquiphellandrene	20.17	1773	37.7 ± 23.6	8.2 ± 3.7	52.4 ± 33.0	34.4 ± 23.3
ar-curcumene	20.25	1777	30.4 ± 12.0	44.2 ± 14.6	43.0 ± 9.9	165.4 ± 91.7
cadina-1,4-diene	20.42	1784	8.2 ± 4.1	7.6 ± 1.9	11.5 ± 3.3	13.8 ± 4.3
unidentified 7	20.51	1789	3.6 ± 2.1	4.8 ± 1.6	4.2 ± 2.2	5.4 ± 2.3
α-cadinene	20.64	1795	11.3 ± 6.8	2.2 ± 1.5	12.6 ± 6.9	10.6 ± 6.1
Oxygenated sesquiterpenes						
caryophyllene oxide isomer 1	23.43	1919	-	13.6 ± 5.5	15.8 ± 7.0	13.1 ± 7.3
caryophyllene oxide isomer 2	24.81	1968	1.6 ± 1.6	13.9 ± 3.8	9.6 ± 4.6	38.7 ± 20.2
caryophyllene oxide isomer 3	25.12	1979	19.8 ± 4.8	88.1 ± 38.6	70.9 ± 25.6	293.4 ± 136.1
β-eudesmol	31.26	2233	16.6 ± 5.5	28.0 ± 14.3	6.0 ± 2.8	23.0 ± 7.9
caryophylla-3,8(13)-dien-5-β-ol	32.81	2347	2.7 ± 2.7	11.7 ± 5.6	4.4 ± 2.7	11.0 ± 7.9
Hydrocarbon homoterpenes						
(*E*)-4,8-dimethyl-1,3,7-nonatriene	9.26	1314	69.6 ± 40.1	2.7 ± 2.7	20.7 ± 9.7	12.9 ± 6.3
Aliphatics						
pentyl acetate	6.14	1182	34.1 ± 11.3	4.2 ± 4.2	17.2 ± 6.3	-
hexyl acetate	8.43	1280	49.1 ± 11.7	14.9 ± 5.1	43.2 ± 12.4	12.0 ± 2.2
octanal	8.80	1295	12.7 ± 6.7	0.6 ± 0.6	11.9 ± 4.2	8.6 ± 4.2
(*Z*)-3-hexenyl acetate	9.55	1326	3036.0 ± 796.4	2227.1 ± 569.0	3645.4 ± 780.5	1286.9 ± 82.9
6-methyl-5-hepten-2-one	9.99	1343	13.3 ± 3.7	9.7 ± 3.6	21.0 ± 4.3	25.8 ± 5.6
(*Z*)-3-hexenol	11.26	1394	102.6 ± 45.4	42.9 ± 13.8	152.9 ± 31.2	63.7 ± 11.4
nonanal	11.38	1399	43.7 ± 3.8	21.5 ± 6.5	41.4 ± 5.7	45.7 ± 1.8
butyl hexanoate	11.91	1420	2.9 ± 1.8	1.0 ± 1.0	4.6 ± 1.3	4.8 ± 2.7

Table 1. (Continued)

Compound	RT[c] (min)	LRI[b]	Morning LS % IS[a] ± SE	Morning MS % IS[a] ± SE	Dusk LS % IS[a] ± SE	Dusk MS % IS[a] ± SE
ethyl octanoate	12.45	1442	2.2 ± 2.2	6.2 ± 3.4	10.6 ± 2.9	18.7 ± 7.2
(Z)-3-hexenyl butanoate	13.08	1468	52.6 ± 15.3	4.1 ± 1.9	68.9 ± 17.0	5.0 ± 1.4
(Z)-3-hexenyl 2-methylbutanoate	13.41	1481	14.6 ± 4.3	7.7 ± 4.1	21.5 ± 10.3	-
decanal	13.96	1503	44.3 ± 6.9	8.1 ± 8.1	32.0 ± 11.2	30.1 ± 2.8
Benzenoids						
1,2,4,5-tetramethylbenzene	12.33	1437	16.2 ± 4.5	25.1 ± 7.3	8.2 ± 4.6	24.7 ± 7.7
dihydrobenzene	12.40	1440	13.1 ± 5.6	5.9 ± 4.6	15.2 ± 2.2	4.6 ± 2.9
methyl salicylate	20.23	1776	87.1 ± 32.1	56.8 ± 18.4	384.1 ± 313.6	26.7 ± 15.6
(Z)-3-hexenyl benzoate	29.34	2126	39.4 ± 14.5	5.0 ± 2.4	105.0 ± 66.4	-
Hydrocarbon monoterpenes	-	-	22544.3 ± 4230.5	14932.6 ± 2113.7	23653.6 ± 3544.1	13084.8 ± 3709.4
Oxygenated monoterpenes	-	-	2481.9 ± 803.9	3249.4 ± 1216.0	3427.0 ± 883.6	4212.2 ± 2017.9
Hydrocarbon sesquiterpenes	-	-	10761.2 ± 5512.8	3445.7 ± 1372.2	17145.5 ± 7209.0	9158.8 ± 5415.9
Oxygenated sesquiterpenes	-	-	40.7 ± 7.6	155.4 ± 45.7	106.7 ± 35.8	379.2 ± 152.6
Hydrocarbon homoterpenes	-	-	69.6 ± 40.1	2.7 ± 2.7	20.7 ± 9.7	12.9 ± 6.3
Aliphatics	-	-	3408.1 ± 845.2	2348.0 ± 588.5	4070.5 ± 796.8	1501.3 ± 104.9
Benzenoids	-	-	155.9 ± 48.1	92.8 ± 27.0	512.5 ± 298.6	56.0 ± 13.3
Total emission	-	-	39461.7 ± 9949.4	24226.5 ± 4177.2	48936.4 ± 11609.3	28405.2 ± 11214.4

LS: late-Spring, MS: mid-Summer. Compounds confirmed by comparison to synthetic compounds are bold-faced. [a] Mean percentages relative to the internal standard area (50 ng heptyl acetate). [b] Linear Retention Index. [c] Retention Time.

Table 2. Mean percentage contribution of the major compounds to the total emission of walnut tree volatiles.

Compound	mLS (% ± SE)	mMS (% ± SE)	dLS (% ± SE)	dLS (% ± SE)
β-pinene	32.6 ± 2.6	41.2 ± 2.0	28.6 ± 3.0	33.7 ± 3.9
(*Z*)-3-hexenyl acetate	9.6 ± 2.6	10.3 ± 2.4	8.6 ± 1.5	6.8 ± 1.8
(*E*)-β-ocimene	11.8 ± 1.2	3.4 ± 1.0	8.9 ± 1.2	3.2 ± 0.6
limonene	5.9 ± 0.6	7.1 ± 0.3	5.7 ± 0.4	5.6 ± 0.3
germacrene D	6.9 ± 1.6	2.6 ± 0.8	9.2 ± 1.7	5.3 ± 1.6
1,8-cineole	4.7 ± 1.2	7.0 ± 2.8	5.0 ± 1.3	6.5 ± 2.5
sabinene	5.1 ± 0.3	6.2 ± 0.8	5.0 ± 0.5	5.4 ± 0.3
(*E*)-β-farnesene	5.2 ± 1.6	3.7 ± 0.9	5.5 ± 1.7	7.3 ± 0.8
(*E*)-β-caryophyllene	4.6 ± 0.9	3.5 ± 0.9	5.8 ± 1.3	5.3 ± 1.5
β-myrcene	2.6 ± 0.3	2.2 ± 0.1	2.5 ± 0.2	1.7 ± 0.1
β-phellandrene	1.5 ± 0.1	1.2 ± 0.1	1.3 ± 0.4	1.0 ± 0.1
other	9.5 ± 1.0	11.6 ± 1.3	13.7 ± 2.1	18.1 ± 1.4

Percentages over total chromatographic area in the different collection periods: mLS, late-Spring period in the morning; mMS, mid-Summer period in the morning; dLS, late-Spring period at dusk; dMS, mid-Summer period at dusk.

Table 3. *F*- and *P*-values of the ANOVA for compounds and chemical groups that showed significant variations.

Compound	*F*	*P*	Compound	*F*	*P*
Significant seasonal variation					
alloaromadendrene[LS]	5.52	0.032	myrtenol[MS]	16.34	0.001
β-bisabolene + unidentified 6[LS]	13.27	0.002	neral[MS]	12.88	0.003
α-campholenal[MS]	26.41	<0.001	neryl acetate[MS]	4.89	0.042
(*E*)-carveol[MS]	21.35	<0.001	nopinone[MS]	60.24	<0.001
caryophyllene oxide isomer 2[MS]	14.77	0.001	(*E*)-β-ocimene[LS]	10.41	0.005
caryophyllene oxide isomer 3[MS]	7.23	0.016	(*Z*)-β-ocimene[LS]	6.06	0.026
β-curcumene[LS]	8.82	0.009	pentyl acetate[LS]	11.97	0.003
γ-curcumene[LS]	5.04	0.039	(*E*)-pinocarveol[MS]	13.04	0.002
p-cymene[MS]	12.08	0.003	pinocarvone[MS]	22.91	<0.001
dihydrobenzene[LS]	6.69	0.020	sabina ketone[MS]	10.11	0.006
(*E*,*E*)-α-farnesene[LS]	15.95	0.001	sabinene hydrate[MS]	7.22	0.016
germacrene D[LS]	8.96	0.009	terpinen-4-ol[MS]	5.46	0.033
β-gurjunene[LS]	6.43	0.022	3,6,6-trimethylnorpinan-2-one[MS]	5.36	0.034
(*Z*)-3-hexenol[LS]	6.63	0.020	unidentified 1[LS]	6.39	0.022
(*Z*)-3-hexenyl acetate[LS]	7.95	0.012	unidentified 3[LS]	6.94	0.018
(Z)-3-hexenyl benzoate[LS]	47.40	<0.001	α-zingiberene[LS]	4.83	0.043
(*Z*)-3-hexenyl butanoate[LS]	44.09	<0.001			
hexyl acetate[LS]	12.43	0.003	Groups		
α-longipinene[LS]	5.35	0.034	*aliphatics*[LS]	8.98	0.009
methyl salicylate[LS]	5.17	0.037	*benzenoids*[LS]	8.86	0.009
β-myrcene[LS]	12.11	0.003	*hydrocarbon monoterpenes*[LS]	6.79	0.019
myrtenal[MS]	38.43	<0.001	*oxygenated sesquiterpenes*[MS]	13.58	0.002
Significant diel variation					
bornyl acetate[D]	5.57	0.031	6-methyl-5-hepten-2-one[D]	7.39	0.015
α-campholenal[D]	5.51	0.032	myrtenal[D]	15.02	0.001
caryophyllene oxide isomer 3[D]	6.98	0.018	ethyl octanoate[D]	9.18	0.008
γ-curcumene[D]	5.27	0.036	neral[D]	4.54	0.049
geranyl acetate[D]	18.40	<0.001	pinocarvone[D]	6.44	0.022
linalool[D]	6.22	0.024	3,6,6-trimethylnorpinan-2-one[M]	7.44	0.015
Significant interaction					
decanal	11.02	0.004	nonanal	7.44	0.015
(*Z*)-3-hexenyl 2-methylbutanoate	4.55	0.049			

n = 5, *df* error = 16, and *df* = 1, for all compounds. [LS] More abundant in late-Spring than in mid-Summer; [MS] More abundant in mid-Summer than in late-Spring; [D] More abundant at dusk than in the morning; [M] More abundant in the morning than at dusk.

GC-EAD with synthetic compounds confirmed the activity of alloocimene, (*Z*)-3-hexenol + nonanal, linalool, (*E*)-β-caryophyllene, (*E*)-β-farnesene, (*E*,*E*)-α-farnesene, and caryophyllene oxide. The average responses to these standards were 0.029, 0.040, 0.038, 0.024, 0.024, 0.047 and 0.043 mV, respectively. No pure synthetics were available for (*E*)-β-ocimene, pinocarvone, and germacrene D.

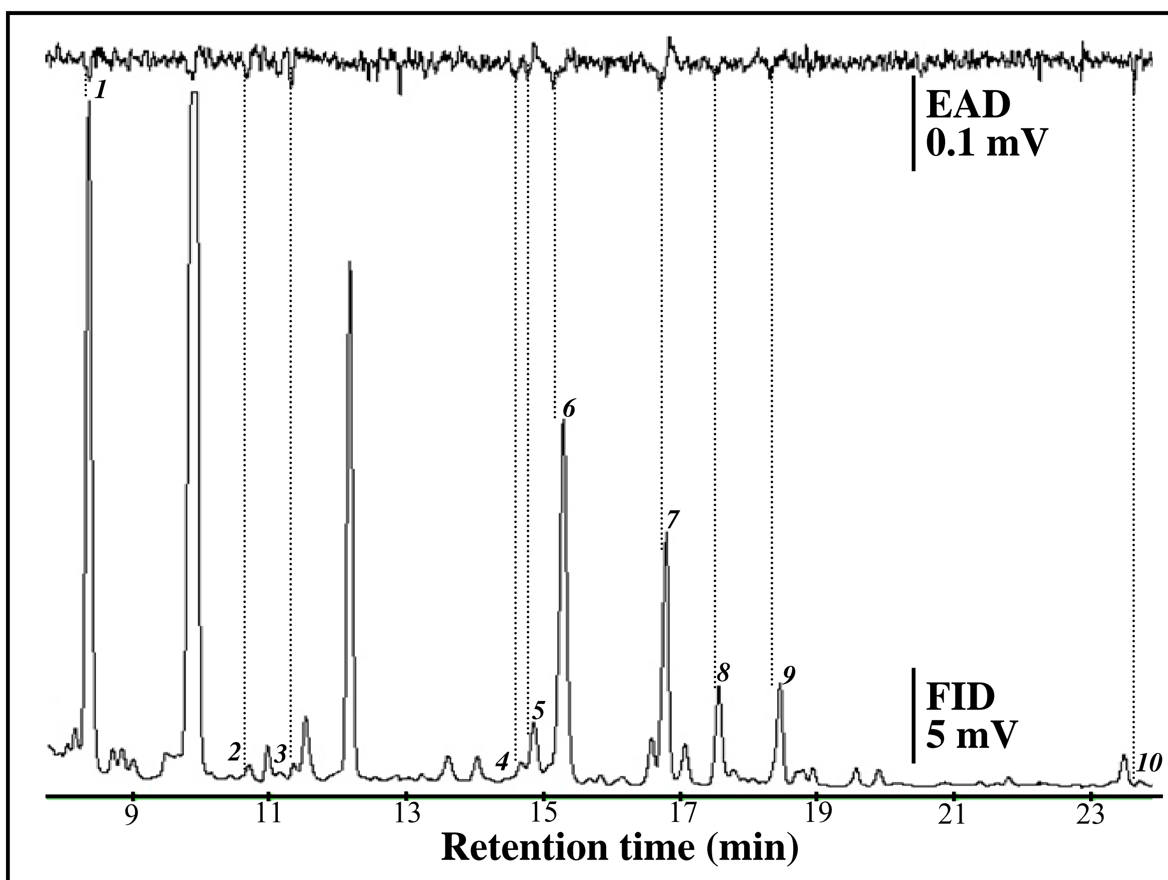

Figure 2. GC-FID (bottom) and GC-EAD (top) traces of a volatile collection from a walnut tree *in situ*, made during the morning in mid-Summer, using the antenna of a male *C. pomonella*. Peaks of compounds that produced discernible and repetitive EAD responses are labeled. 1) (*E*)-β-ocimene, 2) alloocimene, 3) (*Z*)-3-hexenol + nonanal, 4) linalool, 5) pinocarvone, 6) (*E*)-β-caryophyllene, 7) (*E*)-β-farnesene, 8) germacrene D, 9) (*E*,*E*)-α-farnesene + 2-cyclopentylcyclopentanone and 10) caryophyllene oxide.

4. Discussion

The high emission rate of hydrocarbon terpenes by walnut trees that we have observed agrees with the existing literature. β-Pinene was the most emitted compound in our assay, and it also appears as a highly emitted compound in the literature. However caryophyllene, (*E*)-4,8-dimethyl-1,3,7-nonatriene, and a β-farnesene isomer are referred as the main compounds in other studies.[29-31] All these compounds were also abundant in our collections, as well as (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, limonene, germacrene D, 1,8-cineole, sabinene, (*E*)-β-farnesene and (*E*)-β-caryophyllene, which were also present in significant amounts in the previous studies.[29-31]

Differences amongst studies can be due to multiple factors, such as adsorbent affinity, extraction procedure, type and amount of herbivore damage, climatic conditions, or soil substrate.[16,32-37] A close related species, *Juglans nigra* L., emits as main compounds α-pinene, sabinene and β-pinene.[38]

Seasonal period significantly affected volatile emissions from walnut trees, as it has been previously reported for many plant species.[12,19,39-42] Overall variation was mainly qualitative, as no significant variation in total emission was found, though a tendency to decrease from late-Spring to mid-Summer can be perceived. However the emission of many single compounds was affected by the seasonal period, leading to a variation in their relative proportions. Variation in emission rates can be due to several abiotic factors, such as light intensity, photoperiod or temperature, as well as to the tree phenology.[12,43-44]

In our study most of the compounds that showed diel variations were oxygenated both terpenes and aliphatics, and they were usually emitted in higher amounts at dusk. These compounds have a low gas-aqueous-phase partition coefficient and their emission should be strongly affected by stomatal closure.[45] We would expect a higher stomatal closure under high temperatures in Spanish summer midday (temperature ranged from 25.2 to 33.8ºC in our morning-collections) than under the milder early evening temperatures (from 20 to 28.5ºC in our dusk-collections), and this would explain the lower emission of these compounds in our morning samples. However, to confirm this hypothesis stomatal conductance data would be necessary. Two compounds do not fit with this explanation: γ-curcumene, a non-oxygenated sesquiterpene that was found at higher amounts at dusk than in the morning, and 3,6,6-trimethylnorpinan-2-one, an oxygenated monoterpene that was found in lower amounts at dusk than in the morning. Other physiological mechanisms different from stomatal closure should be involved in the emission pattern of these 2 compounds. Similarly, in *Quercus ilex* L., the emission of the main hydrocarbon monoterpenes started to fall down above 40ºC, whereas ocimene isomers dramatically increased from 35 to 45ºC.[46] The authors suggested that an alteration of the product pattern of monoterpene biosynthesis may be involved. Thus, in our case, factors such as light intensity and/or temperature could be involved in the biosynthesis and/or metabolism processes of γ-curcumene and 3,6,6-trimethylnorpinan-2-one.

Variation in emissions between morning and dusk lead to a qualitative variation of the odor-blend, this variation must be taken into account when designing blends to be tested for

insect attraction, as proportions established between ubiquitous volatiles seem to be responsible of host finding by phytophagous insects, rather than species-specific compounds.[47]

The emission profile of walnut tree differs widely from that of apple, the most studied *C. pomonella* host, both qualitatively and quantitatively. In a study similar to the current one, total volatile emission of apple ranged from 2,393.4 to 5,377.9% IS (using the same methodology),[19] and emissions were dominated by aliphatics and two sesquiterpenes: (*E*,*E*)-α-farnesene and germacrene D. In the present study, walnut average total emissions ranged from 24,226.5 to 48,936.4% IS, that is between 10 and 25-fold times apple emissions in our apple study,[19] and hydrocarbon terpenes in walnut were present in higher amounts than in apple. Despite these large differences, many of the major and minor compounds as limonene, (*Z*)-3-hexenyl acetate, (*Z*)-3-hexenol, nonanal, decanal, (*E*)-β-caryophyllene, germacrene D and (*E*,*E*)-α-farnesene are shared by both host species.

We obtained weak responses in our GC-EAD analyses of field collections, ranging between 0.012 and 0.038 mV. This responses were improved when we injected a blend of synthetics, ranging from 0.024 to 0.047 mV, due to the higher amounts present on this blend for most of the compounds. Nevertheless, the responses that have been obtained in similar previous studies are also weak, and of similar order of magnitude.[12,19]

Most of the EAD-active compounds that have been identified in this study were already known to be either electrophysiologically- or behaviorally-active in *C. pomonella*, and they are also emitted by apple. (*E*,*E*)-α-Farnesene is one of the main volatiles of apple headspace,[12-13,19] and it is widely known to generate both antennal[12-13,19] and behavioral responses in *C. pomonella*.[11,48-50] (*E*)-β-Farnesene has been reported to generate significant EAG responses,[12-13,15,19] and to be attractive to males in the wind tunnel when combined with (*E*,*E*)-α-farnesene,[50] and in the field by itself.[50-51] This compound is also known to act as a synergist of the sex pheromone.[17] (*Z*)-3-Hexenol, and linalool are also known as EAD-active compounds.[12,15,19] These 2 compounds synergize the attraction of males by sex pheromone.[51] Nonanal, germacrene D, and (*E*)-β-caryophyllene have been reported to generate significant EAG responses.[12-13,15,19] To our knowledge, no behavioral effects on *C. pomonella* individuals have been reported for germacrene D. (*E*)-β-Caryophyllene has been shown to be attractive for mated females,[18] and nonanal has been reported as a minor component in a *C. pomonella* larval aggregation pheromone,[52] but repellent to mated females.[18]

To our knowledge there are no reports of *C. pomonella* EAG responses to (*E*)-β-ocimene, alloocimene, pinocarvone or caryophyllene oxide. Of these 4 compounds only (*E*)-β-ocimene is also emitted by apple.[12,19] β-Pinene has been recently found to act as a repellent to mated females.[18] Interestingly this is the predominant compound in our walnut tree samples and has been found in high amounts in other similar studies.[29-31] Furthermore we did not found discernible EAD responses in our study to β-pinene, and to our knowledge there are not previous reports in this sense.

Amongst the EAD-active compounds in the current study, linalool, pinocarvone, and caryophyllene oxide were emitted in significantly higher amounts at dusk than in the morning, this was also true for nonanal in the LS period. A tendency to be more emitted at dusk than in the morning was also observed for alloocimene, (*Z*)-3-hexenol, (*E*)-β-caryophyllene, (*E*)-β-farnesene, germacrene D, and (*E*,*E*)-α-farnesene, although there were not significant differences.

In a previous study using the same methodology,[19] we found a compound emitted by the oven bags, 2-cyclopentylcyclopentanone, to generate electroantennographic responses, both in GC-EAD analyses of collections, and in EAG analyses with pure compound. As expected, we also found this compound in the volatile collections in the current study, and EAD-responses to it were observed, too.

Our EAD-data was obtained by using a population of *C. pomonella* native from apple, and this must be taken into account when considering our results. Phillips and Barnes found that wild populations coming from apple strongly preferred apple for oviposition, whereas those coming from walnut and Japanese plum (*Prunus salicina* Lindley) showed a preference for walnut.[53] Furthermore, *C. pomonella* wild populations from pear and walnut increased oviposition in response to walnut volatiles, but a wild population from apple did not.[54] Thus it is possible that a walnut population would have responded differently to our samples. However, apple is the most economically important host of *C. pomonella*, and the responsiveness of apple races to other plant host can be helpful to improve control strategies.

In conclusion, walnut headspace contains a high number of compounds which are not walnut-specific but also emitted by many other plant species, including apple. Walnut volatile blend differs greatly from that of apple, both qualitatively and quantitatively.[19] Despite marked differences between both species, they share an important number of compounds. The antenna of *C. pomonella* males respond to a series of walnut volatiles also emitted by apple, but also to 3

walnut volatiles that are absent in apple emissions: alloocimene, pinocarvone, and caryophyllene oxide. The only commercial kairomone for *C. pomonella* is the pear ester [ethyl (*E*,*Z*)-decadienoate],[20] which is emitted only by pear, nor by apple neither by walnut. When used to capture individuals in the field, this compound works better in walnut (Juglandaceae) than in apple or pear (Rosaceae) orchards.[20] In this sense, alloocimene, pinocarvone and caryophyllene oxide are interesting candidate attractants for *C. pomonella* in apple orchards. The behavioral effects of these 3 compounds must be tested in laboratory and field tests, as a direct relationship between electrophysiological and behavioral responses does not necessarily occur.

Acknowledgements

This work was partially funded by project RTA01-079 from the Programa Nacional de Recursos y Tecnologías Agroalimentarias (Spain) and by projects AGL2003-06599-CO02-02 and AGL2004-05812/AGR from the Plan Nacional de I+D+I (Spain). D. Casado has a fellowship from MECD (Spain). C. Gemeno is funded by the Programa Ramón y Cajal (MCYT, Spain).

5. References

1. Jutsum AR and Gordon RFS, *Insect Pheromones in Plant Protection*. Biddles Ltd., Guildford (1989).

2. Khan ZR, Pickett JA, van den Berg JA, Wadhams LJ and Woodcock CM, Exploiting chemical ecology and species diversity: stem borer and striga control for maize and sorghum in Africa. *Pest Manag Sci* **56**:957-962 (2000).

3. James DG, Field evaluation of herbivore-induced plant volatiles as attractants for benefical insects: methyl salicylate and the green lacewing, *Chrysopa nigricornis*. *J Chem Ecol* **29**:1601-1609 (2003).

4. Reddy GVP, Cruz ZT, Bamba J and Muniappan R, Host adaptation of the fruit piercing moth, *Eudocima fullonia*. *Physiol Entomol* **30**:398-401 (2005).

5. Roelofs W, Comeau A, Hill A and Milicevic G, Sex attractant of the codling moth: characterization with electroantennogram technique. *Science* **174**:297-299 (1971).

6. Charmillot PJ, Mating disruption technique to control codling moth in Western Switzerland, in *Behavior-Modifying Chemicals for Insect Management*, ed. by Ridgway RL, Silverstein RM and Inscoe MN. Marcel Dekker Inc., New York, pp. 165-182 (1990).

7. Pfeiffer DG, Kaakeh W, Killian JC, Lachance MW and Kirsch P, Mating disruption for control of damage by codling moth in Virginia apple orchards. *Entomol Exp Appl* **67**:57-64 (1993).

8. Charmillot PJ, Hofer D and Pasquier D, Attract and kill: a new method for control of the codling moth *Cydia pomonella*. *Entomol Exp Appl* **94**:211-216 (2000).

9. Calkins CO and Faust RJ, Overview of areawide programs and the program for suppression of codling moth in the western USA directed by the United States Department of Agriculture - Agricultural Research Service. *Pest Manag Sci* **59**:601-604 (2003).

10. Gut L and Brunner JF, Implementing codling moth mating disruption in Washington pome fruit orchards. Tree Fruit Research Extension Center Information Series (1). Washington State University, Wenatchee, (1996).

11. Hern A and Dorn S, Sexual dimorphism in the olfactory orientation of adult *Cydia pomonella* in response to α-farnesene. *Entomol Exp Appl* **92**:63-72 (1999).

12. Bengtsson M, Bäckman AC, Liblikas I, Ramirez MI, Borg Karlson AK, Ansebo L, Anderson P, Löfqvist J and Witzgall P, Plant odor analysis of apple: antennal response of codling moth females to apple volatiles during phenological development. *J Agric Food Chem* **49**:3736-3741 (2001).

13. Bäckman AC, Bengtsson M, Borg Karlsson AK, Liblikas I and Witzgall P, Volatiles from apple (*Malus domestica*) eliciting antennal responses in female codling moth *Cydia pomonella* (L.) (Lepidoptera: Tortricidae): effect of plant injury and sampling technique. *Z Naturforsch C* **56**:262-268 (2001).

14. Hern A and Dorn S, A female-specific attractant for the codling moth, *Cydia pomonella* (L.), from apple fruit volatiles. *Naturwissenschaften* **91**:77-80 (2004).

15. Ansebo L, Coracini MDA, Bengtsson M, Liblikas I, Ramírez M, Borg-Karlson AK, Tasin M and Witzgall P, Antennal and behavioural response of codling moth *Cydia pomonella* to plant volatiles. *J Appl Entomol* **128**:488-493 (2004).

16. Hern A and Dorn S, Induction of volatile emissions from ripening apple fruits infested with *Cydia pomonella* and the attraction of adult females. *Entomol Exp Appl* **102**:145-151 (2002).

17. Yang Z, Bengtsson M and Witzgall P, Host plant volatiles synergize response to sex pheromone in codling moth, *Cydia pomonella*. *J Chem Ecol* **30**:619-629 (2004).

18. Vallat A and Dorn S, Changes in volatile emissions from apple trees and associated response to adult female codling moths over the fruit-growing season. *J Agric Food Chem* **53**:4083-4090 (2005).

19. Casado D, Gemeno C, Avilla J and Riba M, Day-night and phenological variation of apple tree volatiles and electroantennogram responses of *Cydia pomonella* (Lepidoptera: Tortricidae). *Environ Entomol* **35**:258-267 (2006).

20. Light DM, Knight AL, Henrick CA, Rajapaska D, Lingren B, Dickens JC, Reynolds KM, Buttery RG, Merrill G, Roitman J and Campbell BC, A pear-derived kairomone with pheromonal potency that attracts male and female codling moth, *Cydia pomonella* (L.). *Naturwissenschaften* **88**:333-338 (2001).

21. Agelopoulos NG, Hooper AM, Maniar SP, Pickett JA and Wadhams LJ, A novel approach for isolation of volatile chemicals released by individual leaves of a plant in situ. *J Chem Ecol* **25**:1411-1425 (1999).

22. Vuorinen T, Nerg AM, Vapaavuori E and Holopainen JK, Emission of volatile organic compounds from two silver birch (*Betula pendula* Roth) clones grown under ambient and elevated CO_2 and different O_3 concentrations. *Atmos Environ* **39**:1185-1197 (2005).

23. Pons S, Eizaguirre M, Sarasúa MJ and Avilla J, Influencia del fotoperiodo sobre la inducción de diapausa de *Cydia pomonella* (Lepidoptera: Tortricidae) en laboratorio y campo. *Invest Agr Prod Prot Veg* **9**:477-491 (1994).

24. Eras J, Méndez JJ, Balcells M and Canela R, Chlorotrimethylsilane: a suitable reagent for synthesis of chlorhydrin esters. *J Org Chem* **67**:8631-8634 (2002).

25. Adams RP, *Identification of Essential Oil Components by Gas Chromatography/Mass Spectroscopy*. Allured Publishing Corporation, Carol Stream, USA (1995).

26. Zheng CH, Kim TH, Kim KH, Leem YH and Lee HJ, Characterization of potent aroma compounds in *Chrysanthemum coronarium* L. (Garland) using aroma extract dilution analysis. *Flavour Frag J* **19**:401-405 (2004).

27. Yu EJ, Kim TH, Kim KH and Lee HJ, Characterization of aroma-active compounds of *Abies nephrolepis* (Khingan fir) needles using aroma extract dilution analysis. *Flavour Frag J* **19**:74-79 (2004).

28. Njoroge SM, Koaze H, Karanja PN and Sawamura M, Essential oil constituents of three varieties of Kenyan sweet oranges (*Citrus sinensis*). *Flavour Frag J* **20**:80-85 (2005).

29. Buttery RG, Flath RA, Mon TR and Ling LC, Identification of germacrene D in walnut and fig leaf volatiles. *J Agric Food Chem* **34**:820-822 (1986).

30. Buttery RG, Light DM, Nam Y, Merrill GB, Roitman JN and Nam YL, Volatile components of green walnut husks. *J Agric Food Chem* **48**:2858-2861 (2000).

31. Henneman ML, Dyreson EG, Takabayashi J and Raguso RA, Response to walnut olfactory and visual cues by the parasitic wasp *Diachasmimorpha juglandis*. *J Chem Ecol* **28**:2221-2244 (2002).

32. Boevé JL, Lengwiler U, Tollsten L, Dorn S and Turlings TCJ, Volatiles emitted by apple fruitlets infested by larvae of the European apple sawfly. *Phytochemistry* **42**:373-381 (1996).

33. Raguso RA and Light DM, Electroantennogram responses of male *Sphinx perelegans* hawkmoths to floral and 'green-leaf volatiles'. *Entomol Exp Appl* **86**:287-293 (1998).

34. Kainulainen P, Nissinen A, Piirainen A, Tiilikkala K and Holopainen JK, Essential oil composition in leaves of carrot varieties and preference of specialist and generalist sucking herbivores. *Agric Forest Entomol* **4**:211-216 (2002).

35. Stashenko EE, Jaramillo BE and René Martínez J, Comparison of different extraction methods for the analysis of volatile secondary metabolites of *Lippia alba* (Mill.) N.E. Brown, grown in Colombia, and evaluation of its in vitro antioxidant activity. *J Chromatogr A* **1025**:93-103 (2004).

36. Vallat A, Gu H and Dorn S, How rainfall, relative humidity and temperature influence volatile emissions from apple trees in situ. *Phytochemistry* **66**:1540-1550 (2005).

37. Ormeño E, Bousquet-Mélou A, Mévy JP, Greff S, Robles C, Bonin G and Fernandez C, Effect of intraspecific competition and substrate type on terpene emissions from some mediterranean plant species. *J Chem Ecol* **33**:277-286 (2007).

38. Buchbauer G, Jirovetz L, Wasicky M and Nikiforov A, Headspace constituents of fresh *Juglans nigra* L. peels. *J Essent Oil Res* **5**:455-457 (1993).

39. Guenther A, Seasonal and spatial variations in natural volatile organic compound emissions. *Ecol Appl* **7**:34-45 (1997).

40. Staudt M, Bertin N, Hansen U, Seufert G, Ciccioli P, Foster P, Frenzel B and Fugit L, Seasonal and diurnal patterns of monoterpene emissions from *Pinus pinea* (L.) under field conditions. *Atmos Environ* **31**:145-156 (1997).

41. Owen SM, Boissard C and Hewitt CN, Volatile organic compounds (VOCs) emitted from 40 Mediterranean plant species: VOC speciation and extrapolation to habitat scale. *Atmos Environ* **35**:5393-5409 (2001).

42. Rapparini F, Baraldi R and Facini O, Seasonal variation of monoterpene emission from *Malus domestica* and *Prunus avium*. *Phytochemistry* **57**:681-687 (2001).

43. Robertson GW, Griffiths DW, Woodford JAT and Birch ANE, Changes in the chemical-composition of volatiles released by the flowers and fruits of the red raspberry (*Rubus idaeus*) cultivar Glen Prosen. *Phytochemistry* **38**:1175-1179 (1995).

44. Hakola H, Rime J and Laurila T, The hydrocarbon emission rates of tea-leafed willow (*Salix phylicifolia*), silver birch (*Betula pendula*) and European aspen (*Populus tremula*). *Atmos Environ* **32**:1825-1833 (1998).

45. Niinemets U, Loreto F and Reichstein M, Physiological and physicochemical controls on foliar volatile organic compound emissions. *Trends Plant Sci* **9**:180-186 (2004).

46. Staudt M and Bertin N, Light and temperature dependence of the emission of cyclic and acyclic monoterpenes from holm oak (*Quercus ilex* L.) leaves. *Plant Cell Environ* **21**:385-395 (1998).

47. Bruce TJA, Wadhams LJ and Woodcock CM, Insect host location: a volatile situation. *Trends Plant Sci* **10**:269-274 (2005).

48. Sutherland ORW and Hutchins RFN, Attraction of newly hatched codling moth larvae (*Laspeyresia pomonella*) to synthetic stereo-isomers of farnesene. *J Insect Physiol* **19**:723-727 (1973).

49. Wearing CH and Hutchins RFN, α-Farnesene, a naturally occurring oviposition stimulant for the codling moth, *Laspeyresia pomonella*. *J Insect Physiol* **19**:1251-1256 (1973).

50. Coracini MDA, Bengtsson M and Witzgall P, Attraction of codling moth males *Cydia pomonella* to apple volatiles. *Entomol Exp Appl* **100**:1-10 (2004).

51. Yang Z, Casado D, Ioratti C, Bengtsson M and Witzgall P, Pheromone pre-exposure and mating modulate codling moth (Lepidoptera: Tortricidae) response to host plant volatiles. *Agric Forest Entomol* **7**:231-236 (2005).

52. Jumean Z, Gries R, Unruh T, Rowland E and Gries G, Identification of the larval aggregation pheromone of codling moth, *Cydia pomonella*. *J Chem Ecol* **31**:911-924 (2005).

53. Phillips PA and Barnes MM, Host race formation among sympatric apple, walnut, and plum populations of the codling moth, *Laspeyresia pomonella*. *Ann Entomol Soc Am* **68**:1053-1060 (1975).

54. Witzgall P, Ansebo L, Yang Z, Angeli G, Sauphanor B and Bengtsson M, Plant volatiles affect oviposition by codling moths. *Chemoecology* **15**:77-83 (2005).

CHAPTER V

Agricultural and Forest Entomology (2005) **7**, 231–236

Pheromone pre-exposure and mating modulate codling moth (Lepidoptera: Tortricidae) response to host plant volatiles

Zhihua Yang, Daniel Casado*, Claudio Ioriatti†, Marie Bengtsson and Peter Witzgall

*Chemical Ecology Group, Department of Crop Science, Swedish University of Agricultural Sciences, Box 44, 230 53 Alnarp, Sweden, *Departament Protecció de Conreus, Centre R+D UdL-IRTA, 25198 Lleida, Spain and †Laboratorio di difesa delle colture, Istituto Agrario, 38010 San Michele a/A, Italy*

Abstract

1 Two codling moth *Cydia pomonella* kairomonal attractants, ethyl (*E,Z*)-2,4-decadienoate (pear ester) and (*E*)-β-farnesene, were tested in an insecticide-sprayed apple orchard and an orchard treated for mating disruption with synthetic pheromone (*E,E*)-8,10-dodecadienol (codlemone). Male captures with pear ester were higher in the pheromone-treated than in the insecticide-treated orchard, whereas captures with (*E*)-β-farnesene were not different. Subsequent wind tunnel experiments confirmed that pre-exposure to sex pheromone codlemone increased the behavioural response of codling moth males to pear ester. This supports the idea that male attraction to the plant volatile pear ester and sex pheromone codlemone is mediated through the same sensory channels.

2 Pear ester is a bisexual codling moth attractant and even captures of female moths were significantly increased in the pheromone-treated orchard. In the laboratory wind tunnel, pheromone pre-exposure had no effect on female response to pear ester, but significantly more mated than unmated codling moth females flew upwind towards a pear ester source. Differences in mating status in insecticide-treated vs. pheromone-treated orchards may thus account for the differences in female trap captures with pear ester.

3 These findings are important with respect to monitoring of codling moth with pear ester in mating disruption orchards. They also emphasize the importance of host plant volatiles in pheromone-mediated mating disruption, which has been neglected to date.

Keywords Host plant volatile, kairomone, Lepidoptera, mating disruption, pheromone pre-exposure, Tortricidae, wind tunnel.

Introduction

Odour cues play an essential role in insect reproductive behaviour. These odours include pheromones released by conspecifics before mating, as well as host plant volatiles that lead the way to suitable mating and oviposition sites. In nature, sex pheromones and plant volatiles are always perceived simultaneously and they interact to enhance mate finding and reproductive isolation (Harrewijn *et al*., 1994; Landolt & Phillips, 1997; Schoonhoven *et al*., 1998).

Codling moth *Cydia pomonella* males show the same upwind flight behaviour in response to pheromone and plant volatiles, and females fly upwind to green apples (Witzgall *et al*., 1999b; Coracini *et al*., 2004). Apple volatiles have also been shown to synergize attraction of codling moth males to the main pheromone compound codlemone (*E,E*)-8,10-dodecadien-1-ol (Yang *et al*., 2004). A similar behavioural sequence in response to plant volatiles and pheromones, and a synergistic effect between them, suggests that common sensory and neural pathways are involved in the perception and processing of these signals (Isman, 1992).

Correspondence: Peter Witzgall, Chemical Ecology Group, Department of Crop Science, Swedish University of Agricultural Sciences, Box 44, 230 53 Alnarp, Sweden. Tel.: +46 40 415307; fax: +46 40 461991; e-mail: peter.witzgall@phero.net

© 2005 The Royal Entomological Society

It is well-known that pre-exposure to female sex pheromone modulates male responsiveness to pheromone point sources (Linn & Roelofs, 1981; Figueredo & Baker, 1992; Liu & Haynes, 1993; Anderson *et al.*, 2003). However, the effect of pheromone pre-exposure on the response to host plant volatiles has not been studied to date. Pear ester, ethyl (*E,Z*)-2,4-decadienoate, is a bisexual codling moth attractant (Light *et al.*, 2001). Attraction of codling moth females makes this compound particularly feasible for monitoring in mating disruption orchards (Ioriatti *et al.*, 2003). The present study reports the effect of pre-exposure to sex pheromone and the effect of mating on the response of codling moth to pear ester and another kairomonal attractant (*E*)-β-farnesene (Coracini *et al.*, 2004).

Materials and methods

Insects

Codling moths reared on semiartificial diet (Mani *et al.*, 1978) were interbred each summer with wild moths collected in apple orchards in Scania (Sweden). Adults were sexed daily, and they were kept in plexiglass cages (33 × 33 × 33 cm) at 22–24 °C, under an LD 18 : 6 h photoperiod. Males and females used in the wind tunnel were 3 days old, and females were mated on the day after eclosion.

Chemicals

Codlemone (*E,E*)-8,10-dodecadien-1-ol (*E*8,*E*10-12OH) was purchased from Pherobank (the Netherlands), chemical and isomeric purity were >99.5 and >99.8%, respectively, by gas chromatography. (*E*)-β-Farnesene (92.4% pure) was purchased from Bedoukian Research Inc. (Danbury, CT). Pear ester ethyl (*E,Z*)-2,4-decadienoate (87.3% pure, containing the other geometric isomers, but without traces of codlemone *E*8,*E*10-12OH) was a gift from P. Kirsch (IPM Technologies Inc., Portland, OR).

Field tests

Codling moths were trapped with two plant volatile compounds in an insecticide-treated and a pheromone-treated orchard. Tetra traps (Arn *et al.*, 1979) baited with rubber septa, containing 10 mg pear ester or 10 mg (*E*)-β-farnesene, were hung at approximately 2 m from the ground to green apple branches. Traps were approximately 10 m apart, and they were placed at random in a line along tree rows ($n = 5$). Traps were checked seven times, during the second codling moth flight period from mid July to beginning of September 2003.

Two orchards were used: (i) a 20-ha conventional, insecticide-treated orchard (cv. Golden Delicous) and (ii) a 6-ha pheromone-treated orchard (cv. Golden Delicous), near Lleida (Spain). The orchard was treated with 300 Checkmate CM WS dispensers/ha (Trécé, Adair, OK), containing 270 mg codlemone per dispenser.

Wind tunnel

Males and unmated and mated females were pre-exposed to pheromone, or to pear ester, and flown to single sources of plant volatiles and sex pheromone. The wind tunnel had a flight section of 63 × 90 × 200 cm and was lit diffusely from above at 6 lux. The upwind end of glass tubes holding the males or females was approximately 180 cm downwind from the source. Wind speed was 30 cm/s, and temperature was in the range 20–22 °C (Witzgall *et al.*, 2001).

Red rubber septa (VWR International, Sweden) were formulated with 1 or 100 μg codlemone, 10 mg pear ester, or 10 mg (*E*)-β-farnesene in a 1 : 1 mixture of heptane and ethanol (VWR International). The septa were kept during 24 h in a hood before tests and were then stored at −20 °C between tests. Rubber septa loaded with pheromone were held in the centre of glass cylinders (10 × 10 cm) covered by a metal screen, at the upwind end of the tunnel, 40 cm from the ground. For tests with two septa, two cylinders were placed side by side.

Males and females were tested on different days. Tests with females started approximately 0.5 h after onset of the dark period and lasted 1 h. Ten females were placed individually in glass tubes (2 × 12.5 cm), stoppered with gauze, approximately 10 min before testing. Females were released individually and allowed 3 min to respond. Each test was replicated six times on different days ($n = 60$). Tests with males started 1 h after lights off and lasted 2–3 h. Males were also released individually. Each test was replicated four times on different days, using 15 males ($n = 60$), which were given 2 min to respond. The following types of behaviour were recorded: activation (walking and wing-fanning) (A), taking flight (F), flying upwind over 50, 100 and 150 cm towards the source (50; 100; 150), touching the source (T) and landing at the source (L).

For pheromone-exposure in the laboratory, one pheromone dispenser, a polyethylene rope containing 113 mg of codlemone, 64 mg of dodecanol and 13 mg of tetradecanol (Shin-Etsu Chemical Co., Japan), was placed into a cage containing males or females, approximately 24 h before the moths were used for wind tunnel tests. The dispenser was enclosed in a metal mesh that precluded insects from touching it. For pre-exposure with pear ester, five rubber septa each containing 20 mg pear ester, were placed into cages containing males or females, approximately 24 h before tests.

Statistical analysis

Trap captures, and the numbers of insects within one test batch of 10 females or 15 males (naive and pheromone-exposed, mated and unmated) responding to a given odour source were transformed to $\log(x + 1)$ and were statistically evaluated by Student's *t*-test (InStat, 2003).

Results

Field trapping test

Codling moth captures in traps baited with 10 mg pear ester or 10 mg (*E*)-β-farnesene were recorded in an apple

© 2005 The Royal Entomological Society,

orchard treated with organophosphate insecticide, and in an apple orchard treated with sex pheromone, at a rate of 81 g codlemone/ha and 300 dispenser/ha, during the second seasonal flight period of codling moth.

With β-farnesene, captures in the two orchards were not significantly different: 0.67 ± 0.43 males/trap/week were trapped in the pheromone-treated orchard vs. 2.6 ± 1.73 males/trap/week in the insecticide-treated orchard ($P = 0.5272$, d.f. = 8, $n = 5$). With pear ester, 9.77 ± 2.86 males/trap/week were captured in the pheromone-treated orchard, which was significantly more than in the conventional orchard (0.27 ± 0.11 males/trap/week; $P < 0.0001$, $t = 7.606$, d.f. = 8, $n = 5$). Codling moth females were not trapped with β-farnesene. Pear ester captured 0.1 ± 0.07 females/trap/week in the insecticide orchard, and significantly more (4.83 ± 1.53 females/trap/week) were captured in the pheromone orchard ($P < 0.0001$, $t = 7.306$, d.f. = 8, $n = 5$).

It is difficult to compare codling moth population densities in orchards under insecticide and pheromone treatment. Pheromone treatment precludes the use of pheromone traps for population monitoring, and sprays with organophosphates target hatching larvae, which are not affected under pheromone treatment. However, the ratio of male captures with β-farnesene and pear ester was 9.75 under insecticide treatment, and 0.07 under mating disruption. This almost inverse ratio of captures with β-farnesene and pear ester, with and without pheromone treatment, is a strong indication that the pheromone treatment affected attractiveness of either one, or of both compounds, disregarding absolute population densities in these two orchards.

Wind tunnel tests

In the wind tunnel, codling moth males started to fly upwind, but were not attracted all the way to a source of pear ester or (*E*)-β-farnesene (Fig. 1A,B). Pre-exposure to sex pheromone codlemone during 24 h significantly increased the number of males that were activated by these two compounds, and 32% pre-exposed males flew upwind over at least 50 cm to pear ester compared with 5% of naïve males ($P = 0.0063$, $t = 4.112$, d.f. = 6; Fig. 1A). By contrast, significantly fewer pre-exposed males flew upwind towards a codlemone source and only 8% males landed compared with 68% control males ($P = 0.0015$, $t = 5.485$, d.f. = 6; Fig. 1C).

Pheromone pre-exposure had no significant effect on the number of females responding to either pear ester or β-farnesene. However, there was a significant difference between the number of unmated and mated females responding to these plant volatiles. Upwind flights over 50 cm, towards pear ester or β-farnesene, increased from 5 and 8% unmated females to 40 and 38% mated females, respectively ($P = 0.0018$, $t = 4.214$, d.f. = 10 and $P = 0.0256$, $t = 2.599$, d.f. = 10; Fig. 1D–G). A few mated females (6%) flew upwind over 150 cm, but none landed at the source.

On the other hand, pre-exposure to pear ester did not have a significant effect on male attraction to pheromone and to a blend of pear ester and pheromone. Blending a 10 000-fold amount of pear ester with codlemone led to a significant decrease in male attraction to the source ($P = 0.0015$, $t = 5.479$, d.f. = 6; Fig. 2). A 1 : 1 and 1 : 100 µg-blend of codlemone and pear ester had no significant effect on male attraction (48 and 43% males landing compared with 45% with 1 µg codlemone alone; $P = 0.3212$, $t = 1.081$, d.f. = 6 and $P = 0.7259$, $t = 0.3674$, d.f. = 6).

Further tests with two sources showed that naïve and pheromone-exposed males were able to distinguish between sources of codlemone and pear ester. Both naïve and pheromone pre-exposed males always landed on the codlemone source (100 µg), which was 10 cm from a pear ester source (10 mg). The presence of the pear ester source slightly augmented the number of males landing, but the difference was not significant (naïve males: 68 and 75% males landing; pre-exposed males: 8 and 10% males landing, without and with a pear ester source, respectively; $P = 0.4082$, $t = 0.8891$, d.f. = 6 and $P = 0.6719$, $t = 0.4450$, d.f. = 6).

Discussion

The plant volatile pear ester, ethyl (*E*,*Z*)-2,4-decadienoate, elicited anemotactic behaviour in codling moth males when presented as a single compound in a wind tunnel. Pre-exposure to sex pheromone increased male response to pear ester in the laboratory (Fig. 1), and a pheromone background in an apple orchard increased male trap captures with pear ester. These results suggest an interconnection of sensory and motor pathways for sex pheromone and plant volatiles in the codling moth (Coracini *et al*., 2004).

Pre-exposure to codlemone during 24 h strongly reduced subsequent upwind orientation to a codlemone source, which is probably due to long-lasting antennal adaptation (Judd *et al*., 2005). However, it is unclear how codlemone-exposure affected the male behavioural response to pear ester. Synergistic interaction of pheromone and a plant volatile compound at the peripheral nervous level has been demonstrated in a noctuid moth (Ochieng *et al*., 2002), and several plant volatiles synergize codling moth attraction to sex pheromone codlemone (Light *et al*., 1993; Yang *et al*., 2004). However, our wind tunnel tests did not show a synergistic effect of pear ester on male attraction to codlemone.

Instead, large amounts of pear ester have a moderate antagonistic effect (Fig. 2), which is in line with the recent finding that there are olfactory neurones on the male antenna that respond to both codlemone and pear ester (De Cristofaro *et al*., 2004; Ansebo *et al*., 2005). A codlemone mimic, *E*10-12OH, which presumably is also perceived via codlemone receptor neurones, has a similar effect on codling moth males as pear ester. It is a weak attractant by itself, but reduces male attraction when blended in large amounts with codlemone. By contrast, other plant volatiles such as α- and β-farnesene, for which

Figure 1 Wind tunnel response of codling moth *Cydia pomonella* males (A–C) and females (D–G) to 10 mg of plant volatiles pear ester ethyl (*E,Z*)-2,4-decadienoate (A, D, F), or (*E*)-β-farnesene (B, E, G), and to 100 μg of sex pheromone codlemone (*E,E*)-8,10-dodecadienol (C). Insects were naïve (shaded line plot; $n = 60$) or pre-exposed to sex pheromone during 24 h (empty bars; $n = 60$). The following behavioural steps were recorded: activation (walking and wing-fanning in release tube, A), taking flight (F), flying upwind over 50, 100 and 150 cm towards the source (50; 100; 150), touching the source (T) and landing at the source (L). Asterisks indicate significant differences within subplots between the response of naïve and pre-exposed insects to the same stimulus; letters indicate differences across subplots D and F, or E and G, between mated and unmated females, for each behaviour ($P < 0.05$, Student's *t*-test).

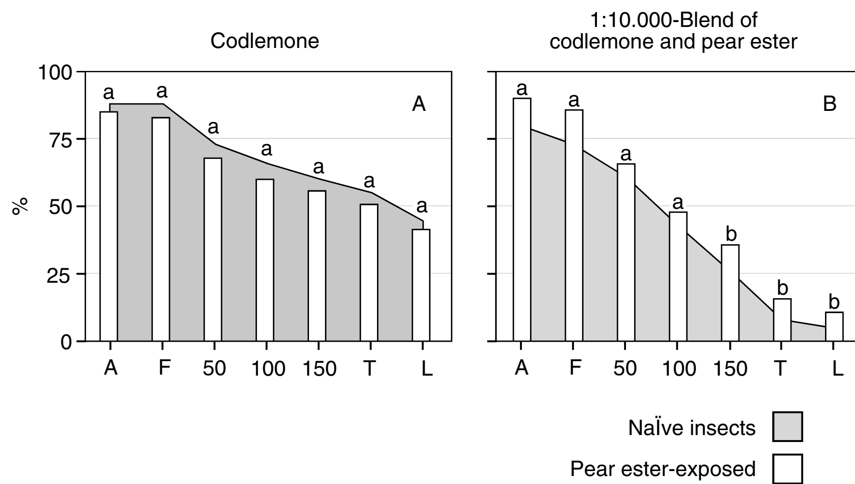

Figure 2 Wind tunnel response of codling moth *Cydia pomonella* males to 1 μg of codlemone (*E,E*)-8,10-dodecadienol (A) and a 1 : 10 000 μg-blend of codlemone and pear ester ethyl (*E,Z*)-2,4-decadienoate (B). Males were naïve (shaded line plot; $n = 60$) or pre-exposed to pear ester during 24 h (open bars; $n = 60$). Letters indicate significant differences between subplots A and B (i.e. the response to codlemone alone and the blend of codlemone and pear ester); the response of naïve and pre-exposed males to the same treatment was not significantly different ($P < 0.05$, Student's *t*-test). The behavioural sequence, from activation (A) to landing (L), is as shown in Fig. 1.

separate receptor neurones have been found, do not reduce upwind flights when blended in large amounts with codlemone (Yang *et al*., 2004; Ansebo *et al*., 2005).

A tentative explanation for the observed increase in trap capture with pear ester in a pheromone-treated orchard and increased upwind flight response in the wind tunnel (Fig. 1) is that exposure to pheromone increases overall sensitivity of codling moth males to plant odours. An octopamine-mediated sensitization of the antennal response to pheromone by previous exposure to plant volatiles has recently been demonstrated in other tortricid moths (Stelinski *et al*., 2003).

Additional sensory cues, presumably other behaviourally active plant volatiles, are necessary to produce male trap captures with pear ester or β-farnesene in the field because these compounds do not attract males all the way to the source in charcoal-filtered wind tunnel air (Coracini *et al*., 2004). This re-emphasizes the role of host volatiles in codling moth mate-finding in pheromone-treated orchards. Aerial concentrations of synthetic codlemone reach approximately 1 ng/m^3, in orchards with very high application rates of 250 g codlemone/ha, which are barely detectable by chemical analysis against a much stronger background of plant volatile compounds (Bäckman, 1997). In pheromone-treated orchards, male codling moths are not attracted to pheromone dispensers, but are observed to fly around the canopy of fruit-bearing apple trees (Witzgall *et al*., 1999a), which clearly indicates that plant volatiles may aid males to locate females in pheromone-permeated orchards. The behavioural mechanisms of mating disruption have attracted much attention (Bartell, 1982; Bengtsson *et al*., 1994; Cardé & Minks, 1995), but the significance of plant volatiles in pheromone-mediated orientation disruption has not been recognized.

Significantly more mated than unmated females responded to pear ester and β-farnesene, whereas pre-exposure to codlemone had no effect at all on the female reponse (Fig. 1). Elevated trap capture of females in the mating disruption orchard compared with the insecticide-treated orchard may reflect different population densities, or differences between populations with respect to mating status and age. The behavioural response of female moths to plant volatiles is intensified by mating and with age (Phelan & Baker, 1987; Rojas, 1999; Mechaber *et al*., 2002), although the underlying mechanism is remains unknown. Volatile host stimuli trigger a pheromone biosynthesis activating neuropeptide, which stimulates pheromone production and release in female moths (Raina, 1993), and also promote ovarian developement (Papaj, 2000). Females in pheromone-treated, unsprayed orchards may, on average, be older, and the proportion of mated females may thus be more elevated than in insecticide-spayed orchards. This needs to be taken into account when interpreting trap captures with pear ester in conventional versus mating disruption orchards.

Acknowledgements

We thank three anonymous reviewers for their constructive critcism. This study was financed by the Carl Trygger Foundation, the Foundation for Strategic Environmental Research (MISTRA), and the Government of the Autonomic Province of Trento, Italy (Bioinnova Research Project).

References

Anderson, P., Sadek, M.M. & Hansson, B.S. (2003) Pre-exposure modulates attraction to sex pheromone in a moth. *Chemical Senses*, **28**, 285–291.

Ansebo, L., Ignell, R., Löfqvist, J. & Hansson, B.S. (2005) Responses to sex pheromone and plant odours by olfactory receptor neurons housed in sensilla auricillica of the codling moth, *Cydia pomonella*. Lepidoptera: Tortricidae). *Journal of Insect Physiology*, doi: 10.1016/j.jinsphys.2005.05.003.

Arn, H., Rauscher, S. & Schmid, A. (1979) Sex attractant formulations and traps for the grape moth *Eupoecilia ambiguella* Hb. *Mitteilungen der Schweizer Entomologischen Gesellschaft*, **52**, 49–55.

Bäckman, A.-C. (1997) Pheromone release by codling moth females and mating disruption dispensers. *IOBC Wprs Bulletin*, **20**, 175–180.

Bartell, R.J. (1982) Mechanisms of communication disruption by pheromone in the control of Lepidoptera: a review. *Physiological Entomology*, **7**, 353–364.

Bengtsson, M., Karg, G., Kirsch, P.A., Löfqvist, J., Sauer, A. & Witzgall, P. (1994) Mating disruption of pea moth *Cydia nigricana* F. (Lepidoptera: Tortricidae) by a repellent blend of sex pheromone and attraction inhibitors. *Journal of Chemical Ecology*, **20**, 871–887.

Cardé, R.T. & Minks, A.K. (1995) Control of moth pests by mating disruption: successes and constraints. *Annual Review of Entomology*, **40**, 559–585.

Coracini, M., Bengtsson, M., Liblikas, I. & Witzgall, P. (2004) Attraction of codling moth males to apple volatiles. *Entomologia Experimentalis et Applicata*, **110**, 1–10.

De Cristofaro, A., Ioriatti, C., Pasqualini, E., Anfora, G., Germinara, G.S., Villa, M. & Rotundo, G. (2004) Electrophysiological responses of codling moth, *Cydia pomonella* to codlemone and pear ester ethyl (*E,IX*)-2,4–decadienoate: peripheral interactions in their perception and evidence for cells responding to both compounds. *Bulletin of Insectology*, **57**, 137–144.

Figueredo, A.J. & Baker, T.C. (1992) Reduction of the response to sex pheromone in the oriental fruit moth, *Grapholita molesta* (Lepidoptera: Tortricidae) following successive pheromonal exposures. *Journal of Insect Behavior*, **5**, 347–363.

Harrewijn, P., Minks, A.K. & Mollema, C. (1994) Evolution of plant volatile production in insect–plant relationships. *Chemoecology*, **5/6**, 55–73.

InStat (2003) *InStat 3 for Macintosh*. GraphPad Software, San Diego, California.

Ioriatti, C., Molinari, F., Pasqualini, E., de Cristofaro, A., Schmidt, S. & Espinha, I. (2003) The plant volatile attractant (*E,IX*)-2,4-ethyl-decadienoate (DA2313) for codling moth monitoring. *Bolletino Di Zoologia Agraria E Di Bachicoltura*, **35**, 127–137.

Isman, M.B. (1992) A physiological perspective. *Insect Chemical Ecology: an Evolutionary Approach* (ed. by by B. D. Roitberg and M. B. Isman), pp. 156–176. Chapman & Hall, New York, New York.

Judd, G.J.R., Gardiner, M.G.T., DeLury, N.C. & Karg, G. (2005) Reduced antennal sensitivity, behavioural response, and

attraction of male codling moths, *Cydia pomonella*, to their pheromone (*E*,*E*)-8,10-dodecadien-1-ol following various pre-exposure regimes. *Entomologia Experimentalis et Applicata*, **114**, 65–78.

Landolt, P.J. & Phillips, T.W. (1997) Host plant influences on sex pheromone behavior of phytophagous insects. *Annual Review of Entomology*, **42**, 371–391.

Linn, C.E. & Roelofs, W.L. (1981) Modification of sex pheromone blend discrimination in male oriental fruit moths by pre-exposure to (*E*)-8-dodecenyl acetate. *Physiological Entomology*, **6**, 421–429.

Light, D.M., Flath, R.A., Buttery, R.G., Zalom, F.G., Rice, R.E., Dickens, J.C. & Jang, E.B. (1993) Host-plant green-leaf volatiles synergize the synthetic sex pheromones of the corn earworm and codling moth (Lepidoptera). *Chemoecology*, **4**, 145–152.

Light, D.M., Knight, A.L., Henrick, C.A. *et al.* (2001) A pear-derived kairomone with pheromonal potency that attracts male and female codling moth, *Cydia pomonella* (L.). *Naturwissenschaften*, **88**, 333–338.

Liu, Y.B. & Haynes, K.F. (1993) Pheromone-mediated responses of male cabbage looper moths, *Trichoplusia ni*, following various exposures to sex pheromone or (*IX*)-7-dodecenol. *Journal of Chemical Ecology*, **19**, 503–515.

Mani, E., Riggenbach, W. & Mendik, M. (1978) Zucht des Apfelwicklers (Laspeyresia pomonella L.) auf künstlichem Nährboden, 1968–1978. *Mitt Schweiz EntoMolecular Ges*, **51**, 315–326.

Mechaber, W.L., Capaldo, C.T. & Hildebrand, J.G. (2002) Behavioral responses of adult female tobacco hornworms, *Manduca sexta*, to hostplant volatiles change with age and mating status. Journal of Insect Science, 2.5. www.insectscience.org/2.5

Ochieng, S.A., Park, K.C. & Baker, T.C. (2002) Host plant volatiles synergize responses of sex pheromone-specific olfactory receptor neurons in male *Helicoverpa zea*. *Journal of Comparative Physiology A*, **188**, 325–333.

Papaj, D.R. (2000) Ovarian dynamics and host use. *Annual Review of Entomology*, **45**, 423–448.

Phelan, P.L. & Baker, T.C. (1987) An attracticide for control of *Amyelois transitella* (Lepidoptera: Pyralidae) in almonds. *Journal of Economic Entomology*, **80**, 779–783.

Raina, A.K. (1993) Neuroendocrine control of sex pheromone biosynthesis in Lepidoptera. *Annual Review of Entomology*, **38**, 329–349.

Rojas, J.C. (1999) Electrophysiological and behavioral responses of the cabbage moth to plant volatiles. *Journal of Chemical Ecology*, **25**, 1867–1883.

Schoonhoven, L.M., Jermy, T. & van Loon, J.J.A. (1998) *Insect-Plant Biology: from Physiology to Evolution*. Chapman & Hall, U.K.

Stelinski, L.L., Miller, J.R., Ressa, N.E. & Gut, L.J. (2003) Increased EAG responses of tortricid moths after prolonged exposure to plant volatiles: evidence for octopamine-mediated sensitization. *Journal of Insect Physiology*, **49**, 845–856.

Witzgall, P., Bäckman, A.-C., Svensson, M. *et al.* (1999a) Behavioral observations of codling moth, *Cydia pomonella*, in orchards permeated with synthetic pheromone. *Biocontrology*, **44**, 211–237.

Witzgall, P., Bengtsson, M., El-Sayed, A. *et al.* (1999b) Chemical communication in codling moth: towards environmentally safe control methods. *IOBC Wprs Bulletin*, **22**, 57–65.

Witzgall, P., Bengtsson, M., Rauscher, S. *et al.* (2001) Identification of further sex pheromone synergists in the codling moth, *Cydia pomonella*. *Entomologia Experimentalis et Applicata*, **101**, 131–141.

Yang, Z., Bengtsson, M. & Witzgall, P. (2004) Host plant volatiles synergize response to sex pheromone in codling moth, *Cydia pomonella*. *Journal of Chemical Ecology*, **30**, 619–629.

Accepted 17 April 2005

CHAPTER VI

Sex pheromone and pear ester upwind attraction of *Cydia pomonella* (L.) males: blend effect and compound discrimination

Casado,D.[1,*]; Gemeno,C[1]; Avilla,J.[1]; Riba,M.[1]; Witzgall,P.[2]

[1] Àrea de Protecció de Conreus, Centre UdL-IRTA de R+D, Av. Alcalde Rovira Roure, 191, E-25198, Lleida, Spain.

[2] Department of Crop Science, Swedish University of Agricultural Sciences, Box 44, 230 53 Alnarp, Sweden.

[*] Corresponding author, e-mail: danicasado@yahoo.es

ABSTRACT. Upwind attraction and discrimination of males of *Cydia pomonella* to sex pheromone (codlemone) and pear ester was studied in the wind tunnel. Codlemone alone and blended with pear ester at 10:10 and 10:1,000 (µg) attracted males upwind and source contact occurred. On the other hand, pear ester alone triggered upwind flight in some males, but not contact. The 10:1,000 blend significantly reduced attraction at the final steps of flight. This antagonism disappeared when the pheromone and the pear ester were presented in two different septa 10 cm apart. Moreover, once flight had been triggered males were unable to distinguish between a source loaded only with codlemone and another loaded with the blends (10:10 or 10:1,000), and contacted equally both sources.

KEYWORDS: Pear ester, sex pheromone, upwind flight, male behavior, *Cydia pomonella*.

1. Introduction

The codling moth, *Cydia pomonella* (L.), is the most serious pest of apples worldwide, and it also attacks other fruit crops such as pears and walnuts (Barnes, 1991). Its sex pheromone (codlemone) was first reported in 1971 (Roelofs *et al.*, 1971), and at present it is widely used to monitor and control pest populations by means of mating disruption (Charmillot, 1990; Pfeiffer *et al.*, 1993; Calkins and Faust, 2003) and attract-and-kill techniques (Charmillot *et al.*, 2000). Nowadays, mating disruption is the most successful technique of control alternative to chemical control, and its use is widely spread around the world (Calkins and Faust, 2003).

The use of mating disruption implies the need of new attractants, alternative to codlemone, for population monitoring that could be successfully used in mating disruption plots. Codling moth males have shown the same upwind behavior in response to plant volatiles than to pheromone (Witzgall *et al.*, 1999; Coracini *et al.*, 2004). In the last years many advances in *C. pomonella* response to host-volatiles have been made, with most of the effort focused on apple (Hern and Dorn, 1999, 2004; Bengtsson *et al.*, 2001; Ansebo *et al.*, 2004; Yang *et al.*, 2004; Casado *et al.*, 2006), and pear (Light *et al.*, 2001).

The most effective plant compound identified is ethyl (*E*,*Z*)-2,4-decadienoate, known as pear ester. This compound was isolated from ripe Bartlett pears and it is a potent attractant for both females and males of *C. pomonella* (Light *et al.*, 2001). The pear ester has been demonstrated as a practical and stable lure, and a successful tool for *C. pomonella* monitoring (Light *et al.*, 2001; Ioratti *et al.*, 2003; Knight and Light, 2004; Trematerra and Sciarretta, 2005). However, its efficacy in the field has been reported to depend on the species of host fruit trees, as well as on the phenological state of the plants (Light *et al.*, 2001; Knight and Light, 2005).

Plant semiochemicals are well known to interact with insect pheromones (Reddy and Guerrero, 2004). There are some studies that rise up the existence of an important interaction between pear ester and codlemone. In the field, a blend of 3 mg of each pear ester and codlemone has been shown to be more attractive than any of the two compounds by themselves (Knight *et al.*, 2005). In a recent study, pre-exposure of *C. pomonella* males to sex pheromone for 24 h, increased their upwind response to pear ester in the wind tunnel, whilst attraction to the sex pheromone was reduced. Similarly, in the same study authors suggested pear ester to be more attractive in the field under

mating disruption conditions than in conventional orchards (Yang *et al.*, 2005). Furthermore, it has been reported the existence in *C. pomonella* antennae of sensilla responding to only pear ester or codlemone, but also sensilla responding to both compounds (De Cristofaro *et al.*, 2004; Ansebo *et al.*, 2005).

The aim of this study was to improve our knowledge about pear ester and codlemone interaction, and determine the ability of males to discriminate between both compounds in the wind tunnel.

2. Material and methods

Insects

All the insects used in the study came from a laboratory colony. This was started in 1992 from insects collected in an abandoned apple orchard in Lleida (Spain), and it has been maintained on a semi-synthetic diet (Pons *et al.*, 1994) under a 16:8 h (L:D) photoperiod at 25 ± 5 ºC. Newly emerged adults were sexed daily, and males were separated from females and supplied with water until the following day, when they were assayed in the first hour of the scotophase.

Chemicals

Hexane purchased from Fluka Chemie (Buchs, Switzerland) was used as solvent (purity > 95%). *C. pomonella* sex pheromone, (*E*,*E*)-8,10-dodecadienol, was purchased from S. Voerman (Institute for Pesticide Research, Wageningen, Holland). Pear ester, ethyl (*E*,*Z*)-2,4-decadienoate, was a gift of P. Kirsch (IPM Technologies Inc., Portland OR, USA). These chemicals had a purity of > 99 % and 87.3 % (respectively, according to gas-chromatographic analysis). Lures solved in hexane, were applied on red rubber septa (ABS, Dietikon, Switzerland), which were kept at -20 ºC between tests.

Wind Tunnel

Assays were conducted in a 63 x 90 x 200 cm wind tunnel. Incoming air was blown through an array of activated charcoal cylinders, and outcoming air passed two sets of similar filters. The wind tunnel was illuminated from above and one of the lateral walls at 20 lux with white light. Wind speed was ca. 30 cm/s and air temperature was 23±1ºC (Witzgall *et al.*, 2001).

One-source assay

Rubber septa were loaded with either codlemone, pear ester or a blend of the two compounds (5 treatments in total, Table 1). A single septum was held at the upwind end, in the centre of a glass cylinder (10 x 10 cm) covered by a metal net, and 40 cm from the tunnel floor.

Two-day-old males were released from glass tubes (2.5 x 15 cm), at the same height and ca. 180 cm downwind, from the odor source. Males were tested individually, in batches of 15. Four replicates were made per treatment in 4 different days (N=60), and only one treatment was assayed per day.

Males were kept in the wind tunnel for 3 min, and the following behaviors were recorded: activation (walking around the glass tube), wing fanning, taking flight (locked on the plume), flying upwind over 50 (F1), 100 (F2) and 150 cm (F3), touching the source, and walking and wing-fanning on the source. We also recorded the time that males took to contact the source, counting from the beginning of the test.

Table 1. Summary of composition of lures formulated and used in the study.

Abbreviation	Codlemone (µg)	Pear ester (µg)
10:0	10	-
10:10	10	10
10:1,000	10	1,000
0:10	-	10
0:1,000	-	1,000

Two-source assay

Two cylinders (see above) were placed side-by-side so that septa were 10 cm apart. One of the septa was always loaded with 10 µg codlemone, and the second with another of the 4 remaining treatments from the previous assay (Table 1). Males were tested individually, in batches of 15. Four replicates were made per treatment in 4 different days (N=60), and only one treatment was assayed per day. The position of the pheromone septa was changed amongst replicates.

The rest of the methodology was as for the one-source assay. However in the recorded behaviors F2, F3, touching, and walking and wing-fanning on the source it was specified the source, or plume, where the insects performed.

Data analysis

To analyze the results an angular transformation (arcsin√x) of the proportion of individuals accomplishing each behavioral step was performed. In the one-source assay an ANOVA was performed for each behavioral category to compare responses amongst treatments.

In the two-source assay an ANOVA was performed for each behavioral category, to compare responses amongst two-source treatments and the one-source pheromone alone treatment. In these analyses it was used the total percentage of insects behaving at each step without differentiate between the two sources. Another group of ANOVAs was performed to compare plume selection in the behavioral steps F2, F3, touching and walking and wing-fanning for each couple of treatments. A last ANOVA with the two-source data was performed to compare amongst treatments the percentage of upwind flying males that changed from one plume to the other while upwind flying.

Another ANOVA was carried out to compare the time that males needed to complete the behavioral sequence from their release till contacting the source, combining the data from both assays. The significance level used was always 0.05, and whenever significant differences were found, a Duncan's Multiple Range Test was performed.

3. Results

One-source assay

Males were highly activated by all treatments. Mean percentage of activation ranged from 59.8 ± 14.6 (1,000 μg pear ester) to 95.1 ± 1.6 (pheromone alone). The mean percentage of males that wing-fanned was also high for the treatments that contained pheromone (over 90%), but it was clearly lower for the other two treatments (around 40%). The mean percentage of males that took flight was around 75 % for the treatments containing pheromone, and it was 10 and 19 % for the treatments that had pear ester only (Figure 1).

The mean percentage of males that contacted the source was 53.8 ± 3.2, 53.0 ± 10.0, and 30.7 ± 5.8 %, for the pheromone, the 10:10 blend and the 10:1,000 blend treatments, respectively. And the mean percentage of males that landed on the source and walked and wing-fanned on it was 52.2 ± 4.1, 53.0 ± 10.0, and 25.8 ± 7.2 %, for the pheromone, the 10:10 blend and the 10:1,000 blend treatments, respectively. Almost all

the males that contacted the source, thereafter landed on it and walked and wing-fanned, but in the 10:1,000 blend the percentage of them that refused the source after touching was slightly higher (Figure 1).

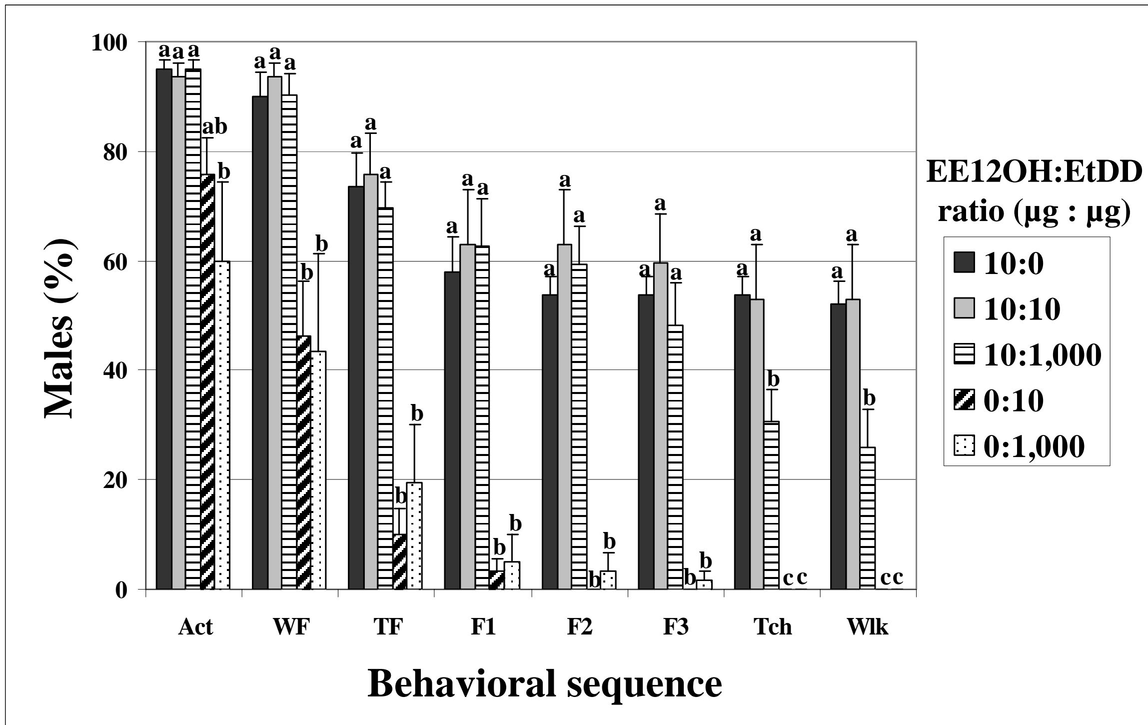

Figure 1. Mean percentage of *Cydia pomonella* males responding to single lures loaded with codlemone (EE12OH), pear ester (EtDD), or both compounds, in the wind tunnel. The following behavioral steps were recorded: activation (walking around the glass tube, Act), wing fanning (WF), taking flight (locked on the plume, TF), flying upwind over 50 (F1), 100 (F2) and 150 cm (F3), touching the source (Tch), and walking and wing-fanning on the source (Wlk). Treatments with different letters within each behavioral category showed significant differences (Duncan's Multiple Range Test, $P<0.05$); n=4 replicates, 15 males each.

There were significant differences in the percentage of males responding amongst treatments in all the behavioral steps recorded: activation ($df = 4$, $F = 5.06$, $P = 0.009$), wing-fanning ($df = 4$, $F = 6.97$, $P = 0.002$), taking flight ($df = 4$, $F = 13.95$, $P < 0.001$), F1 ($df = 4$, $F = 19.95$, $P < 0.001$), F2 ($df = 4$, $F = 36.21$, $P < 0.001$), F3 ($df = 4$, $F = 45.12$, $P < 0.001$), touching ($df = 4$, $F = 55.21$, $P < 0.001$), and walking and wing-fanning ($df = 4$, $F = 44.59$, $P < 0.001$). For all the behavioral steps, a higher percentage of males responded to the treatments containing pheromone than to the others. Activation was an exception, as there was no significant difference between the treatments containing pheromone and the 10 µg pear ester treatment (Figure 1). The response of males to the blend 10:10 of pheromone and pear ester was not different to

that to pheromone alone at any behavioral step. On the other hand, the 10:1,000 blend disrupted the attraction of males at the latest steps: touching, and walking and wing-fanning (Figure 1).

The treatments containing only pear ester stimulated the flight of some males, but none of them resulted in source contact. There were no significant differences in the response of males to the two different dosages of pear ester at any behavioral step (Figure 1).

Two-source assay

The mean percentage of males that oriented upwind until contact, regardless to source choice, ranged from 65.9 ± 7.2 to 33.9 ± 9.0 %, corresponding to the treatments 10 μg of pheromone + 10:10 blend, and 10 μg of pheromone + 10:1,000 blend, respectively. The combination of the pheromone septum and the septum containing the 10:10 blend was the one that elicited a greater response through all the behavioral steps. However there were significant differences amongst treatments only in activation (*df* = 4, $F = 3.18$, $P = 0.045$), F2 (*df* = 4, $F = 4.11$, $P = 0.019$), F3 (*df* = 4, $F = 4.11$, $P = 0.019$), and touching the source (*df* = 4, $F = 3.88$, $P = 0.023$) (Figure 2A). Response to the treatment of pheromone plus the blend 10:10 was significantly higher to all the other treatments in the behavioral steps F2 and F3; in the other two steps with significant differences, those were present only between the treatments pheromone plus the 10:10 blend, and pheromone plus the blend 10:1,000. The weakest responses of males throughout all the behavioral sequence were always to this last treatment except for activation (Figure 2A). Differences in wing-fanning (*df* = 4, $F = 2.01$, $P = 0.145$), taking flight (*df* = 4, $F = 2.79$, $P = 0.065$), F1 (*df* = 4, $F = 2.82$, $P = 0.063$), and walking and wing-fanning on the source (*df* = 4, $F = 1.61$, $P = 0.224$) were not significant, however the same tendencies than in the other behavioral steps can be easily appreciated (Figure 2A).

Males that reached the source did not discriminate between the source containing only pheromone and the 10:10 or 10:1,000 blends (*df* = 1, $F = 0.07$, $P = 0.799$; and *df* = 1, $F = 1.55$, $P = 0.259$, respectively). There was also a lack of discrimination between the pheromone and the blends in F3 (*df* = 1, $F = 0.90$, $P = 0.380$; and *df* = 1, $F = 3.73$, $P = 0.102$), and walking and wing-fanning on the source (*df* = 1, $F = 0.42$, $P = 0.540$; and *df* = 1, $F = 1.55$, $P = 0.259$). However, in F2 males significantly preferred the plume of the 10:1,000 blend rather than the plume of the

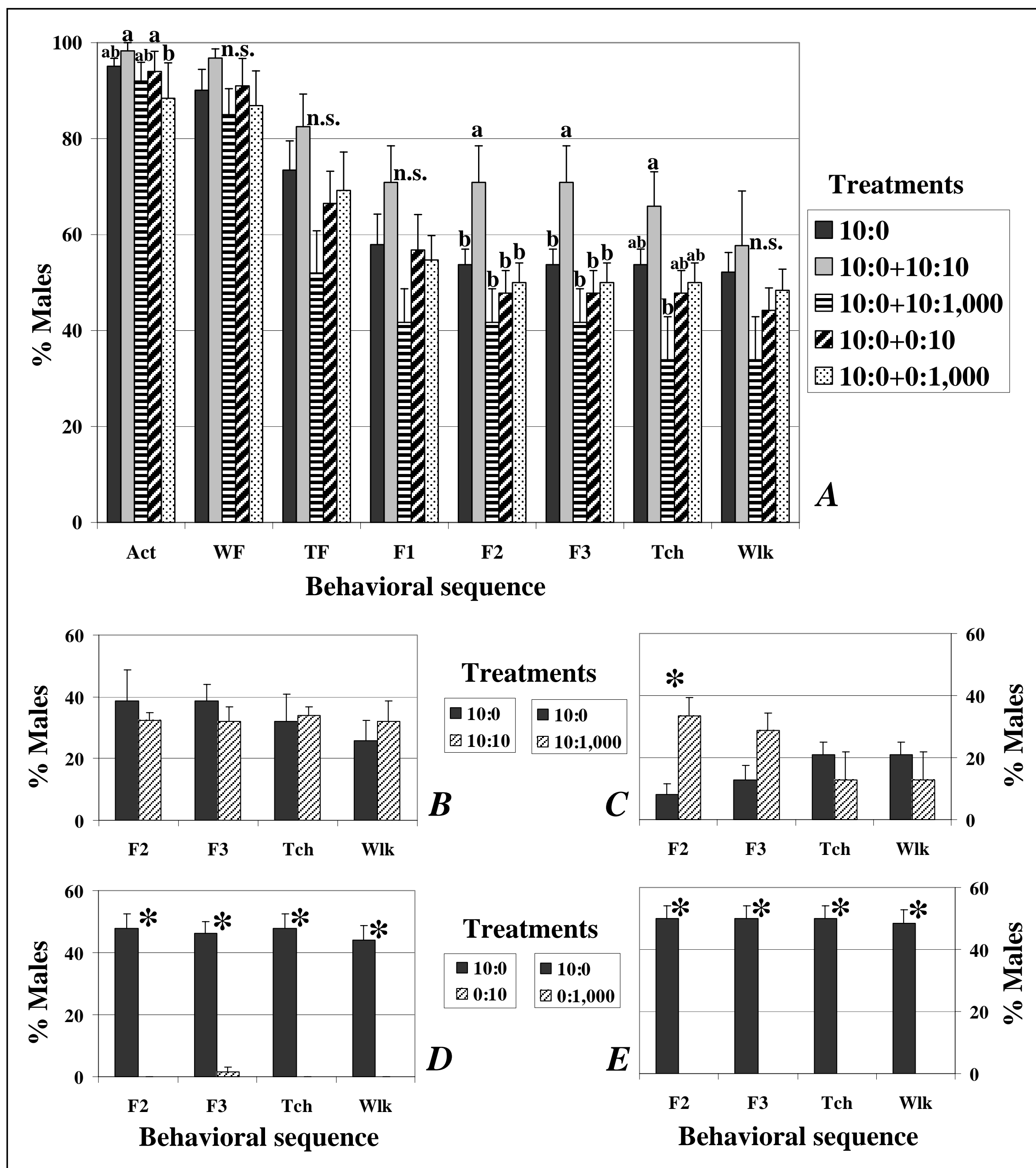

Figure 2. Mean percentage of *Cydia pomonella* male attraction to different lures of codlemone and pear ester in the wind tunnel. The following behavioral steps were recorded: activation (walking around the glass tube, Act), wing fanning (WF), taking flight (locked on the plume, TF), flying upwind over 50 (F1), 100 (F2) and 150 cm (F3), touching the source (Tch), and walking and wing-fanning on the source (Wlk). Treatments are expressed as codlemone:pear ester ratio (μg); '+' indicates two lures being used at the same time. *A*, upwind attraction to a pheromone-loaded single septum, and treatments of two lures, one containing only codlemone and the other containing pear ester, or blended codlemone and pear ester. Total upwind attraction is taken into account, regardless to the plume selected by the insects. Treatments with different letters within each behavioral category showed significant differences (Duncan's Multiple Range Test, $P<0.05$). *B-E*, plume selection by upwind males in the latest behavioral steps (F2 to walking on the source) when exposed simultaneously to two different lures. Asterisks mean significant differences in the percentage of males selecting each plume ($P<0.05$); n=4 replicates, 15 males each.

codlemone (*df* = 1, *F* = 11.15, *P* = 0.016), but they did not differentiate between the 10:10 blend and the sex pheromone (*df* = 1, *F* = 0.31, *P* = 0.598) (Figure 2B,C).

When assayed against the two dosages of pear ester alone, the pheromone source was clearly preferred at all the analyzed behavioral steps: F2 (*df* = 1, *F* = 261.30, *P* < 0.001; and *df* = 1, *F* = 365.14, *P* < 0.001, for 10 and 1,000 µg of pear ester respectively); F3 (*df* = 1, *F* = 83.95, *P* < 0.001; and *df* = 1, *F* = 365.14, *P* < 0.001, for 10 and 1,000 µg of pear ester respectively); touching (*df* = 1, *F* = 261.30, *P* < 0.001; and *df* = 1, *F* = 365.14, *P* < 0.001, for 10 and 1,000 µg of pear ester respectively); and walking and wing-fanning on the source (*df* = 1, *F* = 235.27, *P* < 0.001; and *df* = 1, *F* = 306.23, *P* < 0.001, for 10 and 1,000 µg of pear ester respectively) (Figure 2D,E). Moreover, no males touched or landed on sources containing only pear ester, as it happened in the one-source assay (Figures 1 & 2D,E).

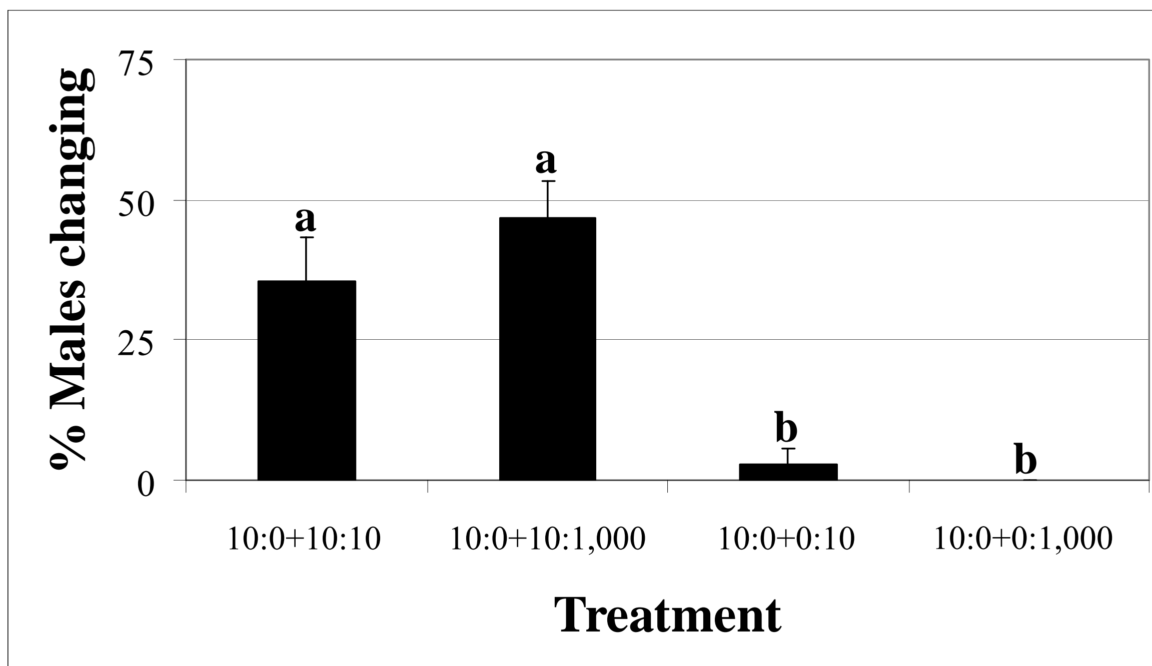

Figure 3. Percentage of upwind flying *Cydia pomonella* males that changed at least once of plume in a two-source wind tunnel assay of attraction to codlemone and pear ester. Treatments are expressed as codlemone:pear ester ratio (µg); '+' separates ratios corresponding to each lure. Treatments with different letters showed significant differences (Duncan's Multiple Range Test, P<0.05).

When males were flown to two sources, there were significant differences amongst treatments in the percentage of them that changed from one plume to another during upwind flight ($df = 3$, $F = 29.99$, $P < 0.001$) (Figure 3). When males were given a choice between the pheromone and one source containing only pear ester, they hardly ever took the plume of the pear ester (Figure 2D,E); almost all of them followed the plume of the pheromone treatment throughout all the flight (Figure 3). On the other hand, when males had to choose between the pheromone and one of the blends, they often changed from one plume to the other (Figures 4). An average of 35.3 ± 8.0 % of males changed at least once when the blend 10:10 was assayed together with the pheromone. In the case of the 10:1,000 blend, this percentage increased to 46.7 ± 6.7 %, but these two values did not differ significantly (Figure 3).

Time of response

The mean time spent to source contact after beginning of test ranged between 0.93 ± 0.13 min, and 2.06 ± 0.17 min, for the single lure 10:1,000 blend and the combination of one lure of 10 μg of pheromone and another of 10 μg of pear ester, respectively. There were significant differences amongst treatments ($df = 6$, $F = 3.98$, $P = 0.001$). Despite the percentage of males that reached the source was lower for the 10:1,000 blend than for the other treatments that elicited complete behavioral responses, the mean time that those males required to reach the target was the lowest (Figures 1 & 2). However, there were not significant differences amongst this average time and those for the 10:10 blend and the combination of one source of codlemone alone and one of the 10:1,000 blend (Figure 4). The mean time needed to reach one source in the combination of one lure of codlemone and one loaded with 10 μg of pear ester was the highest, and significantly higher than in all the other treatments (Figure 4).

4. Discussion

Our data show that, under wind tunnel conditions, pear ester elicits upwind flight of males of *C. pomonella* by itself (Figure 1). However, attraction is not complete as no male reached any source loaded only with pear ester (Figures 1 & 2D,E). This result matches with previous data from the literature on *C. pomonella* male attraction to pear ester (Ansebo *et al.*, 2004; Coracini *et al.*, 2004; Yang *et al.*, 2005). On the other hand, capture of males in the field by traps loaded only with pear ester has been widely reported (Light *et al.*, 2001; Ioratti *et al.*, 2003; Knight and Light, 2005; Knight *et al.*,

2005; Light and Knight, 2005; Trematerra and Sciarretta, 2005; Yang *et al.*, 2005). This suggests that the background of volatiles emitted by trees make pear ester traps fully attractive under field conditions. This can also partially justify the differences in captures by pear ester, that have been observed throughout the season (Light *et al.*, 2001; Knight and Light, 2005), taking into account that volatile emissions from trees vary during season (Bengtsson *et al.*, 2001; Hern and Dorn, 2003; Casado *et al.*, 2006). This variation in attractiveness is however usually attributed to the competition between traps and volatiles emitted by trees. Supporting the background effect hypothesis some complete flights in wind tunnel to pear ester have been achieved when combined with (*E*,*E*)-α-farnesene, or both (*E*,*E*)-α- and (*E*)-β-farnesene (Ansebo *et al.*, 2004; Coracini *et al.*, 2004).

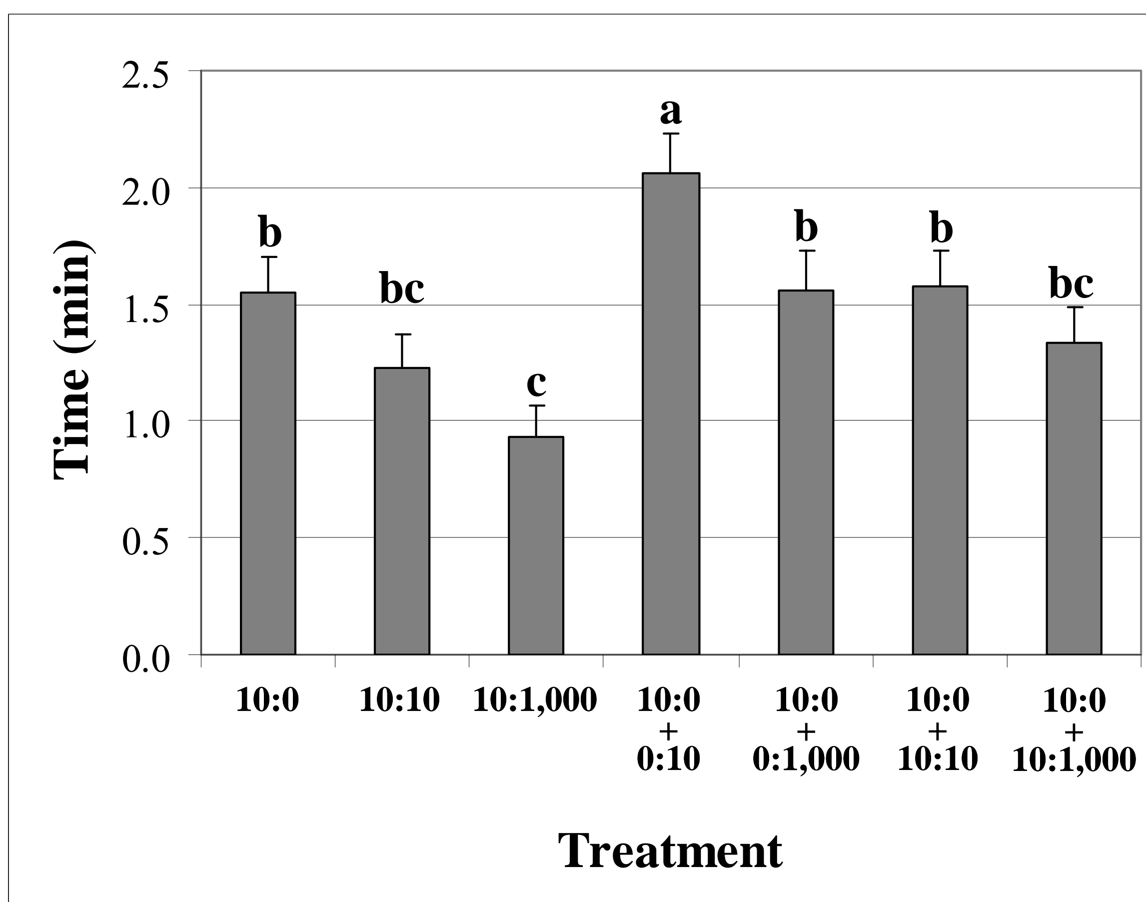

Figure 4. Mean time spent to contact a source by *Cydia pomonella* males in the wind tunnel when flown to one or two sources containing codlemone, pear ester, or a blend of both. Treatments are expressed as codlemone:pear ester ratio (µg); '+' indicates two lures being used at the same time. Treatments with different letters showed significant differences (Duncan's Multiple Range Test, P<0.05).

Recently, females of another Tortricidae species, *Lobesia botrana* Den. et Schiff., have been found to be attracted in wind tunnel to a 3-component blend of grape terpenes, β-caryophyllene, (*E*)-4,8-dimethyl-1,3,7-nonatriene, and (*E*)-β-farnesene (Tasin *et al.*, 2007). Suppression of any of those compounds from the blend dramatically reduced female *L. botrana* attraction to the source. However, substitution of one component by some other grape volatiles partially restored the attractiveness of the blend. Moreover, adequate blends of ubiquitous plant volatiles have been proposed as responsible of phytophagous insect attraction to host plants rather than single species-specific compounds (Bruce *et al.*, 2005).

The effect of the addition of pear ester to codlemone depended on the ratio between both compounds. The 10:10 blend did not have any effect on the attraction of males, as this treatment never differentiated from the 10 μg codlemone lure. On the other hand, when added at high amount (10:1,000), pear ester disrupted the attraction on the last steps of the behavioral sequence, touching, and walking and wing-fanning at the source (Figure 1). In a previous study, Yang *et al.* (2005) found an antagonistic effect of pear ester in wind tunnel when blended with codlemone at 1:10,000 μg, but when blended at 1:10 or 1:100 μg, it did not have any effect. Although in our study antagonism appeared at a lower ratio (10:1,000 μg, 100-fold), in both assays low ratios of pear ester had no effect on attraction, while a high ratio acted as an antagonist, despite different codlemone amounts were assayed, 10 and 1 μg. This antagonism can be related with the occurrence of olfactory neurons on the male antennae that respond to both, codlemone and pear ester (De Cristofaro *et al.*, 2004; Ansebo *et al.*, 2005). A similar effect has been found for a codlemone mimic, (*E*)-10-dodecen-1-ol, which is a weak attractant by itself, but an antagonist when blended in high amounts with codlemone, and is presumably perceived by codlemone receptor neurons.

Under field conditions lures of 3:20 or 3:3 mg of codlemone:pear ester, are more attractive than codlemone alone, both in conventional and mating disrupted orchards, thus showing a synergistic effect (Knight *et al.*, 2005). In wind tunnel, the lack of synergism of 1:1 ratio blends has been reported (Yang *et al.*, 2005). This result emphasizes the importance of background volatiles in field traps baited with pear ester. As in the case of traps baited only with pear ester, the background may play an important role in the synergistic effect of pear ester and codlemone in the field. Multiple plant volatiles, such as (*Z*)-3-hexenyl acetate, (±)-linalool, or (*E*)-β-farnesene, amongst

other have been reported to synergize male attraction to codlemone in wind tunnel (Yang *et al.*, 2004).

Lures of 10 and 1,000 µg of pear ester did not affect attraction to codlemone when they were loaded in adjacent sources, as there were no differences amongst the attractions to single pheromone and to these two treatments (Figure 2A). Furthermore, none of the upwind flying males contacted the sources containing only pear ester (Figure 2D,E). The antagonist effect of 1,000 µg pear ester, disappeared thus when both compounds were spatially separated. The same behavior was reported in the response of *C. pomonella* to codlemone and codlemone acetate, an antagonist of codlemone. This last compound strongly diminishes attraction to codlemone when both compounds are loaded in the same lure, but its antagonistic effect disappears when both compounds are presented in different lures 10 cm apart, and attraction to codlemone is reestablished (Coracini *et al.*, 2003). Spatial and temporal coincidence of substances is needed for antagonist effect to take place (Liu and Haynes, 1992; Baker *et al.*, 1998). Odor filaments of slightly separated sources do not completely intermix, and when emissions of the pheromone and the antagonist have different point sources, attraction is restored (Witzgall and Priesner, 1991; Fadamiro *et al.*, 1999; Coracini *et al.*, 2003). It was found that under field conditions, traps baited with 3 mg pear ester and 3 mg of codlemone were more attractive when both compounds were loaded in the same lure than in separated ones; despite the reduction in captures on the two-lure traps, these traps were still more attractive than traps baited with 3 mg codlemone only (Knight *et al.*, 2005).

Upwind flight and source contact by males were synergized when a lure of the 10:10 blend was placed next to the codlemone lure (Figure 2A). On the other hand, despite the lowest responses in the two-source assay were reached with the combination of codlemone and the blend 10:1,000, its attractiveness was not significantly lower than that of codlemone alone (Figure 2A); probably due to the spatial separation between lures that abolished the antagonistic effect of the 10:1,000 blend. Nevertheless, once upwind behavior was triggered, males were unable to discriminate between the codlemone and the blends, and as many males contacted and landed on the codlemone as on the blends (Figure 2B,C). This suggests that while upwind flying males can not distinguish between the codlemone and the blend 10:1,000, as previously reported in the upwind behavior of *C. pomonella* to codlemone and a blend of codlemone and codlemone acetate (Coracini *et al.*, 2003). However, in the case of pear ester the antagonistic effect at high dosage takes place in the latest steps of the behavioral

sequence, whereas codlemone acetate inhibits initiation of flight (Coracini *et al.*, 2003). As shown in Figure 3, males flown to codlemone and one of the blends often changed from one plume to the other throughout their flight. This reveals the difficulties of males to differentiate between the plumes once the flight sequence had started.

Surprisingly males that contacted one source did it faster when they were flown to the 10:1,000 blend than to most of the other treatments (Figure 4), despite the antagonistic effect that this blend showed (Figure 1). Nevertheless, the blend antagonism did not appear at the initial steps of attraction, and did not affect taking flight or flight throughout the wind tunnel length. Instead, antagonism appeared as a rejection of the source just before touching or landing on it (Figure 1). For this reason antagonism of the 10:1,000 blend did not affect negatively the average time of attraction of upwind flying males (Figure 4).

In our study we have shown that pear ester triggers some upwind flights but not contacts in wind tunnel conditions. This suggests that attractiveness of traps loaded with pear ester alone in the field should be partially due to the background volatiles emitted by trees, as it has been previously proposed for (*E*)-β-farnesene (Coracini *et al.*, 2004); and confirms that attraction of phytophagous to plants is due to blends of ubiquitous volatiles rather than to species-specific single compounds (Bruce *et al.*, 2005). Pear ester showed an antagonistic effect when blended in the same lure than codlemone at 100-fold times, but this antagonism disappeared when both compounds were loaded in separated septa and placed 10 cm apart. Furthermore, once flight had been started males were unable to distinguish between the pheromone and the 10:1,000 blend, contacting them equally. A similar behavior has been reported for the interaction of codlemone and codlemone acetate in *C. pomonella* (Coracini *et al.*, 2003), but codlemone acetate inhibits flight triggering whereas pear ester does not.

Acknowledgements

This work was partially funded by project RTA01-079 from the Programa Nacional de Recursos y Tecnologías Agroalimentarias (Spain), by projects AGL2003-06599-CO02-02 and AGL2004-05812/AGR from the Plan Nacional de I+D+I (Spain), and by the Foundation for Strategic Environmental Research (MISTRA, Sweden). D. Casado had a fellowship from MECD (Spain). Dr. C. Gemeno is funded by the Programa Ramón y Cajal (MCYT, Spain).

5. References

Ansebo, L., R. Ignell, J. Löfqvist, and B. S. Hansson. 2005. Responses to sex pheromone and plant odours by olfactory receptor neurons housed in *sensilla auricillica* of the codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae). J. Insect Physiol. 51:1066-1074.

Ansebo, L., M. D. A. Coracini, M. Bengtsson, I. Liblikas, M. Ramírez, A. K. Borg-Karlson, M. Tasin, and P. Witzgall. 2004. Antennal and behavioural response of codling moth *Cydia pomonella* to plant volatiles. J. Appl. Entomol. 128:488-493.

Baker, T. C., H. Y. Fadamiro, and A. A. Cossé. 1998. Moth uses fine tuning of odour resolution. Nature 393:530.

Barnes, M. M. 1991. Codling moth occurrence, host race formation, and damage, pp. 313-327 In L. P. S. Van Der Geest and H. H. Evenhuis [eds.], Tortricid Pests: Their Biology, Natural Enemies and Control. World Crop Pests. Elsevier, Amsterdam (Holland).

Bengtsson, M., A. C. Bäckman, I. Liblikas, M. I. Ramirez, A. K. Borg Karlson, L. Ansebo, P. Anderson, J. Löfqvist, and P. Witzgall. 2001. Plant odor analysis of apple: antennal response of codling moth females to apple volatiles during phenological development. J. Agric. Food Chem. 49:3736-3741.

Bruce, T. J. A., L. J. Wadhams, and C. M. Woodcock. 2005. Insect host location: a volatile situation. Trends Plant Sci. 10:269-274.

Calkins, C. O. and R. J. Faust. 2003. Overview of areawide programs and the program for suppression of codling moth in the western USA directed by the United States Department of Agriculture - Agricultural Research Service. Pest Manag. Sci. 59:601-604.

Casado, D., C. Gemeno, J. Avilla, and M. Riba. 2006. Day-night and phenological variation of apple tree volatiles and electroantennogram responses of *Cydia pomonella* (Lepidoptera: Tortricidae). Environ. Entomol. 35:258-267.

Charmillot, P. J. 1990. Mating disruption technique to control codling moth in Western Switzerland, pp. 165-182 In R. L. Ridgway, R. M. Silverstein, and M. N. Inscoe [eds.], Behavior-Modifying Chemicals for Insect Management. Marcel Dekker Inc., New York (USA).

Charmillot, P. J., D. Hofer, and D. Pasquier. 2000. Attract and kill: a new method for control of the codling moth *Cydia pomonella*. Entomol. Exp. Appl. 94:211-216.

Coracini, M. D. A., M. Bengtsson, L. Cichón, and P. Witzgall. 2003. Codling moth males do not discriminate between pheromone and a pheromone/antagonist blend during upwind flight. Naturwissenschaften 90:419-423.

Coracini, M. D. A., M. Bengtsson, and P. Witzgall. 2004. Attraction of codling moth males *Cydia pomonella* to apple volatiles. Entomol. Exp. Appl. 100:1-10.

De Cristofaro, A., C. Ioratti, E. Pasqualini, G. Anfora, G. S. Germinara, M. Villa, and G. Rotundo. 2004. Electrophysiological responses of *Cydia pomonella* to codlemone and pear ester ethyl (E,Z)-2,4-decadienoate: peripheral interactions in their perception and evidences for cells responding to both compounds. Bull. Insect. 57:137-144.

Fadamiro, H. Y., A. A. Cossé, and T. C. Baker. 1999. Fine-scale resolution of closely spaced pheromone and antagonist filaments by flying male *Helicoverpa zea* . J. Comp. Physiol. A 185:131-141.

Hern, A. and S. Dorn. 1999. Sexual dimorphism in the olfactory orientation of adult *Cydia pomonella* in response to -farnesene. Entomol. Exp. Appl. 92:63-72.

Hern, A. and S. Dorn. 2003. Monitoring seasonal variation in apple fruit volatile emissions *in situ* using solid-phase microextraction. Phytochem. Anal. 14:232-240.

Hern, A. and S. Dorn. 2004. A female-specific attractant for the codling moth, *Cydia pomonella* (L.), from apple fruit volatiles. Naturwissenschaften 91:77-80.

Ioratti, C., F. Molinari, E. Pasqualini, A. De Cristofaro, S. Schmidt, and I. Espinha. 2003. The plant volatile attractant (E,Z)-2,4-ethyl-decadienoate (DA2313) for codling moth monitoring. Boll. Zool. Agr. Bachic. 35:127-137.

Knight, A. L. and D. M. Light. 2004. Use of ethyl (E,Z)-2,4-decadienoate in codling moth management: kairomone species specificity. J. Entomol. Soc. Brit. Columbia 101:61-67.

Knight, A. L. and D. M. Light. 2005. Dose-response of codling moth (Lepidoptera: Tortricidae) to ethyl (E,Z)-2,4-decadienoate in apple orchards treated with sex pheromone dispensers. Environ. Entomol. 34:604-609.

Knight, A. L., R. Hilton, and D. M. Light. 2005. Monitoring codling moth (Lepidoptera: Tortricidae) in apple with blends of ethyl (*E*,*Z*)-2,4-decadienoate and codlemone. Environ. Entomol. 34:598-603.

Light, D. M., A. L. Knight, C. A. Henrick, D. Rajapaska, B. Lingren, J. C. Dickens, K. M. Reynolds, R. G. Buttery, G. Merrill, J. Roitman, and B. C. Campbell. 2001. A pear-derived kairomone with pheromonal potency that attracts male and female codling moth, *Cydia pomonella* (L.). Naturwissenschaften 88:333-338.

Light, D. M. and A. L. Knight. 2005. Specificity of codling moth (Lepidoptera: Tortricidae) for the host plant kairomone, ethyl (2E,4Z)-2,4-decadienoate: field bioassays with pome fruit volatiles, analogue, and isomeric compounds. J. Agric. Food Chem. 53:4046-4053.

Liu, Y. B. and K. F. Haynes. 1992. Filamentous nature of pheromone plumes protects integrity of signal from background chemical noise in cabbage looper moth, *Trichoplusia ni*. J. Chem. Ecol. 18:299-307.

Pfeiffer, D. G., W. Kaakeh, J. C. Killian, M. W. Lachance, and P. Kirsch. 1993. Mating disruption for control of damage by codling moth in Virginia apple orchards. Entomol. Exp. Appl. 67:57-64.

Pons, S., M. Eizaguirre, M. J. Sarasúa, and J. Avilla. 1994. Influencia del fotoperiodo sobre la inducción de diapausa de *Cydia pomonella* (Lepidoptera: Tortricidae) en laboratorio y campo. Invest. Agr. Prod. Prot. Veg. 9:477-491.

Reddy, G. V. P. and A. Guerrero. 2004. Interactions of insect pheromones and plant semiochemicals. Trends Plant Sci. 9:253-261.

Roelofs, W., A. Comeau, A. Hill, and G. Milicevic. 1971. Sex attractant of the codling moth: characterization with electroantennogram technique. Science 174:297-299.

Tasin, M., A. C. Bäckman, M. Coracini, D. Casado, C. Ioratti, and P. Witzgall. 2007. Synergism and redundancy in a plant volatile blend attracting grapevine moth females. Phytochemistry 68:203-209.

Trematerra, P. and A. Sciarretta. 2005. Activity of the kairomone ethyl (*E*,*Z*)-2,4-decadienoate in the monitoring fo *Cydia pomonella* (L.) during the second annual flight. REDIA 88:57-62.

Witzgall, P. and E. Priesner. 1991. Wind tunnel study on an attraction inhibitor in male *Coleophora laricella* (Lepidoptera: Coleophroidae). J. Chem. Ecol. 17:1355-1362.

Witzgall, P., M. Bengtsson, A. El-Sayed, A. C. Bäckman, S. Rauscher, A. K. Borg-Karlson, C. R. Unelius, and J. Löfqvist. 1999. Chemical communication in codling moth: towards environmentally safe control methods. IOBC/Wprs Bull. 22:57-65.

Witzgall, P., M. Bengtsson, S. Rauscher, I. Liblikas, A. C. Bäckman, M. Coracini, P. Anderson, and J. Lofqvist. 2001. Identification of further sex pheromone synergists in the codling moth, *Cydia pomonella*. Entomol. Exp. Appl. 101:131-141.

Yang, Z., M. Bengtsson, and P. Witzgall. 2004. Host plant volatiles synergize response to sex pheromone in codling moth, *Cydia pomonella*. J. Chem. Ecol. 30:619-629.

Yang, Z., D. Casado, C. Ioratti, M. Bengtsson, and P. Witzgall. 2005. Pheromone pre-exposure and mating modulate codling moth (Lepidoptera: Tortricidae) response to host plant volatiles. Agric. Forest Entomol. 7:231-236.

CHAPTER VII

Effect of temperature and light intensity on *Cydia pomonella* (L.) oviposition and mating diel activity

Casado,D.[1,2,*]; Witzgall,P.[2]; Avilla,J.[1]; Riba,M.[1]; Gemeno,C[1].

[1] Àrea de Protecció de Conreus, Centre UdL-IRTA de R+D, Av. Alcalde Rovira Roure, 191, E-25198, Lleida, Spain.

[2] Department of Crop Science, Swedish University of Agricultural Sciences, Box 44, 230 53 Alnarp, Sweden.

[*] Corresponding author, e-mail: danicasado@yahoo.es

ABSTRACT. Influence of temperature and light intensity on the diel timing of oviposition and mating behavior of *Cydia pomonella* (L.) were investigated under laboratory and semi-field conditions. Semi-field studies showed that diel oviposition and mating activities occurred earlier with respect to dusk in Alnarp (Sweden) than in Lleida (Spain). Temperature sharply decreased in the evening in Alnarp, while it was high and rather constant in Lleida. This suggests that a decrease in temperature could advance oviposition and mating onset with respect to dusk. We tested this hypothesis in the laboratory by placing mated females at 5 constant temperatures (12, 17, 22, 27 and 32 ºC) and counting hourly the number of eggs around the beginning of the scotophase. In this assay, 50 % of oviposition was reached before as lower was the temperature, but for most of the assayed temperatures, oviposition in laboratory peaked in the first hour of the scotophase. However, this peak was less prominent as lower the temperature and at 17 ºC was completely absent. Therefore temperature has an important role on the periodicity of oviposition behavior in *C. pomonella*.

We also obtained data about the role of temperature and age in fecundity. In the field, female oviposition was found to be maximal in the third and fourth days of life, smaller in the second and fifth, and almost inexistent in the first. Under laboratory conditions, there was not oviposition when insects were kept at 12 ºC, and maximum oviposition was reached at 22 and 27 ºC.

KEYWORDS: Temperature, oviposition, mating, diel activity, light intensity, *Cydia pomonella* (L.)

1. Introduction

The codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), is the most serious pest of apples worldwide, and it also attacks other fruit crops such as pears, walnuts, quinces and plums (Barnes, 1991). Because of its great economic importance, this species has been intensively studied. Despite the huge amount of publications concerning the species, there are still some gaps on the knowledge of its biology.

Temperature is an important factor in the regulation of physiology of poikilothermic organisms (Sharpe & DeMichele, 1977), such as insects. Although temperature has been shown to influence development (Pitcairn *et al.*, 1991; Ferreira *et al.*, 1994), thermoregulation behavior (Kührt *et al.*, 2006), male flight activity (Sæthre & Hofsvang, 2005), female calling behavior (Castrovillo & Cardé, 1979), and fecundity (Isely, 1938; Hagley, 1976; Sæthre & Hofsvang 2002), little is known about the influence of temperature on the diel oviposition timing in *C. pomonella*.

Temperature is known to modulate diel timing of circadian reproductive behaviors such as female calling in *C. pomonella* (Castrovillo & Cardé, 1979), and several other crepuscular and nocturnal moth species, such as *Holomelia immaculata* (Rearkirt) (Cardé & Roelofs, 1973), *Cydia molesta* (Busck) (Baker & Cardé, 1979), *Pseudaletia unipuncta* (Haworth) (Delisle & McNeil 1987a,b), or *Choristoneura rosaceana* (Harris) (Delisle, 1992). It is also known that *C. pomonella* is an insect of crepuscular activity (Collins & Machado, 1935), but little or nothing is known about diel activity of ovipositing females, and how temperature and light intensity interact to regulate this behavior.

The main aim of the present study was to improve our knowledge on female oviposition behavior focusing on the effect of temperature on oviposition diel timing, which should be an important factor to take into account when designing behavioral assays for female attractants or oviposition stimulants. The influence of age and temperature on fecundity, and the influence of temperature and light intensity on mating diel activity have been also studied. To reach these objectives two assays were conducted. The first one was a semi-field assay conducted in Spain and Sweden, and the second one was a laboratory assay to determine the effect of temperature on the oviposition behavior.

2. Material and methods

Insects

All the insects used in the study came from a laboratory colony, which was started in 1992 from insects collected in an abandoned apple orchard in Lleida (Spain) and has been maintained on a semi-synthetic diet (Pons *et al.*, 1994) under a 16:8 h (L:D) photoperiod at 25 ± 5 ºC.

Semi-field assay

In the semi-field assay mating and oviposition under natural conditions of light and temperature were studied. In this assay couples of codling moth adults (one male and one female) were placed the day of emergence inside glass tubes (15 cm length x 2.5 cm diameter) closed by gauze pieces at the two ends, with no food supply. Tubes were brought, earl in the morning, to an apple orchard, and placed under the tree shade, in a platform ca. 50 cm above ground.

The behavior of the couples was observed for 5 days. During this period observations were made at 9:00 in the morning, and every hour from 17:00 to 23:00 h. The number of eggs laid was counted and marked with an indelible pen in the glass at each observation. Mating was recorded if observed, and air temperature and light intensity were measured near the test insects. After sunset, observations were made with the help of a flashlight, lighting the tubes one by one and for as short time as possible.

The test was reproduced in two different locations, Alnarp (Southern Sweden, 55º 55' N and 13º 37' E) in the second half of June 2005, and Lleida (North-eastern Spain, 41º 37' N and 0º 38' E) in middle-September 2005. Fifty-five couples were observed at each location. During the time-curse of the tests, dusk in Alnarp took place between 21:54 and 21:51 h, and between 20:12 and 19:43 h in Lleida (local times, GMT+2). And sun rose between 4:19 and 4:20 h in Alnarp, and between 7:35 and 7:52 h in Lleida (local times, GMT+2) (ROA 2006).

The following statistical comparisons were made by ANOVA: a) in each location independently, the hourly percentage of oviposition at each evening-observation time (from 17:00 to 23:00 h) during the 5-days period of study; b) number of eggs present in the morning observation (at 9:00 h) between both locations; and c) daily oviposition amongst the 5 ages and the 2 locations. Significance level was 0.05 and when significant differences occurred a Duncan's Multiple Range Test was

performed. Only females that laid at least 10 eggs throughout the 5-day period were considered in the analyses.

Laboratory assay

In the laboratory assay oviposition behavior at 5 different constant temperatures was studied. In this assay mating boxes (cylindrical, 31 cm length x 16 cm diameter) were lined with wax paper (Cut-Rite®, Reynolds®, Richmond, Virginia, USA), which is an oviposition substrate suitable for *C. pomonella*. The ends had polyester covers lined with adhesive non-woven fabric (Fixomull ® stretch, BSN medical GmbH & Co. KG, Hamburg, Germany), which is unsuitable for oviposition.

Groups of between 10 and 12 females, and 12 and 15 males were placed in the mating boxes on the day of their emergence. They were kept in a climatic chamber at 22 ± 1 ºC under a 16:8 h (L:D) photo regime for 2 days to allow them to mate. At the onset of the third photophase the mating boxes were moved to other climatic chambers under the same photo regime, but at either 12, 17, 22, 27 or 32 ±1 ºC, depending on the treatment. Light intensity in these chambers was of ca. 2500 lux.

In the day that insects were changed to the new conditions, the wax paper of the mating boxes was removed every hour from 4 hours before to 4 hours after the onset of the scotophase. The number of eggs laid during each hour was recorded. One hour before the first control, the wax paper was removed to eliminate eggs laid during the two earlier days. After the end of the assay females were dissected to determine their mating status. Five mating boxes (replicates) were used per treatment.

Two unifactorial ANOVA and Duncan's Multiple Range Tests were performed to analyze differences amongst treatments in total fecundity, and percentage of mated females. A third bifactorial ANOVA and a pairwise comparison of least squared means were performed to compare the percentage of oviposition amongst the temperatures where oviposition occurred, at the different times of observation. An angular transformation ($\arcsin(\sqrt{x})$) of the data was required for this last analysis. A significance level of 0.05 was used.

3. Results

Semi-field assay

Light intensity and mean temperature recorded in the fields clearly differed between both locations through the assayed period (Figure 1C,F). From 17:00 to 23:00 h the average temperature ranged between 23.1 and 22.0 ºC in Lleida (Spain) (Figure 1C), and between 23.6 and 16.9 ºC, falling down rapidly after 19:00 h, in Alnarp (Sweden) (Figure 1F). Mean light intensity next to insects was higher at any time of the assay in the orchard in Alnarp than in the orchard in Lleida (Figure 1C,F). In the period of the assays, nautical dusk in Alnarp happened between 21:51 and 21:54 h, whilst it did between 19:43 and 20:12 h Lleida (local time, GMT+2) depending on the concrete day (ROA 2006). At dusk time mean temperatures were around 23 ºC and 19 ºC in Lleida and Alnarp, respectively; and mean light intensity was less than 500 lux in both locations (Figure 1C,F).

Forty-four females in Lleida and 41 in Alnarp, out of 55, laid 10 or more eggs through the studied period (including the morning control), the threshold for the data to be included in the analyses. One-day-old females did not oviposit in Lleida, and laid few eggs in Alnarp (Figure 2). Daily oviposition increased in the second day, reached a maximum on the third and fourth days, and decreased again on the last day in both locations (Figure 2 and 3). There was no significant interaction between location and age ($df = 4$, $F = 1.25$, $P = 0.288$), and there was neither a significant effect of location ($df = 1$, $F = 0.04$, $P = 0.834$), on the fecundity of females. On the other hand, there were significant differences on fecundity amongst ages ($df = 4$, $F = 36.24$, $P < 0.001$) (Figure 3).

The mean number of eggs in the morning (9:00 h) was significantly higher in Lleida (7.5 ± 1.1) than in Alnarp (0.6 ±0 .2) ($df = 1$, $F = 37.19$, $P < 0.001$). Significant differences in the percentage of oviposition amongst evening observations (from 17:00 to 23:00 h) were found in both locations ($df = 6$, $F = 18.96$, $P < 0.001$, and $df = 6$, $F = 14.09$, $P < 0.001$, in Lleida and Alnarp, respectively). Oviposition was concentrated in the observations from 19:00 to 21:00 h in Lleida (76.5% of oviposition), and from 18:00 to 20:00 h in Alnarp (78.9% of oviposition) (Figure 1 A,D). In Lleida oviposition peaked in the observation at 21:00 h (31.8 % of total), and in Alnarp oviposition was maximum at 18:00 and 20:00 h (26.9 and 27.7 %, respectively). Oviposition activity

was concentrated around the nautical dusk time in Lleida, whereas in Alnarp it was almost over before dusk (Figure 1A,D).

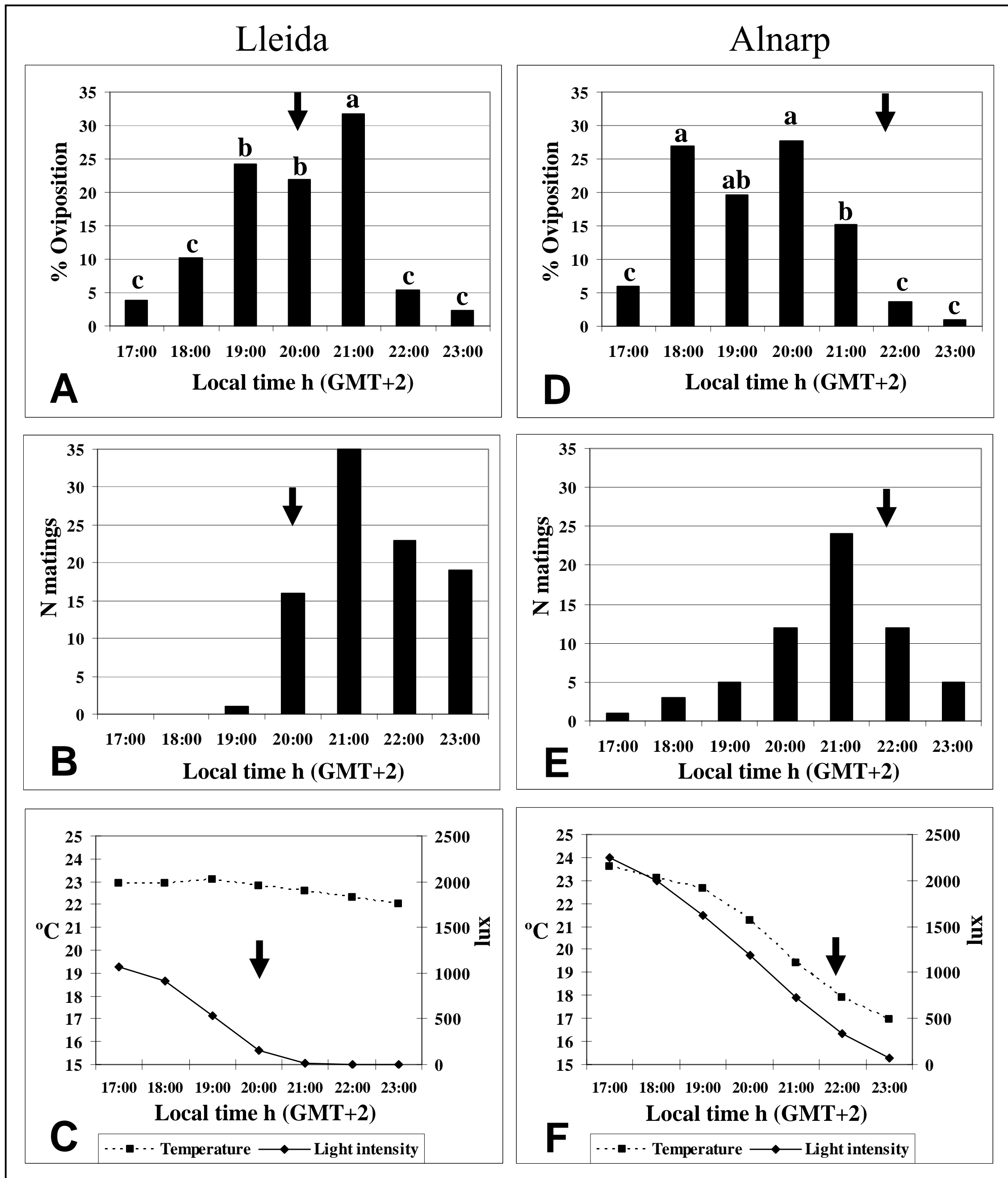

Figure 1. Oviposition and mating by codling moth females under natural conditions of light and temperature in two different European locations. A-C: Data recorded in Lleida (41º 37' N, 0º 38' E). D-F: Data recorded in Alnarp (55º 55' N, 13º 37' E). A and D: mean percentage of oviposited eggs in the different observations; different letters mean significant differences between observations within each location. B and E: total number of couples observed mating in the observations. C and F: mean recorded temperature and light intensity through the period assayed. Arrows indicate mean time of dusk at the different locations, Lleida 19:57 h and Alnarp 21:53 h.

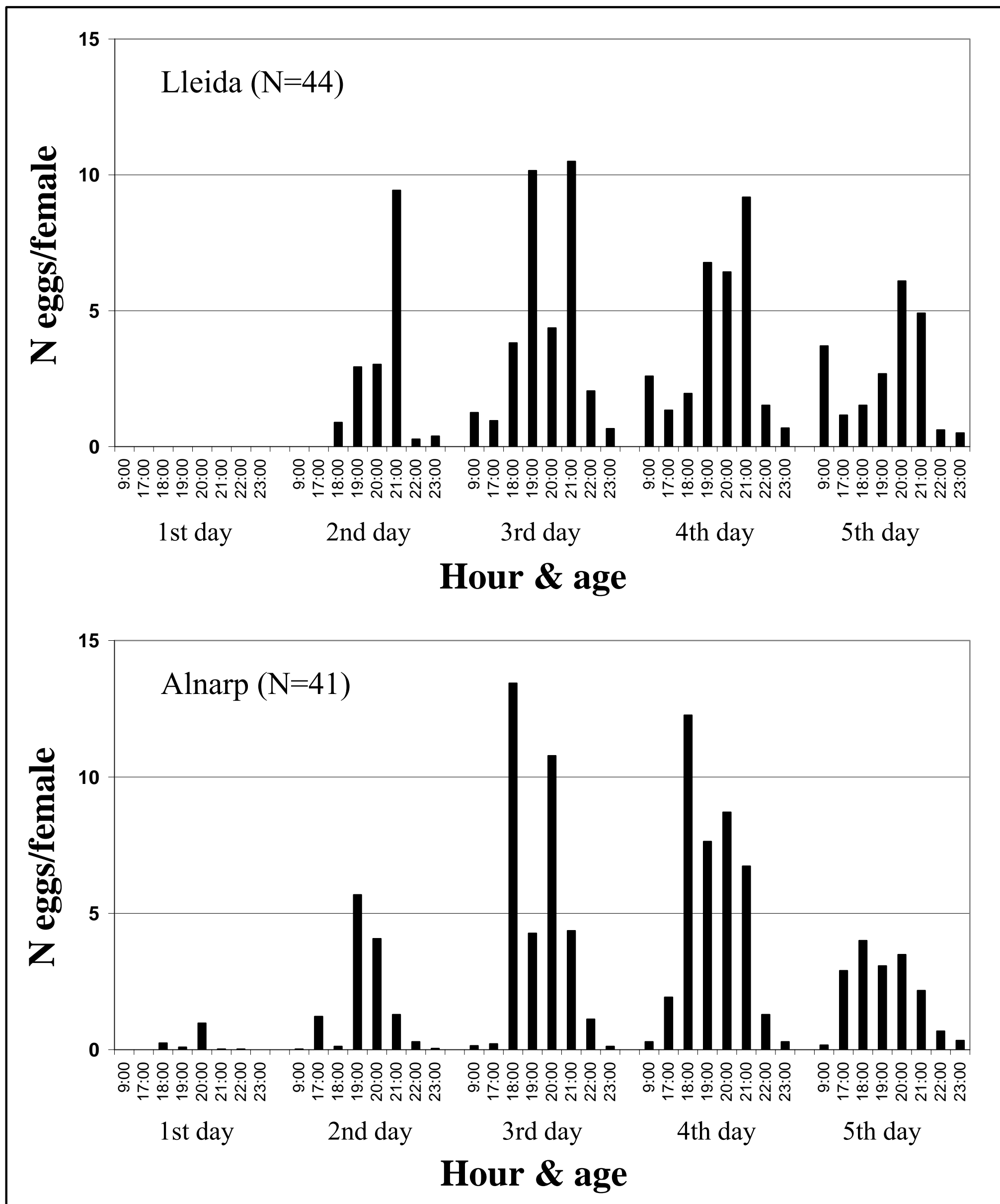

Figure 2. Oviposition pattern of *Cydia pomonella* females within the first five days of life, under natural conditions of light and temperature in two different locations, Lleida (41º 37' N, 0º 38' E) and Alnarp (55º 55' N, 13º 37' E).

With regard to the mating activity, it was scarce in both locations before 20:00 h, increased afterwards, peaked at 21:00 h, and decreased after this observation. The decrease in matings observed was sharper in Alnarp than in Lleida (Figure 1 B,E). Although mating activity timing was similar in both locations with respect to local time, differences were apparent when solar time was considered. In Lleida most of the matings and the mating activity peak were observed after dusk, which happened between 20:12 and 19:43 h during the period of assay. On the other hand, in Alnarp oviposition activity peaked before dusk, and ca. 75 % of matings were observed before

sunset, which occurred at approximately 22:00 h during the period of assay (Figure 1B,E).

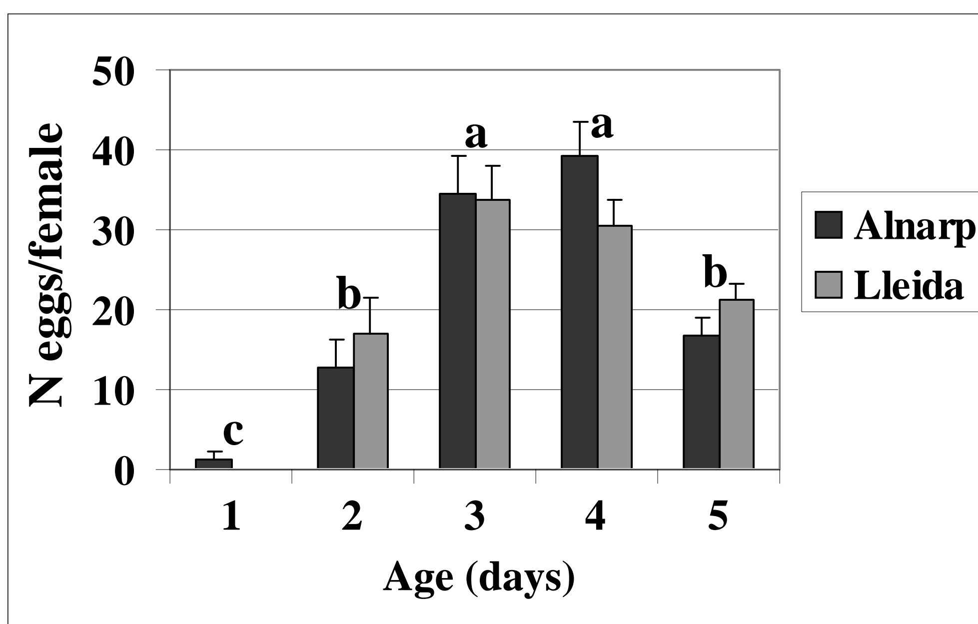

Figure 3. Daily mean oviposition of *Cydia pomonella* females within the first five days of life, under natural conditions of light and temperature in two different locations, Lleida (Spain, 41º 37' N, 0º 38' E) in mid-September, and Alnarp (Sweden, 55º 55' N, 13º 37' E) in late-Sweden. Different letters mean significant differences amongst days (Duncan's Multiple Range Test, $P<0.05$).

Laboratory assay

The average percentage of mated females ranged from 74.3 to 78.7 %, and there were not significant differences amongst treatments ($df = 4$, $F = 0.08$, $P = 0.986$). On the contrary, there was a significant difference in the average total fecundity per female amongst treatments ($df = 4$, $F = 8.37$, $P < 0.001$). No oviposition occurred at 12 ºC, and maximum oviposition took place at 22 and 27 ºC (Figure 4).

As there was no oviposition at 12 ºC, this temperature was discarded for the analysis of the interaction between temperature and observation time. This interaction was significant ($df = 21$, $F = 4.69$, $P < 0.001$), which means that the distribution of oviposition through the period studied varied depending on the temperature. At 27 and 32 ºC, oviposition concentrated on the first hour of the scotophase (54.4 and 59.5 % of total oviposition, respectively), and it was scarce at all other observation times (Figure 5C,D). At 22 ºC, oviposition also peaked during the first hour of the scotophase, but this peak was much smaller (33.4 % of total oviposition), and there were no significant

differences between the mean percentage of oviposition in this observations and observations at 4, 3, and 2 hours before the onset of the scotophase (P = 0.398, P = 0.213, and P = 0.167, respectively) (Figure 5B). At 17 ºC oviposition was rather constant throughout all the period studied. Under this temperature oviposition was maximum 3 hours before the onset of the scotophase, and during its first hour (24.9 and 21.0 % of total oviposition, respectively) (Figure 5A). However the differences between these two observations and the rest were not significant, except for the third hour of the scotophase (P = 0.006, and P = 0.030 for 3 hours before scotophase and its first hour respectively) (Figure 5A).

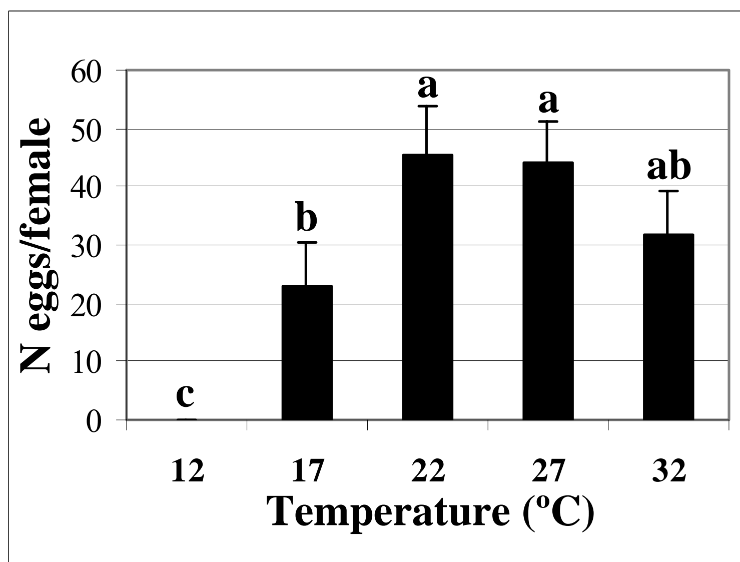

Figure 4. Mean female fecundity of *Cydia pomonella* under laboratory conditions, at five different constant temperatures, and a light intensity of ca. 2500 lux. Different letters mean significant differences amongst temperatures (Duncan's Multiple Range Test, $P<0.05$).

There was also a difference for the time of 50% of oviposition. This was reached earlier the lower was the temperature. At 17 and 22 ºC 50% of oviposition was overcome 3 and 2 hours before scotophase onset, respectively, while at 27 and 32 ºC 50 % of oviposition was not overcome until the first hour of the scotophase (Figure 6).

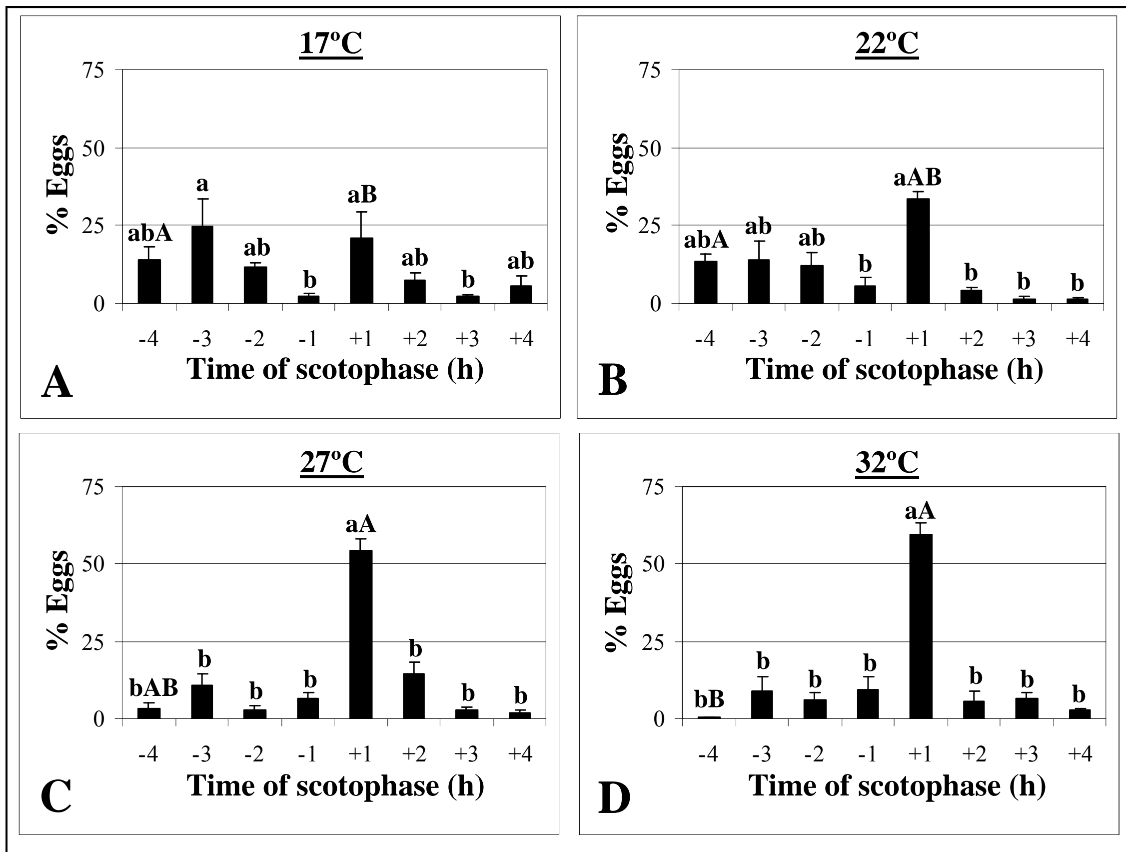

Figure 5. Mean percentage of oviposition each hour from 4 hours before to 4 hours after scotophase onset, at 4 different constant temperatures. Different small letters show significant differences between observations at different times within each temperature, and capital letters show significant differences amongst temperatures for a given time (pairwise comparison of least squared means, $p<0.05$).

4. Discussion

Under semi-field conditions diel oviposition activity took place earlier in Alnarp (Sweden) than in Lleida (Spain), despite dusk happened earlier in Lleida than in Alnarp. In Lleida oviposition activity was concentrated in a few hours around dusk, whereas in Alnarp it was almost ended when dusk happened (Figure 1A,D). On the other hand, mean temperature was rather high and rather constant during the diel period studied in Lleida, whilst it was milder in Alnarp, falling sharply after 19:00 h (Figure 1C,F). Thus our data suggest that temperature is an important factor in the modulation of *C. pomonella* diel oviposition behavior, and a decrease of temperature advances oviposition activity in the day respect dusk. Despite there is no doubt that oviposition is a periodic behavior controlled by light-dark cycles (Riedl & Loher, 1980).

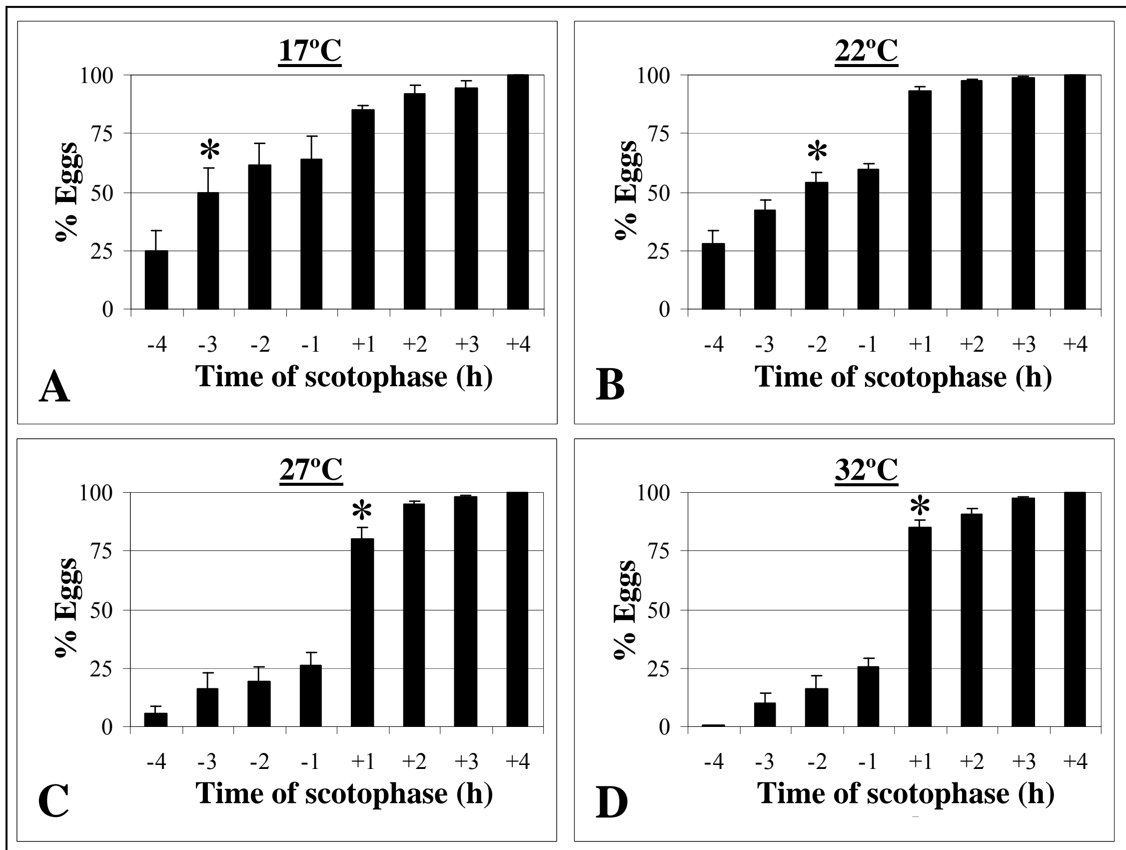

Figure 6. Mean cumulated percentage of oviposition each hour from 4 hours before to 4 hours after scotophase onset, at 4 different constant temperatures. Asterisks mark first control where 50% of oviposition was raised at the different temperatures.

A similar behavior has been observed in the calling diel activity not only in *C. pomonella* (Castrovillo & Cardé, 1979), but also in multiple other moth species, which under the same photoperiod rhythm advance their calling onset and mean hour of calling when temperature is decreased (Cardé & Roelofs, 1973; Baker & Cardé, 1979; Delisle & McNeil, 1987a,b; Delisle, 1992). The onset of mating activity of females of *C. rosaceana*, another tortricid, has been also found to be advanced in the day by low temperatures, both in the field and in the laboratory under constant and fluctuant temperature conditions (Delisle, 1995). However, activity of moth is not modulated by temperature in all the species. In example, onset of flight activity of *Lymantria dispar* (L.) females has been shown to be triggered by a critical light intensity independently of ambient temperature (Charlton *et al.*, 1999).

In our study, when kept at constant temperatures and under a full light/full darkness photoperiod, temperature also affected oviposition timing. Under these conditions time for 50% of oviposition was delayed as the temperature increased

(Figure 6). Furthermore, at 17 ºC there was not a clear peak of oviposition, and at the other temperatures oviposition peaked during the first hour of the scotophase, although this peak was moderate and did not significantly differ from several other times at 22 ºC, and more prominent at 27 and 32 ºC (Figure 5). In previous studies maximum oviposition in *C. pomonella* has been reported to occur at different times around lights-off. Riedl and Loher (1980) reported that oviposition peaked three hours before scotophase onset at 21 ºC under a 17:7 photoperiod (L:D). Weissling and Knight (1996), in an assay conducted at 22 ºC and under a 15:9 photoperiod (L:D) simulating twilight, found oviposition to peak during the two first hours of the scotophase.

It has been reported that onset of male activity in *C. pomonella* is triggered by a decrease in light intensity, together with the reach of a temperature threshold (Witzgall *et al.*, 1999). Authors suggested that on warm days, males become active well after sunset, while on colder days they do during the last sunshine. This agrees with our data as in the semi-field assay, matings were observed earlier respect sunset in Alnarp than in Lleida. Matings observed in Lleida were scarce before dusk, whilst a high proportion of observed matings took place before it in Alnarp (Figure 1B,E). The importance of temperature as a limiting factor for male activity has been also reported in several works. Batiste *et al.* (1973) reported that low temperatures were limiting for the diel flight during early season, while high temperatures delayed initiation of diel flight late in the season. Furthermore, Sæthre and Hofsvang (2005) suggested that temperature is the limiting factor for male flight and oviposition activity in Norway, since light conditions are suitable for moth activity the whole night.

The semi-field assay also showed that, in a given day, *C. pomonella* oviposition activity takes place mainly before mating activity (Figure 1A,B,C,D). Witzgall *et al.* (1999) observed that females flew and oviposited mostly before, and towards the end of male activity. Their observations partially agree with ours, as we observed that female oviposition was concentrated mostly before matings. We observed very little or no oviposition at all by 1-day-old females. Females have been reported capable of mating in the first 12 hours after emergence, while there is a preoviposition period of about one day (Gehring & Madson, 1963). It seems that oviposition may not occur in the same day as mating, and females wait to the day after mating before initiating oviposition.

Eggs were found in the morning observation (9:00 h) in the semi-field assay. These eggs could be laid during night after the last observation (23:00 h), but at this time oviposition was already very low (Figure 1A,D). Those eggs are more likely to had

been laid around dawn when twilight takes place again and temperature increases. The presence of a second peak of activity at the end of the scotophase has been reported for *C. pomonella* in several works (Borden, 1931; Cutright, 1964; Song & Riedl, 1985), although it has also been absent in some other studies (Wong *et al.*, 1971; Batiste *et al.*, 1973; Keil *et al.*, 2001). In Lleida the number of eggs at 9:00 h was higher than in Alnarp, and temperatures are higher in Spanish than in Swedish dawns. These differences in temperature could favor a second peak of oviposition activity at dawn in Spain, as previously suggested (Knight *et al.*, 1994; Song & Riedl, 1985).

In the laboratory assay, there was not a difference in the percentage of mated females amongst treatments, probably because most of the matings occurred within the first 2 days that insects spent in the mating boxes at 22 ºC. On the other hand, differences occurred in the total fecundity per female. Oviposition did not take place at 12 ºC, it reached the maximum at 22 and 27 ºC, and took intermediate values at 17 and 32 ºC (Figure 4). Temperature is well known to influence fecundity in *C. pomonella* (Isely, 1938; Sæthre & Hofsvang, 2002), and many other moth species (e.g., Henneberry & Clayton, 1991; McAvoy & Kok, 1992; Milonas & Savopoulou-Soultani, 2000). The lower oviposition threshold reported for *C. pomonella* varies amongst authors from bellow 10 ºC (Sæthre and Hofsvang, 2002) to 18 ºC (Borden, 1931), and it may vary depending on the population origin (Sæthre & Hofsvang, 2002). Isely (1938) studied the influence of mean diel temperature on different oviposition parameters in *C. pomonella*, and he found that daily oviposition reached its maximum when mean temperature was 27 ºC, and fecundity decreased for temperatures above and below this.

In our semi-field study we observed that most of the oviposition took place at the age of 3 and 4 days, and it decreased on day 5 (Figure 3). Gehring and Madsen (1963) reported that most active oviposition occurred between the second and fourth days and moths were reproductively spent after the sixth day, in laboratory at 21 ºC. On the other hand, Riedl and Loher (1980) found oviposition to be maximum in the first day and then decline specially after the fifth day, also at 21 ºC.

Our study shows that diel female oviposition takes place before than male activity in a given day, and at temperatures higher than male flight does. Oviposition of *C. pomonella* is concentrated in the first hour of the scotophase, under laboratory conditions, for temperatures ranging from 22 to 32 ºC, and it is maximum in 3- and 4-day-old females. In consequence, we suggest that laboratory behavioral assays with mated females should be conducted soon after lights-off, with 3- to 4-day-old

individuals, and at temperatures slightly higher than those used for male assays, which usually range between 22 and 24 ºC (e.g., McDonough *et al.*, 1993; Witzgall *et al.*, 2001).

Acknowledgements

This work was partially funded by project RTA01-079 from the Programa Nacional de Recursos y Tecnologías Agroalimentarias (Spain), by projects AGL2003-06599-CO02-02 and AGL2004-05812/AGR from the Plan Nacional de I+D+I (Spain), and by the Foundation for Strategic Environmental Research (MISTRA, Sweden). D. Casado had a fellowship from MECD (Spain). Dr. C. Gemeno is funded by the Programa Ramón y Cajal (MCYT, Spain).

5. References

Baker, T.C. & Cardé, R.T. (1979) Endogenous and exogenous factors affecting periodicities of female calling and male sex pheromone response in *Grapholitha molesta* (Busck). *Journal of Insect Physiology*, **25**, 943-950.

Barnes, M.M. (1991) Codling moth occurrence, host race formation, and damage. *World Crop Pest: Tortricid Pests* (ed. by L.P.S. Van Der Geest & H.H. Evenhuis), pp. 313-327. Elsevier, Amsterdam.

Batiste, W.C., Olson, W.H. & Berlowitz, A. (1973) Codling moth: Influence of temperature and daylight intensity on periodicity of diel flight in the field. *Journal of Economic Entomology*, **66**, 883-892.

Borden, A.D. (1931) Some field observations on codling moth behavior. *Journal of Economic Entomology*, **24**, 1137-1145.

Cardé, R.T. & Roelofs, W. (1973) Temperature modification of male sex pheromone response and factors affecting female calling in *Holomelina immaculata* (Lepidoptera: Arctiidae). *The Canadian Entomologist*, **105**, 1505-1512.

Castrovillo, P.J. & Cardé, R.T. (1979) Environmental regulation of female calling and male pheromone response periodicities in the codling moth (*Laspeyresia pomonella*). *Journal of Insect Physiology*, **25**, 659-667.

Charlton, R.E., Cardé, R.T. & Wallner, W.E. (1999) Synchronous crepuscular flight of female Asian gypsy moths: relationships of light intensity and ambient and body temperatures. *Journal of Insect Behavior*, **12**, 517-531.

Collins, D.L. & Machado, W. (1935) Comments upon phototropism in the codling moth with reference to the physiology of the compound eyes . *Journal of Economic Entomology*, **28**, 103-106.

Cutright, C.R. (1964) The codling moth in Ohio: historical and ecological aspects. *Ohio Agricultural Experiment Station Research Bulletin*, **969**.

Delisle, J. & McNeil, J.N. (1987) Calling behaviour and pheromone titre of the true armyworm *Pseudaletia unipuncta* (Haw.) (Lepidoptera: Noctuidae) under different temperature and photoperiodic conditions. *Journal of Insect Physiology*, **33**, 315-324.

Delisle, J. & McNeil, J.N. (1987) The combined effect of photoperiod and temperature on the calling behaviour of the true armyworm, *Pseudaletia unipuncta*. *Physiological Entomology*, **12**, 157-164.

Delisle, J. (1992) Age related changes in the calling behaviour and the attractiveness of obliquebanded leafroller virgin females, *Choristoneura rosaceana*, under different constant and fluctuating temperature conditions. *Entomologia Experimentalis et Applicata*, **63**, 55-62.

Delisle, J. (1995) Effect of male and female age on the mating success of the obliquebanded leafroller *Choristoneura rosaceana* (Lepidoptera: Tortricidae) under different ecological conditions. *Journal of Insect Behavior*, **8**, 781-799.

Ferreira, V.S., Riedl, H., Sarasúa, M.J. & Avilla, J. (1994) Modelización del desarrollo y mortalidad de *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) a temperatura constante. *Investigación Agraria*, **2**, 79-86.

Gehring, R.D. & Madsen, H.F. (1963) Some aspects of the mating and oviposition behavior of the codling moth, *Carpocapsa pomonella*. *Journal of Economic Entomology*, **56**, 140-143.

Hagley, E.A.C. (1976) Effect of rainfall and temperature on codling moth oviposition (Lepidoptera: Olethreutide). *Environmental Entomology*, **5**, 967-969.

Henneberry, T.J. & Clayton, T.E. (1991) Tobacco budworm (Lepidoptera: Noctuidae): temperature effects on mating, oviposition, egg viability, and moth longevity. *Journal of Economic Entomology*, **84**, 1242-1246.

Isely, D. (1938) Codling moth oviposition and temperature. *Journal of Economic Entomology*, **31**, 356-359.

Keil, S., Dorn, S. & Gu, H. (2001) Diel patterns of locomotor activity in Cydia pomonella: age and sex related differences and effects of insect hormone mimics. *Physiological Entomology*, **26**, 306-314.

Knight, A.L., Weiss, M. & Weissling, T. (1994) Diurnal patterns of adult activity of four orchard pests (Lepidoptera: Tortricidae) measured by timing trap and actograph. *Journal of Agricultural Entomology*, **11**, 125-136.

Kührt, U., Samietz, J. & Dorn, S. (2006) Thermal response in adult codling moth. *Physiological Entomology*, **31**, 80-88.

McAvoy, T.J. & Kok, L.T. (1992) Development, oviposition, and feeding of the cabbage webworm (Lepidoptera: Pyralidae). *Environmental Entomology*, **21**, 527-533.

McDonough, L.M., Davis, H.G., Chapman, P.S. & Smithhisler, C.L. (1993) Response of male codling moths (*Cydia pomonella*) to components of conspecific female sex pheromone glands in flight tunnel tests. *Journal of Chemical Ecology*, **19**, 1737-1748.

Milonas, P.G. & Savopoulou-Soultani, M. (2000) Development, survivorship, and reproduction of *Adoxophyes orana* (Lepidoptera: Tortricidae) at constant temperatures. *Annals of the Entomological Society of America*, **93**, 96-102.

Pitcairn, M.J., Pickel, C., Falcon, L.A. & Zalom, F.G. (1991) Development and survivorship of *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) at ten constant temperatures. *Pan-Pacific Entomologist*, **67**, 189-194.

Pons, S., Eizaguirre, M., Sarasúa, M.J. & Avilla, J. (1994) Influencia del fotoperiodo sobre la inducción de diapausa de *Cydia pomonella* (Lepidoptera: Tortricidae) en laboratorio y campo. *Investigación Agraria: Producción y Protección Vegetal*, **9**, 477-491.

Riedl, H. & Loher, W. (1980) Circadian control of oviposition in the codling moth, Laspeyresia pomonella, Lepidoptera: Olethreutidae. *Entomologia Experimentalis et Applicata*, **27**, 38-49.

ROA (Real Instituto y Observatorio de la Armada en San Fernando) (2006) *Fenómenos de sol.* http://www.roa.es.

Sæthre, M.G. & Hofsvang, T. (2002) Effect of temperature on oviposition behavior, fecundity, and fertility in two northern populations of the codling moth (Lepidoptera: Tortricidae). *Environmental Entomology*, **31**, 804-815.

Sæthre, M.G. & Hofsvang, T. (2005) Diurnal activity of codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae) males in relation to temperature and twilight. *Norwigian Journal of Entomology*, **52**, 75-90.

Sharpe, P.J.H. & DeMichele, D.W. (1977) Reaction kinetics of poikilotherm development. *Journal of Theoretical Biology*, **64**, 649-670.

Song, Y.H. & Riedl, H. (1985) Effects of temperature and photoperiod on male moth activity of *Laspeyresia pomonella* (L.) in New York. *Korean Journal of Plant Protection*, **24**, 71-77.

Weissling, T.J. & Knight, A.L. (1996) Oviposition and calling behavior of codling moth (Lepidoptera: Tortricidae) in the presence of codlemone. *Annals of the Entomological Society of America*, **89**, 142-147.

Witzgall, P., Bäckman, A.C., Svensson, M., Koch, U., Rama, F., El Sayed, A., Brauchli, J., Arn, H., Bengtsson, M. & Lofqvist, J. (1999) Behavioral observations of codling moth, Cydia pomonella, in orchards permeated with synthetic pheromone. *BioControl*, **44**, 211-237.

Witzgall, P., Bengtsson, M., Rauscher, S., Liblikas, I., Bäckman, A.C., Coracini, M., Anderson, P. & Lofqvist, J. (2001) Identification of further sex pheromone synergists in the codling moth, Cydia pomonella. *Entomologia Experimentalis et Applicata*, **101** , 131-141.

Wong, T.T.Y., Cleveland, M.L., Ralston, D.F. & Davis, D.G. (1971) Time of sexual activity of codling moths in the field. *Journal of Economic Entomology*, **64**, 553-554.

CHAPTER VIII

MAIN RESULTS
AND
GENERAL DISCUSSION

1. Diel and seasonal variations of volatile emissions by *Cydia pomonella* (L.) host plants and electroantennogram responses

The emissions of volatile organic compounds (VOCs) by two hosts of *Cydia pomonella* (L.) and the electroantennographic response of the antenna of the insect to them were studied. Emissions were sampled at different times of the growth season as well as at two different daytimes, morning and dusk; the latest being the period of activity of the insect. Apple (*Malus domestica* Borkh.) is considered the preferred host of *C. pomonella*, it is an important crop in Spain and many other countries worldwide, and can suffer very serious economic losses by *C. pomonella* (Barnes 1991). Walnut (*Juglans regia* L.) is not as cultivated as apple worldwide, and economic losses caused by *C. pomonella* are much less important than in apple. Walnut belongs to the family Juglandaceae, whereas all the other hosts of *C. pomonella* belong to the family Rosaceae, and therefore it is an interesting host species for comparative studies.

Our data show that VOC emissions from apple and walnut differ widely, both quantitatively and qualitatively. Forty-four compounds have been detected in the emissions of apple branches, bearing leaves and fruits. Those are mainly aliphatic compounds, especially alcohols, aldehydes and esters, although a few terpenes, such as (*E*,*E*)-α-farnesene, germacrene D, and (*E*)-β-caryophyllene, are emitted in considerable amounts. The most abundant compound in apple emissions is by far (*Z*)-3-hexenyl acetate, and it is followed by (*Z*)-3-hexenol, and (*E*,*E*)-α-farnesene (Table 1, Chapter III). In contrast, walnut emits large amounts of terpenes, especially hydrocarbon monoterpenes (Figure 1, Chapter IV). Ninety compounds have been detected in emissions from walnut branches, bearing fruits and leaves; β-pinene is the most abundant one, followed by (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, limonene, and germacrene D, amongst others (Table 2, Chapter IV). Our results agree in general with previous studies in apple (Mattheis *et al.* 1991, Bengtsson *et al.* 2001, Hern and Dorn 2003, Vallat and Dorn 2005) and in walnut (Buttery *et al.* 1986, 2000, Henneman *et al.* 2002). Emissions of both host species differ not only in the identity and quantity of single compounds, but also in the total emission of volatiles, which is around 10-fold times higher in walnut than in apple (Table 1, Chapters III & IV).

Significant seasonal variations on the emission of VOC occur in both tree species. In apple differences amongst seasonal periods occurred for 26 compounds. Most of them were emitted in greater amounts in mid-Spring and/or earl-Summer than

in late-Summer (Table 2, Chapter III). All saturated aldehydes appeared in smaller amounts in late- than in early-Summer, which is related with their reduction to alcohols, and later esterification during fruit ripening (Mattheis *et al.* 1991). In relation with this process, several aliphatic esters also show a tendency to increase in late-Summer (Table 1, Chapter III). In walnut, seasonal variations were found for 38 compounds. Twenty-two of them were more abundant in late-Spring and the remaining in mid-Summer (Table 3, Chapter IV). Plants change their emissions through the season due to environmental and phenological causes (e.g. Guenther 1997, Staudt *et al.* 1997, Bengtsson *et al.* 2001, Rapparini *et al.* 2001).

VOC emissions have been also found to vary between morning and dusk periods in both plant species. In apple, (*E*)-β-carypophyllene and an unidentified compound are emitted in higher amounts in the morning, hexyl 2-methylbutanoate has not been detected at dusk, and 2-hexanone, octanal, and (*Z*)-3-hexenol are emitted at larger amounts at dusk. Differences between the two diel periods in walnut have been found for bornyl acetate, α-campholenal, caryophyllene oxide, γ-curcumene, geranyl acetate, linalool, 6-methyl-5-hepten-2-one, myrtenal, ethyl octanoate, neral, pinocarvone, and 3,6,6-trimethylnorpinan-2-one. All these compounds except 3,6,6-trimethylnorpinan-2-one are emitted in larger amounts at dusk than in the morning. Apart from these compounds, nonanal, decanal, and (*Z*)-3-hexenyl 2-methylbutanoate have been also found in higher amounts at dusk than in the morning, but only in one of the seasonal periods sampled. Many other compounds show a tendency to vary amongst morning and dusk in both hosts (Table 1, Chapters III & IV), but differences are not significant. Diel variation of VOC emissions from plants is widely documented (e.g. Kesselmeier *et al.* 1996, Staudt *et al.* 1997, Staudt and Bertin 1998). Recently it has been shown that diel variations in volatile emission, despite depend on other factors, are largely controlled by the inherent physicochemical properties of the different VOC and some physiological controls (Niinemets *et al.* 2002, 2004, Niinemets and Reichstein 2003a,b). Stomatal closure is particularly important in the control of emissions of VOC with a low gas-aqueous-phase partition coefficient. This is the case of oxygenated volatile terpenes and short-chain aliphatics (Niinemets *et al.* 2002, 2004, Niinemets and Reichstein 2003a,b).

Repetitive and consistent responses of *C. pomonella* adult antennae in the gas chromatography-electroantennodetection (GC-EAD) analyses have been detected, in this work, for hexyl acetate, (*Z*)-3-hexenol + nonanal, (*E*)-4,8-dimethyl-1,3,7-

nonatriene, hexyl butanoate, and (*E*,*E*)-α-farnesene in apple collections; and for (*E*)-β-ocimene, alloocimene, (*Z*)-3-hexenol + nonanal, linalool, pinocarvone, (*E*)-β-caryophyllene, germacrene D, (*E*,*E*)-α-farnesene, and caryophyllene oxide in walnut (Figure 1, Chapter III, & Figure 2, Chapter IV). These responses were confirmed by the injection in the GC-EAD of synthetic alloocimene, (*Z*)-3-hexenol + nonanal, linalool, (*E*)-β-caryophyllene, (*E*)-β-farnesene, (*E*,*E*)-α-farnesene, and caryophyllene oxide. Strong EAG-responses were also recorded to many of these compounds (Table 3, Chapter III). Furthermore, all the compounds that elicited EAD consistent responses in apple collections are also present in walnut emissions except for hexyl butanoate; and all the EAD-active compounds from walnut collections are also emitted by apple, except for alloocimene, pinocarvone and caryophyllene oxide (Table 1, Chapters III & IV). The lack of responsiveness to compounds shared by both species that have been found EAD-active in the collections from one host but not from the other, should be due to lower amounts of these compounds in the collections from the host which did not elicit EAD response, as the same *C. pomonella* population was used to detect EAD responses to volatiles of both hosts.

Most of the EAD-active compounds had been already reported as EAD-active and/or as behavior modifying semiochemicals for *C. pomonella*. EAG activity in *C. pomonella* antenna has been already reported for hexyl acetate, (*Z*)-3-hexenol, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*,*E*)-α-farnesene, (*E*)-β-farnesene, (*E*)-β-caryophyllene, germacrene D, and linalool (Bengtsson *et al.* 2001, Bäckman *et al.* 2001, Avilla *et al.* 2003, Ansebo *et al.* 2004). But, to our knowledge, we report for the first time EAG activity for nonanal, alloocimene, (*E*)-β-ocimene, pinocarvone, and caryophyllene oxide. From a behavioral point of view, (*E*,*E*)-α-farnesene is known to stimulate oviposition (Wearing and Hutchins 1973), and to influence adult upwind attraction (Hern and Dorn 1999, Coracini *et al.* 2004) and larval behavior (Sutherland and Hutchins 1973, Landolt *et al.* 2000). Hexyl acetate and nonanal have been reported as repellents to mated females in olfactometer (Hern and Dorn 2004, Vallat and Dorn 2005), and nonanal combined with decanal failed to attract adults in the field (Light and Knight 2005). Interestingly, they are minor components of a larval aggregation pheromone (Jumean *et al.* 2005). (*Z*)-3-Hexenol and linalool have been reported to synergize male attraction to pheromone in wind tunnel (Yang *et al.* 2004). Finally, (*E*)-β-caryophyllene attracts mated females (Vallat and Dorn 2005).

The EAG responses of male and female *C. pomonella* to a wide series of synthetic plant volatiles have been also studied, and EAG-responses to many of them do not differ from these of pear ester [ethyl (*E*,*Z*)-2,4-decadienoate], the only commercial kairomone of *C. pomonella*. The highest responses are elicited by some aliphatic esters, nonanal, and decanal in both sexes. However, linalool also elicits especially high responses in females (Table 3, Chapter IV). Males respond equal or higher than females to all assayed compounds, except β-myrcene. Thus the use of male antennae is recommended in GC-EAD analysis of volatile collections were the amounts of the different compounds are usually small, and antennal responses are weak.

Pear ester is a compound of pear origin that can be used to monitor *C. pomonella* in the field. However, it performs better in walnut than in apple or pear orchards; this is thought to be due to the background effect of other plant volatiles (Light *et al.* 2001). In this sense, it may be interesting to test caryophyllene oxide, pinocarvone, and alloocimene, which are walnut-specific, as lures in apple orchards, where they will not be present in the background emissions. In our samples 2-cyclopentylcyclopentanone was present and elicited EAD-responses (Figure 1 & Table 3, Chapter III, & Figure 2, Chapter IV). This compound is emitted by the bags used for volatile collection (Gramshaw and Soto-Valdez 1998), and interestingly it is used in fragrance industry because of its fruity aroma (ZEON Corp. 2005). Although 2-cyclopentylcyclopentanone is a non-naturally occurring compound, it may be also interesting to test it as a lure for *C. pomonella* trapping.

Our data show that despite large differences between apple and walnut emissions occur, both hosts share many compounds that in our study or in previous ones have been found to be either electrophysiologically- and/or behaviorally-active in *C. pomonella*. Furthermore, these compounds not only are emitted by apple and walnut, but by many other plant species. This supports the hypothesis that phytophagous insects use appropriate blends of ubiquitous compounds to guide themselves to their hosts rather than single species-specific compounds (Bruce *et al.* 2005). Our data also show that daytime affects apple and walnut VOC emissions. Stomatal closure and physicochemical properties of the different compounds are key in the daytime variation of VOC, being oxygenated both terpenes and short-chain aliphatics especially sensible to this physiological mechanism (Niinemets *et al.* 2004).

Amongst the electrophysiologically- and behaviorally-active compounds affecting *C. pomonella* there are several compounds that can be affected by stomatal

closure. It is necessary thus to take into account the period of insect activity to establish the ratios of blends responsible to attract *C. pomonella* to their hosts. Nevertheless, these ratios can not be as strict as in the case of pheromone components because phytophagous are able to attack a range of host species and varieties, and emissions may vary amongst them (e.g. Sutherland *et al.* 1977, Kainulainen *et al.* 2002, Njoroge *et al.* 2005). Plasticity on upwind attraction to plant volatiles has been recently showed for *Lobesia botrana* Den. & Schiff., which is attracted to a 3-component blend of *Vitis vinifera* L. volatiles. Suppression of any of these compounds leads to a dramatic decrease or even abolition of upwind attraction of females, but attraction can be restored (at least partially) by addition of some other compounds that are redundant when added to the 3-component blend (Tasin *et al.*, 2007).

2. Behavioral responses to pear ester, (*E*)-β-farnesene, and codlemone

Pear ester and (*E*)-β-farnesene elicit upwind flight of *C. pomonella* males and females in wind tunnel, but attraction is not complete, because no odor-source contact at all was reached in the wind tunnel. Mated females are more attracted than virgin ones (Figure 1, Chapter V, & Figure 1 Chapter VI). These compounds are fully attractive under field conditions (i.e., Light *et al.* 2001, Coracini *et al.* 2004, Knight and Light 2005); which suggests that additional sensory cues such as other behaviorally active plant volatiles are necessary to produce *C. pomonella* attraction to traps baited with them in the field. This emphasizes the importance of the background odors in *C. pomonella* attraction to single-component-baited traps. This is again in line with the hypothesis that phytophagous insects find their host by appropriate multicomponent blends of common plant volatiles, rather than by single species-specific ones (Bruce *et al.* 2005).

Addition of large amounts of pear ester to the sex pheromone of *C. pomonella* (codlemone) results in an antagonistic effect on male attraction to codlemone in the last steps of the upwind sequence (Figure 2, Chapter V, & Figure 1, Chapter VI). Olfactory receptor neurons on male antenna responding to both codlemone and pear ester have been recently reported (De Cristofaro *et al.* 2004, Ansebo *et al.* 2005). (*E*)-10-Dodecen-1-ol, a codlemone mimic presumably perceived by codlemone neuron receptors, has a similar antagonistic effect on *C. pomonella* attraction. It is a weak attractant by itself, but reduces attraction to codlemone when blended at high rates (Dr. Peter Witzgall,

personal communication). On the contrary, plant volatiles that are perceived by separated olfactory receptor neurons, such as α- and β-farnesene (Ansebo *et al.* 2005), not only do not reduce upwind male attraction when blended in large amounts with codlemone, but may also synergize attraction (Yang *et al.* 2004).

The antagonistic effect of high amounts of pear ester disappears when it is loaded in a separated septum and placed 10 cm apart from codlemone (Figure 2A, Chapter VI). Spatial and temporal coincidence of substances is needed for antagonistic effect to take place (Liu and Haynes 1992, Baker *et al.* 1998). Odor filaments of slightly separated sources do not completely intermix, and separation of antagonist and pheromone restores attraction (Witzgall and Priesner 1991, Fadamiro *et al.* 1999, Coracini *et al.* 2003).

Furthermore, when a septum loaded with the antagonistic blend is tested against codlemone alone, males are unable to discriminate amongst both sources and they land in the similar proportions in both sources (Figure 1C, Chapter VI). This lack of preference between codlemone, and a blend of codlemone and pear ester is also observed for a blend with low pear ester rate, which has nor antagonistic neither synergistic effect at all when assayed alone in wind tunnel (Figures 1 & 2A,B, Chapter VI). Moreover, a high proportion of upwind-flying males alternated between the plume of codlemone, and another of a blend of codlemone and pear ester (Figure 3, Chapter VI). The same behavior has been reported for the behavioral response of *C. pomonella* males to codlemone acetate [(*E*,*E*)-8,10-dodecadienyl acetate], which is a codlemone antagonist. This last compound strongly diminishes attraction to codlemone when both are loaded onto the same septum, but antagonism disappears when they are loaded onto different septa, and they contact per equal both sources (Coracini *et al.* 2003). The authors hypothesized that triggering and maintenance of a behavioral response may be controlled by different neural pathways.

Pre-exposure to codlemone increases male *C. pomonella* upwind response to pear ester, strongly diminishes attraction to codlemone, and has no effect at all on female behavior (Figure 1, Chapter V). Reduction of attraction to codlemone of pre-exposed males is probably due to long-lasting antennal adaptation (Judd *et al.* 2005), but it is unclear how pre-exposure to codlemone affected male response to pear ester. The increase of response of pre-exposed males to pear ester suggests an interconnection of sensory and motor pathways for sex pheromone and plant volatiles in *C. pomonella*, as previously suggested by Coracini *et al.* (2004). Synergistic interaction of pheromone

and a plant volatile compounds at the peripheral nervous level has been demonstrated in a Noctuidae moth (Ochieng *et al.* 2002), and several plant volatiles synergize male attraction to sex pheromone in *C. pomonella* (Light *et al.* 1993, Yang *et al.* 2004). However, our results do not show a synergism between pear ester and codlemone at any assayed rate (Figure 2, Chapter V, & Figure 1, Chapter VI); although synergism between both compounds has been reported in field trapping (Knight *et al.* 2005).

We found increased captures with pear ester under mating disruption. A tentative explanation for this increase in field captures, and increased response of males to pear ester in wind tunnel after codlemone pre-exposure, is that pheromone exposure increases overall sensitivity of *C. pomonella* males to plant odors. An octopamine-mediated sensitization of antennal response to plant volatiles has been demonstrated in other Tortricidae moths (Stelinski *et al.* 2003). This increase in sensitivity to plant odors may play a role in mate finding by *C. pomonella* in orchards under mating disruption. Visual observations in pheromone-treated orchards have revealed that male are not attracted to pheromone dispensers, but they are observed flying around the canopy of fruit-bearing apple trees (Witzgall *et al.* 1999), where the likelihood of finding females should be higher.

3. Influence of temperature and light intensity on oviposition and mating behaviors

Oviposition in *C. pomonella* follows a circadian rhythm controlled by light-dark cycles (Riedl and Loher 1980), but this study shows that temperature is an important factor in the modulation of its diel periodicity. Under semi-field conditions we observed diel oviposition to take place earlier respect dusk time in Sweden (Alnarp) than in Spain (Lleida). On the other hand, temperature decreased at a faster rate in Swedish than in Spanish evenings (Figure 1A,C,D,F, Chapter VII). This indicates that by decreasing the temperature, oviposition is advanced in the day. A similar response to temperature has been reported for calling activity not only in *C. pomonella* (Castrovillo and Cardé 1979), but also in multiple other moth species (Cardé and Roelofs 1973, Baker and Cardé 1979, Delisle and McNeil 1987a,b, Delisle 1992). Studies on pupil movement in *C. pomonella* suggest that flight activity can be triggered only by changes in temperature under complete darkness (Nordström and Warrant 2000).

Adults were maintained at several constant temperatures to determine the effect of temperature on diel oviposition timing. Time to 50 % of oviposition is delayed as the

temperature increases (Figure 6, Chapter VII). On the other hand, oviposition peaks on the first hour of the scotophase in most of the temperatures assayed, and this peak is more prominent at the higher temperatures. For the lowest temperature with oviposition activity (17 ºC), there is not a peak of oviposition; instead oviposition is rather constant throughout the diel period studied. On the other hand, the peak of oviposition at the highest temperatures represents more than 50 % of total oviposition (Figure 5, Chapter VII).

We found a clear second peak of oviposition at the end of the scotophase in Spain that was almost inexistent in Sweden. This difference can be due to higher temperatures at Spanish than at Swedish dawns. This second peak of oviposition has been reported or not by different authors, and temperature has been suggested as a factor favoring or suppressing it (Song and Riedl 1985, Knight *et al.* 1994).

Our data also suggests that oviposition takes place earlier in the day than mating (Figure 1A,B,C,D, Chapter VII). Witzgall *et al.* (1999) observed in the field that female flights, which are related with oviposition activity, take place mostly before and toward the end of male flights, which are related with mating activity.

Fecundity was maximum at 22 and 27ºC, intermediate at 17 and 32ºC, and absent at 12ºC (Figure 4, Chapter VII). The lower oviposition thresholds reported range from bellow 10ºC (Sæthre and Hofsvang 2002) to 18ºC (Borden 1931), and it has been suggested to depend on geographical origin of *C. pomonella* origin (Sæthre and Hofsvang 2002). Optimum temperature for fecundity has been reported as 27ºC (Isely 1938). We found oviposition to be maximum in the 3rd and 4th days of life (Figures 2 & 3, Chapter VII). This agrees with the study of Gehring and Madsen (1963), in which most oviposition occurred between the 2nd and 4th days of life.

Summarizing, our study shows that diel female oviposition takes place before than mating activity, and suggest that the former occurs at higher temperature than male flight. Oviposition of *C. pomonella* is concentrated in the first hour of the scotophase, under laboratory conditions, for temperatures ranging from 22 to 32ºC. We suggest that future behavioral assays with females of *C. pomonella* should be made soon after lights-off, with 3- or 4-day-old females, and at temperatures slightly higher than those used for male bioassays, that usually range between 22 and 24ºC (i.e. McDonough *et al.* 1993, Witzgall *et al.* 2001).

4. Final conclusions

We have shown that *C. pomonella* antenna can perceive many plant volatiles emitted by apple and walnut trees. These two hosts emit a widely different blend of VOCs, but they share most of the compounds that are perceived by *C. pomonella* adults. Alloocimene, pinocarvone, and caryophyllene oxide are compounds only emitted by walnut, and are perceived by *C. pomonella*. As background seems to be an important factor conditioning trap captures in field by host volatiles, these three compounds should be assayed in apple orchards as trapping lures. *C. pomonella* also strongly responded to 2-cyclopentylcyclopentanone, a non-naturally occurring compound, which is used as green fruit odorant in the perfume industry, and may also be an interesting candidate as trapping lure in further behavioral assays.

Pear ester and (*E*)-β-farnesene are fully attractive lures in the field, whilst they do not stimulate source contact in wind tunnel, despite upwind flight is triggered. This emphasizes the importance of background volatiles in field captures of these two compounds, concept expressed in the theory that phytophagous insects are attracted to their hosts by means of multicomponent blends of common plant volatiles, rather than by species-specific single compounds.

There are significant diel variations in the VOC emissions of apple and walnut trees. These differences should be taken into account when establishing ratios in volatile compound blends to be used in further *C. pomonella* bioassays of attraction to host volatiles. However, these ratios do not need to be highly precise, because *C. pomonella* is able to attack different species and varieties of hosts, which differ in their emissions, and plasticity in its attraction to plant hosts should occur.

Pre-exposure to sex pheromone increases male upwind to pear ester in wind tunnel, and captures of *C. pomonella* by pear ester are also increased under mating disruption. This suggests an interconnection of sensory and motor pathways for sex pheromone and plant volatiles in *C. pomonella*. Pheromone pre-exposure may increase male response to plant volatiles, and this can help male in mate finding under mating disruption conditions. Blending pear ester at high amounts with codlemone reduces male upwind response. This effect disappears when both compounds are loaded onto different sources 10 cm apart, and males do not discriminate between the codlemone alone and the antagonistic blend when they are presented at the same time. This

suggests that *C. pomonella* triggering and maintenance of a behavioral response may be controlled by different neural pathways.

Temperature modulates diel timing of *C. pomonella* oviposition and mating activities. Under semi-field conditions, oviposition and mating take place later with respect to dusk the higher the ambient temperature. Under laboratory conditions, oviposition is concentrated in the first hour of the scotophase for temperatures ranging from 22 to 32 ºC, but this concentration is more pronounced at the higher temperatures. On the other hand, 50 % of oviposition occurs earlier as the temperature decreases.

In the future, bioassays must be made with the compounds that have been identified as EAG-active in these studies. Further studies on codlemone and plant volatile interconnected sensory and motor pathways and pear ester perception are required. And our findings in temperature influence on oviposition behavior must be taken into account when designing behavioral assays in female response to plant volatiles.

5. References

Ansebo, L., M. D. A. Coracini, M. Bengtsson, I. Liblikas, M. Ramírez, A. K. Borg-Karlson, M. Tasin, and P. Witzgall. 2004. Antennal and behavioural response of codling moth *Cydia pomonella* to plant volatiles. J. Appl. Entomol. 128:488-493.

Ansebo, L., R. Ignell, J. Löfqvist, and B. S. Hansson. 2005. Responses to sex pheromone and plant odours by olfactory receptor neurons housed in *sensilla auricillica* of the codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae). J. Insect Physiol. 51:1066-1074.

Avilla, J., D. Casado, N. Varela, D. Bosch, and M. Riba. 2003. Electrophysiological response of codling moth (*Cydia pomonella*) adults to semiochemicals. IOBC/Wprs Bull. 26:1-7.

Baker, T. C. and R. T. Cardé. 1979. Endogenous and exogenous factors affecting periodicities of female calling and male sex pheromone response in *Grapholitha molesta* (Busck). J. Insect Physiol. 25:943-950.

Baker, T. C., H. Y. Fadamiro, and A. A. Cossé. 1998. Moth uses fine tuning of odour resolution. Nature 393:530.

Barnes, M. M. 1991. Codling moth occurrence, host race formation, and damage, pp. 313-327 In L. P. S. Van Der Geest and H. H. Evenhuis [eds.], Tortricid Pests: Their Biology, Natural Enemies and Control. World Crop Pests. Elsevier, Amsterdam (Holland).

Bäckman, A. C., M. Bengtsson, A. K. Borg Karlsson, I. Liblikas, and P. Witzgall. 2001. Volatiles from apple (*Malus domestica*) eliciting antennal responses in female codling moth *Cydia pomonella* (L.) (Lepidoptera: Tortricidae): effect of plant injury and sampling technique. Z. Naturforsch. C 56:262-268.

Bengtsson, M., A. C. Bäckman, I. Liblikas, M. I. Ramirez, A. K. Borg Karlson, L. Ansebo, P. Anderson, J. Löfqvist, and P. Witzgall. 2001. Plant odor analysis of apple: antennal response of codling moth females to apple volatiles during phenological development. J. Agric. Food Chem. 49:3736-3741.

Borden, A. D. 1931. Some field observations on codling moth behavior. J. Econ. Entomol. 24:1137-1145.

Bruce, T. J. A., L. J. Wadhams, and C. M. Woodcock. 2005. Insect host location: a volatile situation. Trends Plant Sci. 10:269-274.

Buttery, R. G., R. A. Flath, T. R. Mon, and L. C. Ling. 1986. Identification of germacrene D in walnut and fig leaf volatiles. J. Agric. Food Chem. 34:820-822.

Buttery, R. G., D. M. Light, Y. Nam, G. B. Merrill, J. N. Roitman, and Y. L. Nam. 2000. Volatile components of green walnut husks. J. Agric. Food Chem. 48:2858-2861.

Cardé, R. T. and W. Roelofs. 1973. Temperature modification of male sex pheromone response and factors affecting female calling in *Holomelina immaculata* (Lepidoptera: Arctiidae). Can. Entomol. 105:1505-1512.

Castrovillo, P. J. and R. T. Cardé. 1979. Environmental regulation of female calling and male pheromone response periodicities in the codling moth (*Laspeyresia pomonella*). J. Insect Physiol. 25:659-667.

Coracini, M. D. A., M. Bengtsson, L. Cichón, and P. Witzgall. 2003. Codling moth males do not discriminate between pheromone and a pheromone/antagonist blend during upwind flight. Naturwissenschaften 90:419-423.

Coracini, M. D. A., M. Bengtsson, and P. Witzgall. 2004. Attraction of codling moth males *Cydia pomonella* to apple volatiles. Entomol. Exp. Appl. 100:1-10.

De Cristofaro, A., C. Ioratti, E. Pasqualini, G. Anfora, G. S. Germinara, M. Villa, and G. Rotundo. 2004. Electrophysiological responses of *Cydia pomonella* to codlemone and pear ester ethyl (E,Z)-2,4-decadienoate: peripheral interactions in their perception and evidences for cells responding to both compounds. Bull. Insect. 57:137-144.

Delisle, J. and J. N. McNeil. 1987. Calling behaviour and pheromone titre of the true armyworm *Pseudaletia unipuncta* (Haw.) (Lepidoptera: Noctuidae) under different temperature and photoperiodic conditions. J. Insect Physiol. 33:315-324.

Delisle, J. and J. N. McNeil. 1987. The combined effect of photoperiod and temperature on the calling behaviour of the true armyworm, *Pseudaletia unipuncta*. Physiol. Entomol. 12:157-164.

Delisle, J. 1992. Age related changes in the calling behaviour and the attractiveness of obliquebanded leafroller virgin females, *Choristoneura rosaceana*, under different constant and fluctuating temperature conditions. Entomol. Exp. Appl. 63:55-62.

Fadamiro, H. Y., A. A. Cossé, and T. C. Baker. 1999. Fine-scale resolution of closely spaced pheromone and antagonist filaments by flying male *Helicoverpa zea* . J. Comp. Physiol. A 185:131-141.

Gehring, R. D. and H. F. Madsen. 1963. Some aspects of the mating and oviposition behavior of the codling moth, *Carpocapsa pomonella*. J. Econ. Entomol. 56:140-143.

Gramshaw, J. W. and H. Soto-Valdez. 1998. Migration from polyamide 'microwave and roasting bags' into roast chicken. Food Addit. Contam. 15:329-335.

Henneman, M. L., E. G. Dyreson, J. Takabayashi, and R. A. Raguso. 2002. Response to walnut olfactory and visual cues by the parasitic wasp *Diachasmimorpha juglandis* . J. Chem. Ecol. 28:2221-2244.

Hern, A. and S. Dorn. 1999. Sexual dimorphism in the olfactory orientation of adult *Cydia pomonella* in response to α-farnesene. Entomol. Exp. Appl. 92:63-72.

Hern, A. and S. Dorn. 2003. Monitoring seasonal variation in apple fruit volatile emissions *in situ* using solid-phase microextraction. Phytochem. Anal. 14:232-240.

Hern, A. and S. Dorn. 2004. A female-specific attractant for the codling moth, *Cydia pomonella* (L.), from apple fruit volatiles. Naturwissenschaften 91:77-80.

Isely, D. 1938. Codling moth oviposition and temperature. J. Econ. Entomol. 31:356-359.

Judd, G. J. R., M. G. T. Gardiner, N. C. DeLury, and G. Karg. 2005. Reduced antennal sensitivity, behavioural response and attraction of male codling moths, *Cydia pomonella*, to their pheromone (E,E)-8,10-dodecadien-1-ol following various pre-exposure regimes. Entomol. Exp. Appl. 114:65-78.

Jumean, Z., R. Gries, T. Unruh, E. Rowland, and G. Gries. 2005. Identification of the larval aggregation pheromone of codling moth, *Cydia pomonella*. J. Chem. Ecol. 31:911-924.

Kainulainen, P., A. Nissinen, A. Piirainen, K. Tiilikkala, and J. K. Holopainen. 2002. Essential oil composition in leaves of carrot varieties and preference of specialist and generalist sucking herbivores. Agric. Forest Entomol. 4:211-216.

Kesselmeier, J., L. Schäfer, P. Ciccioli, E. Brancaleoni, A. Cecinato, M. Frattoni, P. Foster, V. Jacob, J. Denis, J. L. Fugit, L. Dutaur, and L. Torres. 1996. Emission of monoterpenes and isoprene from a Mediterranean oak species Quercus ilex L measured within the BEMA (Biogenic Emissions in the Mediterranean Area) project. Atmos. Environ. 30:1841-1850.

Knight, A. L., M. Weiss, and T. Weissling. 1994. Diurnal patterns of adult activity of four orchard pests (Lepidoptera: Tortricidae) measured by timing trap and actograph. J. Agric. Entomol. 11:125-136.

Knight, A. L. and D. M. Light. 2005. Dose-response of codling moth (Lepidoptera: Tortricidae) to ethyl (E,Z)-2,4-decadienoate in apple orchards treated with sex pheromone dispensers. Environ. Entomol. 34:604-609.

Knight, A. L., R. Hilton, and D. M. Light. 2005. Monitoring codling moth (Lepidoptera: Tortricidae) in apple with blends of ethyl (*E*,*Z*)-2,4-decadienoate and codlemone. Environ. Entomol. 34:598-603.

Landolt, P. J., J. A. Brumley, C. L. Smithhisler, L. L. Biddick, and R. W. Hofstetter. 2000. Apple fruit infested with codling moth are more attractive to neonate codling moth larvae and possess increased amounts of (E,E)-alpha-farnesene. J. Chem. Ecol. 26:1685-1699.

Light, D. M., R. A. Flath, R. G. Buttery, F. G. Zalom, R. E. Rice, J. C. Dickens, and E. B. Jang. 1993. Host-plant green-leaf volatiles synergize the synthetic sex pheromones of the corn earworm and codling moth (Lepidoptera). Chemoecology 4:145-152.

Light, D. M., A. L. Knight, C. A. Henrick, D. Rajapaska, B. Lingren, J. C. Dickens, K. M. Reynolds, R. G. Buttery, G. Merrill, J. Roitman, and B. C. Campbell. 2001. A pear-derived kairomone with pheromonal potency that attracts male and female codling moth, *Cydia pomonella* (L.). Naturwissenschaften 88:333-338.

Liu, Y. B. and K. F. Haynes. 1992. Filamentous nature of pheromone plumes protects integrity of signal from background chemical noise in cabbage looper moth, *Trichoplusia ni*. J. Chem. Ecol. 18:299-307.

Mattheis, J. P., J. K. Fellman, P. M. Chen, and M. E. Patterson. 1991. Changes in headspace volatiles during physiological development of Bisbee Delicious apple fruit. J. Agric. Food Chem. 39:1902-1906.

McDonough, L. M., H. G. Davis, P. S. Chapman, and C. L. Smithhisler. 1993. Response of male codling moths (Cydia pomonella) to components of conspecific female sex pheromone glands in flight tunnel tests. J. Chem. Ecol. 19:1737-1748.

Niinemets, U., M. Reichstein, M. Staudt, G. Seufert, and J. D. Tenhunen. 2002. Stomatal constraints may affect emission of oxygenated monoterpenoids from the foliage of Pinus pinea. Plant Physiol. 130:1371-1385.

Niinemets, U. and M. Reichstein. 2003. Controls on the emission of plant volatiles through stomata: Differential sensitivity of emission rates to stomatal closure explained. J. Geophys. Res. 108:4208, DOI: 10.1029/2002JD002620.

Niinemets, U. and M. Reichstein. 2003. Controls on the emission of plant volatiles through stomata: A sensitivity analysis. J. Geophys. Res. 108.

Niinemets, U., F. Loreto, and M. Reichstein. 2004. Physiological and physicochemical controls on foliar volatile organic compound emissions. Trends Plant Sci. 9:180-186.

Njoroge, S. M., H. Koaze, P. N. Karanja, and M. Sawamura. 2005. Essential oil constituents of three varieties of Kenyan sweet oranges (*Citrus sinensis*). Flavour Frag. J. 20:80-85.

Nordström, P. and E. J. Warrant. 2000. Temperature induced pupil movements in insect superposition. J. Exp. Biol. 203:685-692.

Ochieng, S. A., K. C. Park, and T. C. Baker. 2002. Host plant volatiles synergize responses of sex pheromone-specific olfactory receptor neurons in male *Helicoverpa zea*. J. Comp. Physiol. A 188:325-333.

Riedl, H. and W. Loher. 1980. Circadian control of oviposition in the codling moth, Laspeyresia pomonella, Lepidoptera: Olethreutidae. Entomol. Exp. Appl. 27:38-49.

Sæthre, M. G. and T. Hofsvang. 2002. Effect of temperature on oviposition behavior, fecundity, and fertility in two northern populations of the codling moth (Lepidoptera: Tortricidae). Environ. Entomol. 31:804-815.

Song, Y. H. and H. Riedl. 1985. Effects of temperature and photoperiod on male moth activity of *Laspeyresia pomonella* (L.) in New York. Korean J. Plant. Prot. 24:71-77.

Staudt, M., N. Bertin, U. Hansen, G. Seufert, P. Ciccioli, P. Foster, B. Frenzel, and L. Fugit. 1997. Seasonal and diurnal patterns of monoterpene emissions from *Pinus pinea* (L.) under field conditions. Atmos. Environ. 31:145-156.

Staudt, M. and N. Bertin. 1998. Light and temperature dependence of the emission of cyclic and acyclic monoterpenes from holm oak (*Quercus ilex* L.) leaves. Plant Cell Environ. 21:385-395.

Stelinski, L. L., J. R. Miller, and L. J. Gut. 2003. Presence of long-lasting peripheral adaptation in oblique-banded leafroller, *Choristoneura rosaceana* and absence of such adaptation in redbanded leafroller, *Argyrotaenia velutinana*. J. Chem. Ecol. 29:405-423.

Sutherland, O. R. W. and R. F. N. Hutchins. 1973. Attraction of newly hatched codling moth larvae (*Laspeyresia pomonella*) to synthetic stereo-isomers of farnesene. J. Insect Physiol. 19:723-727.

Sutherland, O. R. W., R. F. N. Hutchins, and C. H. Wearing. 1974. The role of the hydrocarbon α-farnesene in the behaviour of codling moth larvae and adults, pp. 249-263 In L. Barton Brown [ed.], Experimental Analysis of Insect Behaviour. Springer-Verlag, Berlin (Germany).

Sutherland, O. R. W., C. H. Wearing, and R. F. N. Hutchins. 1977. Production of alpha -farnesene, an attractant and oviposition stimulant for codling moth, by developing fruit of ten varieties of apple. J. Chem. Ecol. 3:625-631.

Tasin, M., A. C. Bäckman, M. Coracini, D. Casado, C. Ioratti, and P. Witzgall. 2007. Synergism and redundancy in a plant volatile blend attracting grapevine moth females. Phytochemistry 68:203-209.

Vallat, A. and S. Dorn. 2005. Changes in volatile emissions from apple trees and associated response to adult female codling moths over the fruit-growing season. J. Agric. Food Chem. 53:4083-4090.

Wearing, C. H. and R. F. N. Hutchins. 1973. α-Farnesene, a naturally occurring oviposition stimulant for the codling moth, *Laspeyresia pomonella*. J. Insect Physiol. 19:1251-1256.

Witzgall, P. and E. Priesner. 1991. Wind tunnel study on an attraction inhibitor in male *Coleophora laricella* (Lepidoptera: Coleophroidae). J. Chem. Ecol. 17:1355-1362.

Witzgall, P., A. C. Bäckman, M. Svensson, U. Koch, F. Rama, A. El Sayed, J. Brauchli, H. Arn, M. Bengtsson, and J. Löfqvist. 1999. Behavioral observations of codling moth, Cydia pomonella, in orchards permeated with synthetic pheromone. BioControl 44:211-237.

Witzgall, P., M. Bengtsson, S. Rauscher, I. Liblikas, A. C. Bäckman, M. Coracini, P. Anderson, and J. Löfqvist. 2001. Identification of further sex pheromone synergists in the codling moth, *Cydia pomonella*. Entomol. Exp. Appl. 101:131-141.

Yang, Z., M. Bengtsson, and P. Witzgall. 2004. Host plant volatiles synergize response to sex pheromone in codling moth, *Cydia pomonella*. J. Chem. Ecol. 30:619-629.

ZEON Corp. 2005. Specialty chemicals product list. http://www.zeon.co.jp/business_e/enterprise/spechemi/index.html