Comunicación química intraespecífica de *Coroebus spp.* (Coleoptera: Buprestidae) y *Dociostaurus maroccanus* (Orthoptera: Acrididae), dos insectos plaga de la Península Ibérica

Benjamin Fürstenau

The Chemical Ecology of four insect pests was investigated in this work. Eavesdropping of their intraspecific communication by means of pheromones or other volatile organic compounds provide important details that could be used to control them in a environmentally-friendly way.

With the aim to find possible pheromones of three pest species of the Iberian Peninsula, analytical studies of the emitted volatiles were accomplished. In behavioral and electrophysiological bioassays the activity of the identified compounds was determined, and additionally, field trapping experiments were initiated.

The two jewel beetles, *Coreobus undatus* and *C. florentinus* (Coleoptera: Buprestidae), represent serious threats to the cork oak, *Quercus suber*, causing severe damage to the cork, exclusively produced by this endemic tree. Further pest species investigated include the Moroccan locust, *Dociostaurus maroccanus*, (Orthoptera: Acrididae). This polyphagous pest of crops and pastures causes high economic damage in many countries of the Mediterranean Basin.

In addition, this work filled a knowledge gap of the desert locust, *Schistocerca gregaria* (Orthoptera: Acrididae), one of the most destructive crop pests in Africa and Asia, concerning the maturation response of gregarious nymphs to the presence of pheromone producing conspecific mature adults.

Altogether, this study provides the basis for following research and may be helpful to curtail the reproduction and spread of these pests.

Comunicación química intraespecífca de *Coroebus spp.* y *D. maroccanus*

2011

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Memoria presentada por **Benjamin Fürstenau**
para optar al título de Doctor por la Universidad de Barcelona

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To my family

Wie schon mein Biologielehrer, Herr Alheit, mir mit auf den Weg gab,

„Nichts ist überzeugender als Erfolg"

(Leopold von Ranke, 1795-1886)

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It started at the age of 5, when I was obsessed with the idea to become the future head of the Berlin Zoo. Together with my grandmother we called Prof. Klös, who was the zoo director back then. He explained me that for fulfilling my dream, first of all, I had to respect animals and nature, and necessarily study biology. That is what I did.

En primer lugar quería dar las gracias a los dos directores de mi tesis, Dr. Ángel Guerrero y Dra. Gloria Rosell. Ángel ha tenido la confianza en ofrecerme éstos proyectos novedosos y prometedores para realizar el presente trabajo. Con las ayudas, las ideas y los ánimos de ambos he podido conseguir este resultado. Gracias también a Pilar.

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Ahora me dirijo a los otros compañeros, los cuales me han acompañado y apoyado en todos los momentos: alegres y también algunos duros durante los últimos años. Ha sido un placer ir cada día a trabajar, sólo para veros un rato. Gracias Anna, por tu organización, tu compañía y tus pastelitos. Lourdes, por tu profesionalidad y tu ayuda en cualquier momento. Tú has podido identificar el BP84. Gracias Patricia, por discutir conmigo muchos aspectos biológicos y por plancharme mis camisetas en Jena. Gracias Sara, por llenar los días con alegría y gastarme el dinero en Privalia.

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ABBREVIATIONS

Abbreviation	Meaning
AL	antennal lobe
BP	base peak
C_6	fatty acid (hydrocarbon) with 6 carbons
DCM	dichloromethane
DDT	dichlorodiphenyltrichloroethane
EAB	emerald ash borer (*Agrilus planipennis*)
EAG	electroantennogram
EI	electron impact
eV	electron volt
FID	flame ionization detector
GC	gas chromatography
GC-EAD	gas chromatography-electroantennographic detection
GC-MS	gas chromatography-mass spectrometry
GLVs	green leaf volatiles
GOBP	general odorant binding protein
ID	inner diameter
IPM	integrated pest management
IS	internal standard
JH (III)	juvenile hormone (III)
MF	methyl farnesoate
M_r	relative molecular mass
MS	mass spectra
m/z	ion mass/ion charge
OBP	odorant binding protein
OBB	oak branch borer
OFB	oak flathead borer
OD	outer diameter
ODE	odorant degrading enzyme
OR	olfactory receptor
ORN	olfactory receptor neuron
PAN	phenylacetonitrile
PBP	pheromone binding protein
RI	retention index
SEM	scanning electron microscope
SPME	solid phase microextraction
VOC	volatile organic compound

Statistical abbreviations and symbols

α	significance level
F	variance of means
N	number of replicates
n.s.	not significant
P	probability value
S.E.M.	standard error of mean
χ^2	chi-square goodness-of-fit test
*	significance level 5%
**	significance level 1%
***	significance level 0.1%

GENERAL INTRODUCTION

A) BIORATIONAL CONTROL OF INSECT PESTS

Insects are among the most diverse group of animals on earth with more than a million described species. Furthermore, they are involved in various vital 'ecosystem services', such as pollination, decomposition, biological control, etc. and they also contribute directly to human-based economies through silk and honey production (Murugan, 2006). On the other hand, insects account for the biggest part of herbivore organisms, executing a strong selection pressure on all living plants, and thereby they affect negatively to worldwide nutrition, economy and finally human population.

During evolution plants developed a multiplicity of defense mechanisms as adaptation against its numerous enemies. This includes, on the one hand, morphological characteristics such as hairs, thorns and a thick cuticle, and on the other hand, specific chemical mechanisms (Harborne, 1995). In most cases there is a combination of both features (Howe and Westley, 1993). Ehrlich and Raven (1964), among others, proposed the theory of a biochemical co-evolution between plants and its herbivores, in which e.g. the synthesis of secondary plant metabolites by host plants is closely related to the pattern of utilization of phytophagous insects. Thus, herbivores were also capable to evolve new strategies to avoid the different plant defense types, and therefore the number and variety of insect pests rose in a drastic manner. The consequences were and still are a strongly enhanced damage and subsequently loss of crops, forest stands and other plants used by humans. Natural defense mechanisms of plants alone were not sufficient anymore to guarantee its consistency. Additionally, the dramatically increase of human population required the exploitation of a faultless agricultural flora to secure food and to ensure great economic interests. Finally, the excessive proliferation of insect pests combined with the need to dispose of healthy plants evoked the development and application of chemical treatments to control and/or kill the pests. Another great problem is that some insects can be used as vectors of human and animal diseases, like malaria, dengue or leishmaniasis, for what chemical control strategies have become essential.

The first mass-produced chemical compound, dichlorodiphenyltrichloroethane (DDT), was initially applied during the Second World War to eliminate lice and organisms that are used as vectors of typhus and malaria (Wheeler and Aus, 1946). After the discovery by Nobel Prize winner Paul H. Müller of its high efficiency as a contact poison against several arthropods, DDT was used as a global agricultural pesticide. Over the last 60 years, utilization of pesticides such as insecticides, herbicides, fungicides and growth regulators to reduce herbivore produced damage,

diseases and weeds, has represented a basic requirement for food production in developing and less developed countries (Barr and Needham, 2002). In a recent report of the EPA (Environmental Protection Agency) it was shown that ca. 2.5 million of metric tons of pesticides as active ingredients were applied in agriculture worldwide in 2001. Only in US households the use of pesticides reached an amount of up to 0.5 million metric tons (Gilden, et al., 2010). Nonetheless, although effective at short notice, chemical treatments are very aggressive to the environment, provoking loss of circumfluent biodiversity, ambiance breakdown and contamination of soil, water and air. Furthermore, pesticides that demonstrate low or null specificity display an evident risk on the health of potential consumers of the treated crops (Byers, 1991), provoking acute and/or chronic intoxications (Yarsan and Cakir, 2006). For example, 10 of the 12 most dangerous and persistent organic chemicals are considered pesticides (Gilden, et al., 2010). The massive utilization of products like DDT caused great damage to the environment and human health, particularly the decline of bird and fish populations (Carson, 1962) and the correlation between the exposure to this product and numerous types of cancer (Flodin, et al., 1988). Under the Stockholm Convention on Persistent Organic Pollutants its use in agriculture was forbidden worldwide, meanwhile its application in disease vector control, especially in less developed countries, continues. Another great problem of pesticides is that insects dispose of a great ability to adapt and to create resistance against the applied chemicals.

For all these reasons, it has become indispensible to develop new, more specific and less contaminant alternatives for pest control for a sustainable agriculture. An effective and environmentally sensitive approach is the Integrated Pest Management (IPM) (Dreistadt, et al., 2004), whose program is based on a combination of reasonable practices including a series of pest management evaluations, decisions and control methods (Bennett, et al., 2005). At first it is necessary to evaluate if the pest population or the environmental conditions require a specific pest control. For this, regular observations should be carried out to identify the pest damage and outline the infestation level. The following preventive step is using mechanical control measures with little or no risk to people or the environment, like barriers, rotating between crops, selecting pest-resistant varieties, and planting of pest-free rootstock. Once identification and action thresholds indicate that a specific pest control is required, and the former preventive methods are no longer effective or available, IPM programs help to evaluate a proper control method. Knowledge of the interaction between insects and host plants and the way insects communicate with each other is significant for the development of effective, environmentally-friendly control strategies. Among the

different control methods we can include, on the one hand, the application of synthetic insecticides based in plant natural products like pyrethroids (**chemical control**) (Reigart and Roberts, 1999), and, on the other hand, the use of genetically modified plants, microbial pesticides such as *Bacillus thuringiensis* or baculoviruses and natural enemies of the pest (**biological control**), and in particular semiochemicals, a class of specific volatile compounds, which will be discussed in this work.

Semiochemicals (derived from *semeo,* in Greek = sign), also called infochemicals (Dicke and Sabelis, 1988), are organic compounds (natural or synthetic origin) that carry a message and regulate intra- and interspecific interactions between organisms and their environment (Isman, 2004). Unlike visual or auditory signals, semiochemicals are mostly volatile and perceived through olfaction. They are emitted by a plant or an animal and the messages that they convey generally result in specific behaviors or physiological responses in another organism (Harborne, 1995). Activity of semiochemicals depends on their molecular structure, type of functional group, grade of unsaturation and chirality (Tumlinson and Teal, 1987). Contingent upon its function and the benefiting organism, semiochemicals are divided into several subgroups (Fig. 1). When the compound produced by an insect affects an individual of the same species (*intraspecific signal*), it is called pheromone (Francke and Schulz, 1999; Mori, 2010), whereas semiochemicals that affect an individual of a different species (*interspecific signal*) are called allelochemicals (allelomones) (Tumlinson, et al., 1993; Petroski, et al., 2005).

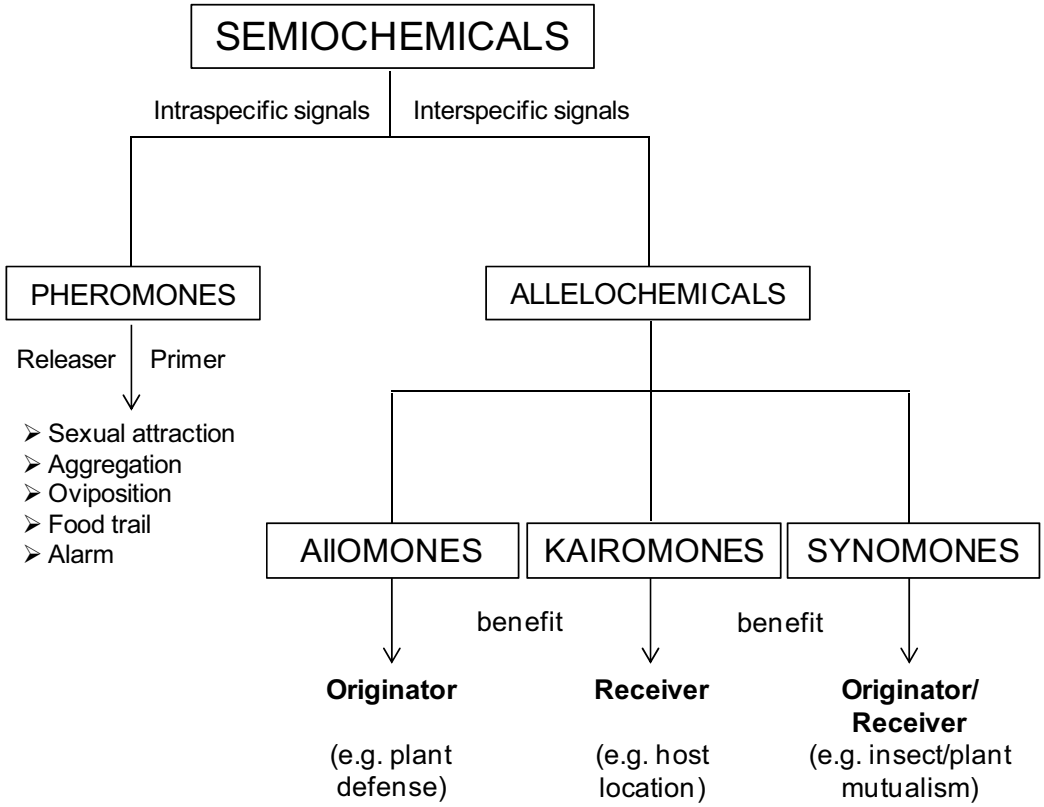


Fig. 1. Modified classification and functions of infochemicals (semiochemicals) (Mori, 2010).

The term "pheromone" was introduced by Peter Karlson and Martin Lüscher (Karlson and Lüscher, 1959), based on the Greek word *pherein* (to transport) and *hormone* (to stimulate). Except for most contact pheromones, insect pheromones are highly volatile chemicals produced by a variety of glands located in different parts of the insects body (Kaissling, 2004). Since the first identification of such chemicals by German biochemist Adolf Butenandt (Butenandt, et al., 1959), called bombykol ((*E,Z*)-10,12-hexadecadienol), a great number of pheromonal compositions of different insect species were determined (Mayer and McLaughlin, 1991; Hardie and Minks, 1999). Bombykol is a sex pheromone secreted by the abdominal *sacculi lateralis* (Steinbrecht, 1964) of the female silkworm *Bombyx mori* to attract their mates. Basic research on olfaction and odor-induced behavior from the bombykol analysis allowed scientist to discover new attractants or repellents for a great variety of insect pest species (Schneider, 1999).

Pheromones are classified into two categories: releaser pheromones and primer pheromones. A releaser (or signaler) pheromone causes a change of behavior in the receiver while a primer pheromone has a physiological impact on the receiver (Borden, 1993), like maturation in nymphs and young hoppers of the gregarious desert locust

Schistocerca gregaria (Assad, et al., 1997; Mahamat, et al., 2000). Releaser pheromones can be further classified as sex pheromones, aggregation pheromones, trail pheromones, etc., according to the type of behavioral change induced (Mori, 2010). Generally, pheromones are released in minute quantities and they consist of more than one particular chemical (Silverstein, 1981). Dependent on species and function pheromones can be produced by both sexes. For example, sex pheromones are emitted frequently by females attracting males (Barclay and Van den Driessche, 1983), although in some species males can be also the producers (Wang and Millar, 2000; Narayanan and Nadarajan, 2005; Fonseca, et al., 2010).

Allelochemicals (derived from *allelon,* in Greek = of each other), which mediate the chemical communication between individuals of different species, comprise allomones, kairomones and synomones (Headrick and Gordh, 2001). Allomones (derived from *allos,* in Greek = other) are biofunctional molecules which evoke advantageous reactions in their producers, such as defense compounds, whereas kairomones (from *kairo,* in Greek = opportune) evoke advantageous reactions in their receivers. Foraging kairomones, for example, are used by insects to reach the habitat of the food source (host habitat-location) and to locate the food source within the habitat (host location) (Ruther, et al., 2002).

Synomones (derived from *syn,* in Greek = together with), in turn, are biofunctional molecules which evoke advantageous reactions in both, their producers and receivers (Birch and Haynes, 1990). Examples are, inter alia, herbivore-induced volatiles emitted by damaged maize plants (*Zea mays*) that attract parasitoids of the herbivore. Thus, the plant benefits from the parasitoid which kills the enemy and the parasitoid is able to locate its host (Turlings and Tumlinson, 1991; Turlings, et al., 1990; 1991; 1995).

As the Chemical Ecology progressed along the 1970's, practical applications from the emerging technology were envisioned, with the emphasis to interfere with the chemical communication of the studied organisms. Thus, semiochemicals, with their capacity to modify organism behavior, attract, repel or confuse, have been considered in many cases as tools for integrated pest management, with the main objective to minimize the damage of insect pest species by reducing and/or regulating their populations. Unlike pesticides, semiochemicals are naturally occurring compounds, not toxic, with a high degree of species specificity and low environmental impact (Silverstein, 1981).

In practice, first of all semiochemicals have to be located and identified from the pest before starting structural elucidation and synthesis of the components. In

subsequent behavioral and electrophysiological bioassays the possible activity has to be confirmed. Finally, the selected and active compounds can be applied in different ways in IPM programs (Renou and Guerrero, 2000).

The most important class of semiochemicals used in biorational pest control is represented by the pheromones (Barclay and Van den Driessche, 1983). In many studies it has been demonstrated that application of pheromones or a combined treatment of pheromones and kairomones in IPM programs has permited to control a variety of insect pest species (Ridgway, et al., 1990), including Lepidoptera (moths and butterflies) (Tingle and Mitchell, 1982; Mitchell, et al., 1983), beetles (Bordon, 1993; Oehlschlager, et al., 2002; Brockerhoff, et al., 2006a) and locusts (Hunter, 2004; Simpson, et al., 2005; Lecoq, 2010;).

Synthetic semiochemicals (generally pheromones and/or kairomones) are applied onto specially designed traps to attract individuals of the studied pest with the aim to estimate its presence, population size, expansion and grade of infestation (**monitoring**). This technique helps to choose the right control measures against the insect, and monitoring of insect pest populations with sex pheromone baited traps has become a routine part in the control of severed crop, green house and forest pests (Wall, 1989; Bordon, 1993). For **mass trapping**, highly specific attractants are deployed in an elevated number of traps to catch a big number of individuals, so that population size of the next generations can be reduced to economically acceptable levels (Oehlschlager, et al., 2002). To accomplish this, in the case of lepidopteran pests these levels are estimated at around 80-95% of captures (Knipling and McGuire, 1966). One disadvantage of the utilization of pheromones in mass trapping experiments is that they are only efficient at low pest densities, since at high concentrations they either confuse or repel the target species (Jacobsen and Beroza, 1964). On the contrary, this fact can be exploited by using pheromones to disrupt mating or to redirect pests to an inappropriate host for oviposition (Barclay and Van den Driessche, 1983). For this alternative method, the so-called **mating disruption**, a high number of volatile releasing dispensers baited with sex pheromones are placed in the herbivore infested area. Emission of great quantities of synthetic pheromones leads to saturation of the environment, masking the natural pheromone plume and thereby confusing the searching insects to locate their mates (Welter, et al., 2005). Thus, the number of copulations decreases provoking reduction of the population levels of successive generations. The California Department of Pesticide Regulation, the California Department of Food and Agriculture, and the United States Environmental Protection Agency (EPA) consider mating disruption to be among the most useful

environmentally friendly treatments to eradicate pest infestations. Therefore, the use of sex pheromones has proven as a potentially effective mean for controlling certain species (Barclay and Judd, 1995).

As noticed above, before starting an IPM program to control a pest, it is essential to understand the complete biology, physiology and specific behavior of the insect. If we want to interfere with the chemical communication of an animal it is necessary to evaluate an important part of this process, the detection of odors.

B) OLFACTORY PERCEPTION

Basically, insects perceive the world through small volatile molecules (semiochemicals), which carry information of potential sex mates, possible predators and specific characteristics of the environment, like suitable food sources, oviposition sites, etc. (Visser, 1986). Hence, for most insects, olfactory signals are more essential in the moment of valuating the environment than the visual or auditory ones (Schoonhoven, 1990). Interruption and/or exploitation of the olfaction process are considered to be very useful for the establishment of alternative pest control methods. It should be outlined that an insect searching for mates and food or avoiding natural enemies is facing a complex chemical world with the “obligation” to take the right decision (Visser and de Jong, 1988).

The main organ of the insects to perceive odorant molecules is the antenna, which possess hair-like sensory organs called *sensilla*. They are morphologically and physiologically well-defined units responsible for the detection and recognition of the odors (Steinbrecht, 1996; Keil, 1999). A sensillum is a hollow cuticular structure, covered by a waxy layer and implemented by pores and pore tubules, which is innervated by more or less branched dendrites of one to several sensorial cells (Fig. 2 D) (Ernst, 1969; Steinbrecht, 1973). It has been demonstrated that olfactory systems of different insect species are equipped with a multitude of these olfactory receptor neurons (ORNs), enabling them to detect and perceive different semiochemicals from their surrounding environment (Hansson, 1995; de Bruyne, et al., 2001). The cell bodies of ORNs are located in the tissue under the cuticle (Keil, 1997; 1999). A bigger antennal surface generally corresponds to a higher number of receptors, and consequently, the insect should be more sensitive to pheromone or other attractant molecules. Nevertheless, it was shown that the sensitivity depends not only on the

number of olfactory receptor neurons (sensorial cells) but also on the number of dendrite ramifications (Davies, 1988).

Volatile molecules penetrate through the above mentioned pores into the cavity of the antennal sensilla, filled with lymph (Fig. 2 E) and initiate the odor perception (Kanaujia and Kaissling, 1985). The antennal lymph is an aqueous solution rich in enzymes and other proteins able to bind different kinds of molecules (Vogt and Riddiford, 1981). It contains an unusual ion composition (200 mM of K^+, 40 mM of Na^+), which is regulated by three types of auxiliary cells (*tormogen*, *trichogen* and *thecogen*) positioned at the base of the sensilla (Fig. 2 D) (Vogt, 2005). Once in the sensillar lymph, the odors are bound to special proteins called *odorant binding proteins* (OBPs) (Breer, et al., 1990a; Steinbrecht, et al., 1996; Steinbrecht, 1998) that are divided into two groups depending on the ligand nature. If the molecule to be bound is a pheromone, it is called *pheromone binding protein* (PBP) (Vogt, et al., 1985; Raming, et al., 1990), and if it is a more general odor, like host plant volatiles, the corresponding protein is a *general odorant binding protein* (GOBP) (Breer, et al., 1990b). The binding proteins are thought to transport odor molecules from the inner openings of the sensillum wall, through the lymph, to the dendritic membrane where receptor proteins receive the odor stimuli (and determine the physiological specificity of the ORN) (Vogt and Riddiford, 1981; Vosshall, et al., 2000). The final role of these proteins is not clear yet. They may a) act as carriers of odorants, b) work as selective filters, c) present the molecule to the receptor in the dendritic membrane, d) clean the hemolymph or e) could deactivate the odorants after the transduction (Kaissling, 1996; Steinbrecht, 1998). When the odor molecule is docked to the ORNs, the chemical stimulus is transformed to an electric current which after a cascade of transformations finally provokes the specific behavior (Fig. 3). This so-called receptor potential reflects a change in the membrane potential and may be recorded extra- or intracellularly. The electroantennogram (EAG) is very useful for measuring the receptor potentials induced by an odor and to determine the type of receptor cells that are tuned to a specific key compound, respectively (Kaissling, 2004).

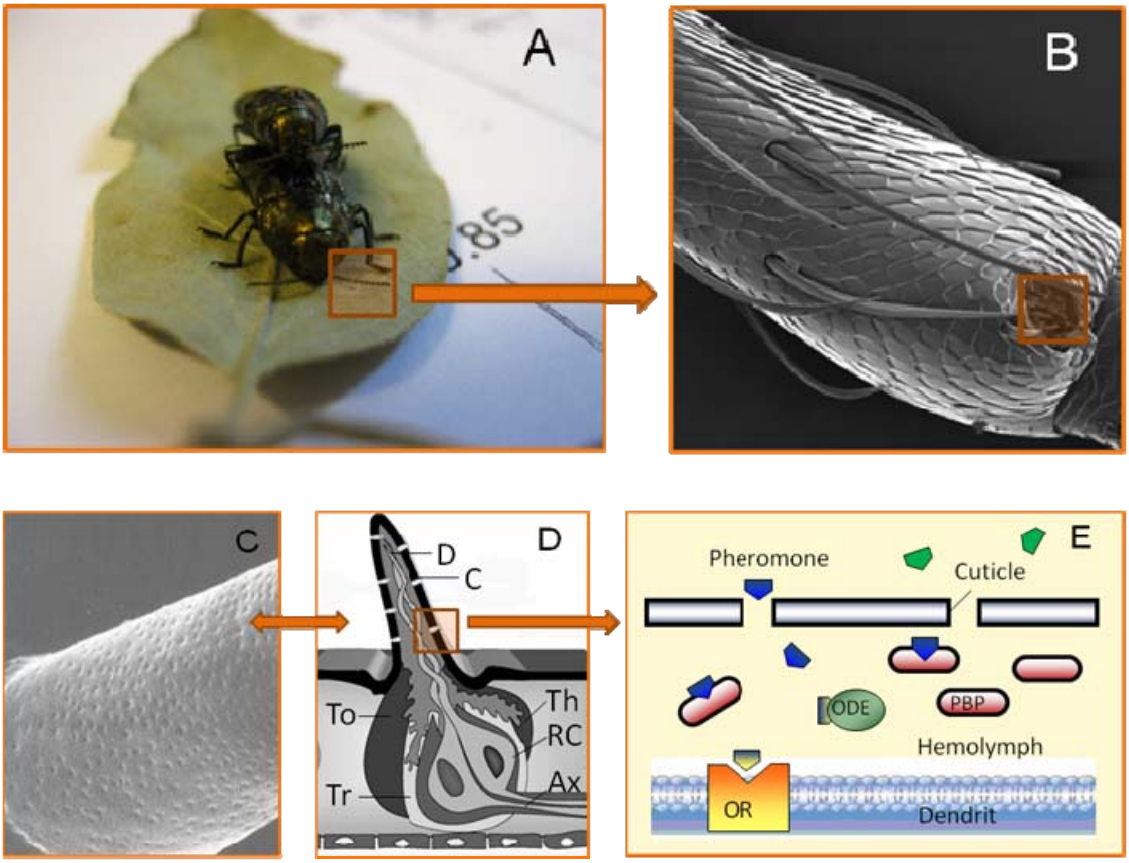


Fig. 2. Pheromone perception in insects exemplary displayed by adults of (A) *Coroebus undatus*. The SEM micrograph of a male antenna (B) shows the presence of different types of sensilla; one type is a trichoid sensillum (C). (D) Scheme of an olfactory *sensillum trichodeum* (general morphology) C: cuticle, D: dendrite of a receptor neuron, RC: receptor cell, Ax: Axon, To: tormogen cell, Tr: trichogen cell, Th: thecogen cell. (E) Model of the pheromone transport, OR: olfactory receptor, ODE: odorant degrading enzyme, PBP: pheromone binding protein.

Interaction of the olfactory receptor with odor molecules leads to the opening of ion channels followed by a voltage change across the cell membrane (Stengl, et al., 1999). Simultaneous occurring receptor potentials (electric flow) can be combined and transferred as action potentials down the neuron axons to the insects' brain (antennal lobe, AL) (Anderbrant, et al., 1995) (Fig. 3). The AL represents the principal centre of the olfactory processing (Hildebrand, 1996) and consists of several glomeruli (Koontz and Schneider, 1987). One or more olfactory receptors (OR) are stimulated by each odor molecule (Hallem, et al., 2004) and ORNs express the same type of OR converge to one glomerulus (Vosshall, et al., 2000). Through projection neurons, the olfactory signals are passed to the protocerebrum, where the stimulus is received (Fig. 3). Thus, the motor system is induced and as final result a nerve impulse provokes the specific behavior, action, or movement (Hildebrand, 1997). Finally, the signal is inactivated by *odorant degrading enzymes* (ODEs), present in the sensillar lymph that relieve the

receptor of the molecule by binding the active product and/or its metabolic material (Fig. 2 E) (Prestwich, et al., 1989; Ishida and Leal, 2005). In this way saturation of the antenna is avoided and new volatile molecules or rather environmental information can be processed (Leal, 2005).

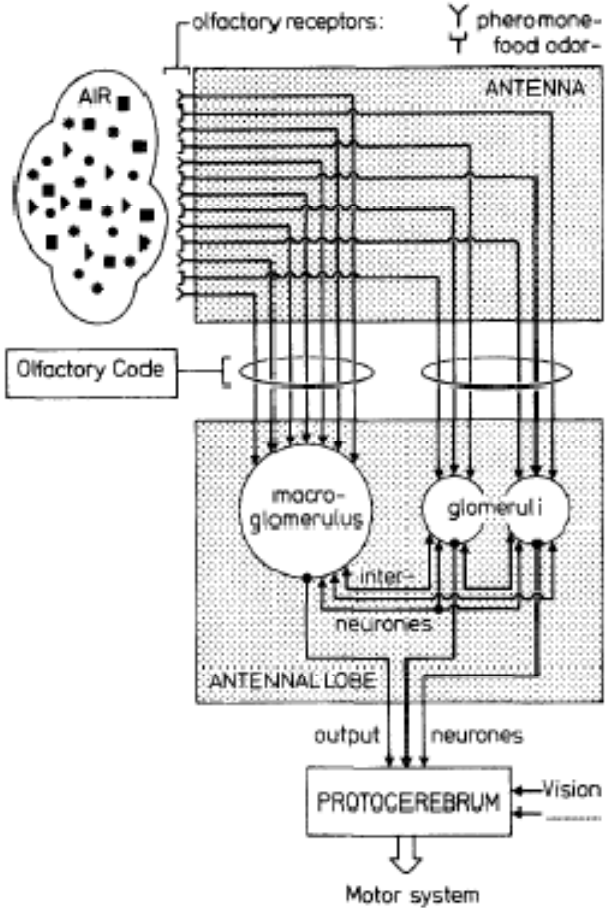


Fig. 3. The olfactory pathway in adult insects is displayed according to Visser and de Jong (1988). Semiochemicals in the air are detected by antennal sensilla and the olfactory message is transformed by receptor neurons to bioelectrical responses. Through axons the signal is passed to different glomeruli of the antennal lobe where a synapses between the inter neurons is produced. After processing, the information is relayed to the protocerebrum and the original message will be translated to a specific behavior evoked by the motor system.

The insect sensory sensilla are of different morphological types and display different functions ranging from chemoreceptivity (gustatory and olfactory), mechano- and thermoreceptivity to hygro- and CO_2 receptivity (Keil, 1997; 1999).

Olfactory sensilla are mainly distributed on the antennae (Fig. 2 B) or the maxillary palps. They are responsible for the above described odor detection in insects and are

characterized by the following types: *trichodea, basiconica, coeloconica* and *auricilica* (Grant, et al., 1989; Steinbrecht, 1996; Pophof, 1997; Anderson, et al., 2000). Generally, perception of pheromones and plant odors is done in *s. trichodea* (Fig. 2 C), which can differ in form and number of pores between different species (Steinbrecht, 1987). Within the same species sexual dimorphism can exist, reflected in the number and distribution of sensilla (Flower and Helson, 1974). Gustatory/taste sensilla can be found all over the body (antennae, legs, wings, mouthparts) (Dahanukar, et al., 2005). In some species, this sensilla type can intervene in the chemical communication by detecting for example, contact pheromone cues (Crook, et al., 2008a, b). A typical mechanoreceptor in nearly all insects is the *sensilla chaetica* (van der Pers and den Otter, 1978). Analysis of the antennal morphology and identification and arrangement of the sensilla types present in a specific insect are usually carried out by scanning electron microscopy (SEM).

OBJECTIVES

In the present work different aspects of the chemical communication of four insect pest species, which belong to the order Coleoptera (Family: Buprestidae) and Orthoptera (Family: Acrididae), are investigated. The final aim of the project is to establish a biorational control of these insects using the identified compounds with pheromonal and/or kairomonal activity as ecological tools. To do this, in this PhD thesis the following objectives will be pursued:

Chapter 1 Analytical and behavioral studies on the oak branch borer (OBB) *Coroebus florentinus* (Coleoptera: Buprestidae)

- Morphological analysis of insect antennae and identification of the antennal sensilla by SEM.
- Identification of volatile organic compounds (VOCs) produced and emitted by adult individuals of both sexes and the host plant *Quercus suber*.
- Determination of the activity of these compounds in electrophysiological and behavioral bioassays.

Chapter 2 Analytical studies and field trapping experiments on the oak flathead borer (OFB) *Coroebus undatus* (Coleoptera: Buprestidae)

- Morphological analysis of insect antennae and identification of the antennal sensilla by SEM.
- Analysis and identification of VOCs produced and emitted by adult individuals of both sexes.
- Confirmation of the biological activity of the different compounds and influence of trap design in field trapping experiments.

Chapter 3 Analytical and electrophysiological studies on the Moroccan locust *Dociostaurus maroccanus* (Orthoptera: Acrididae), pest of the Iberian Peninsula

- Morphological analysis of insect antennae and identification of the antennal sensilla by SEM.

- Identification of VOCs produced and emitted by nymphs and adult individuals of both sexes and egg pods/froth laid by mature females.
- Determination of activity of these compounds in electrophysiological and oviposition bioassays.

Chapter 4 Maturation studies in gregarious nymphs of the desert locust *Schistocerca gregaria* (Orthoptera: Acrididae), a destructive crop pest in Africa

- Determination of the effect of grouping nymphs with conspecific adults on the maturation of nymphs.
- Influence of the male produced pheromone PAN on the maturation process of nymphs.

HYPOTHESES

From several studies carried out on the biology and chemical communication of insect pests and subsequent application of the results in IPM programs, quite a few reasons exist for the research of the unexplored pests species outlined in this thesis. Different studies on jewel beetles (Buprestidae) and on locusts (Acrididae) cited in the following chapters provide the basis for our objectives and assumptions.

Therefore, in the present work different aspects of the chemoecology of these insect pest species will be investigated, based in the following hypothesis.

Chapter 1 Analytical and behavioral studies on the oak branch borer (OBB) *Coroebus florentinus* (Coleoptera: Buprestidae)

- The volatile composition of male and female *C. florentinus* adults differs in quantity and quality (indicating presence of a sex-specific pheromone).
- Female produced volatiles elicit activity in males, whereas male produced volatiles don't exhibit activity in conspecific individuals (indicating presence of a female-specific sex-pheromone).
- Host plant volatiles of *Q. suber* elicit activity in live adults in laboratory bioassays (indicating presence of a foraging, host finding or oviposition kairomone).

Chapter 2 Analytical studies and field trapping experiments on the oak flathead borer (OFB) *Coroebus undatus* (Coleoptera: Buprestidae)

- The volatile composition of both sexes of *C. undatus* adults differs in quantity and quality (indicating presence of a sex-specific pheromone).
- The volatile composition of *C. undatus* adults is different to that of *C. florentinus* (indicating presence of species-specific compounds).
- Trap design and type of semiochemical lures exercise strong influence on the capture of *C. undatus* adults in field trapping experiments.

Chapter 3 Analytical and electrophysiological studies on the Moroccan locust *Dociostaurus maroccanus* (Orthoptera: Acrididae), pest of the Iberian Peninsula

- The volatile composition of male and female *D. maroccanus* nymphs and adults differs in quantity and quality (indicating presence of an age-dependent and sex-specific pheromone).
- Male/female produced volatiles elicit activity in conspecific adults in the laboratory (indicating presence of a sex-specific pheromone).
- Sand treated with extracts of gravid females attracts conspecific females to lay egg pods (indicating presence of a female-specific oviposition/aggregation pheromone).

Chapter 4 Maturation studies in gregarious nymphs of the desert locust *Schistocerca gregaria* (Orthoptera: Acrididae), important crop pest in Africa

- The presence of *S. gregaria* adults and/or their volatiles lead to accelerated development and maturation in conspecific gregarious nymphs (indicating presence of pheromones that could procure synchronous maturation in gregarious populations).

GENERAL METHODS & MATERIALS

C) SPECIMENS OF STUDY

In this PhD thesis different aspects of the chemical communication and behavior of four insect pest species have been studied. The work was implemented on two jewel beetles, *Coroebus florentinus* (Fig. 4 A) and *Coroebus undatus* (Fig. 4 B) (Coleoptera: Buprestidae), pests of the cork oak, *Quercus suber* in the Mediterranean region (Bachiller et al., 1981; Cobos, 1986; Soria, et al., 1992; Suñer and Abós, 1994; Evans, et al., 2007). Specimens of *C. florentinus* were obtained by field collections of infested branches of *Q. suber*, and live adults of *C. undatus* were captured with transparent mosquito nets placed around the trunk of infested trees, hence for both species no rearing system have been established up to now. Emergency of adult *C. florentinus* individuals was carried out with specially designed cardboard-boxes in the laboratory (Fig. 5).

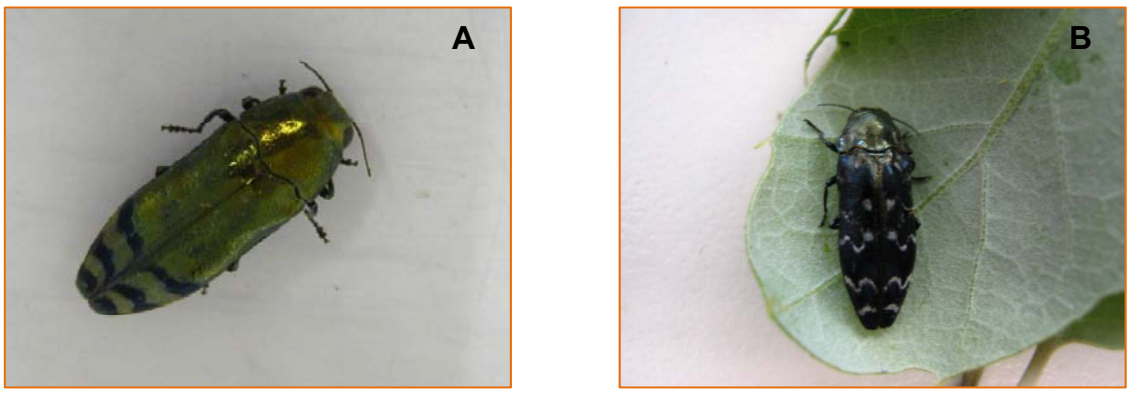


Fig. 4. Adult females of the oak flathead borer (A) *C. florentinus* and (B) *C. undatus*, pest of the cork oak *Q. suber*.

On the other hand, the bioecology of two locust species, *Dociostaurus maroccanus* (Fig. 7 A) and *Schistocerca gregaria* (Fig. 7 B) (Orthoptera: Acrididae) was investigated. The Moroccan locust, *D. maroccanus*, a crop pest in the Iberian Peninsula (Uvarov, 1966; Latchinisky, 1998) and the desert locust, *S. gregaria*, present in Africa, the Middle East and Asia, represent two important insect pest species of agricultural crops (Roffey and Popov, 1968; Enserink, 2004). The latter species was reared at the Insect and Animal Breeding Unit (IABU) of the International Centre of Insect Physiology and Ecology (ICIPE; Nairobi, Kenya) (Fig. 6). The following techniques were used in the study of the Chemical Ecology of these four insect pest species.

Fig. 5. Emergence boxes for *C. florentinus.*

Fig. 6. Rearing cages for *S. gregaria.*

Fig. 7. Adult males and females of: (A) the Moroccan locust *D. maroccanus* and (B) the desert locust *S.gregaria*.

D) SCANNING ELECTRON MICROSCOPE (SEM)

For a better knowledge of the morphology and surface of insect antennae and to detect possible differences between species and/or sexes, a scanning electron microscope (SEM) was applied. This technique, developed in the early 1950s, allows scanning the sample with a high-energy beam of electrons in a raster scan pattern instead of light, used in an ordinary microscope. These electrons interact with the sample atoms producing signals which contain information about the topography, composition and other properties of the sample surface. Antennae of the studied species were cut beneath the first segment near the head and cleaned with gradient concentrations of ethanol in Eppendorf® vials. To obtain SEM micrographs, antennae were dehydrated and placed on a metallic holder, fixed with an adhesive double-sided tape before they were covered with a thin layer of a gold solution under vacuum

conditions. The last step (covering the antennae) is important to achieve a good conductivity for electron transmission, a high resolution and a better contrast of the micrographs. Sample preparation was realized at the Electron Microscope Service of the University of Barcelona (Servicios Científicos Técnicos, Barcelona, Spain). The microscope employed for the images capture was a Stereoscan S-360 (Leica) at 15 kV.

E) VOLATILE COLLECTION AND STRUCTURAL CHARACTERIZATION

Semiochemicals emitted by plants and animals convey the necessary information for the chemical communication between organisms and guarantee consistency of their biocenosis (Harborne, 1995). Investigation of the chemical ecology of insect pests requires foremost identification of the released semiochemicals and therefore, it is essential to collect and analyze their volatiles. For collection of volatiles produced by the studied insects and in the case of *Coroebus spp.* by the host plant *Q. suber*, two different methods were applied: the dynamic headspace and the solid phase microextraction (SPME).

Dynamic headspace extraction

The dynamic headspace extraction permits to collect the volatiles emitted by an organism or a synthetic sample enclosed in an air-tight container, generally made out of glass or plastic. The headspace samples reflect most realistically the odors (quantity and quality) that an animal or plant produces in a certain time (Nuñez, et al., 1984; Jakobsen, 1997). The dynamic headspace method used in this work was based on the closed-loop-stripping analysis described by Grob and coworkers (1975) and Boland and coworkers (1984). One pump pushes charcoal-filtered air through a Teflon tube in a closed chamber, where the test organisms are placed. A second pump purges the air, accumulated with insect volatiles, and passes it through an adsorbent filter placed at the outcome of the trapping chamber, where the produced odors are trapped (Fig. 8).

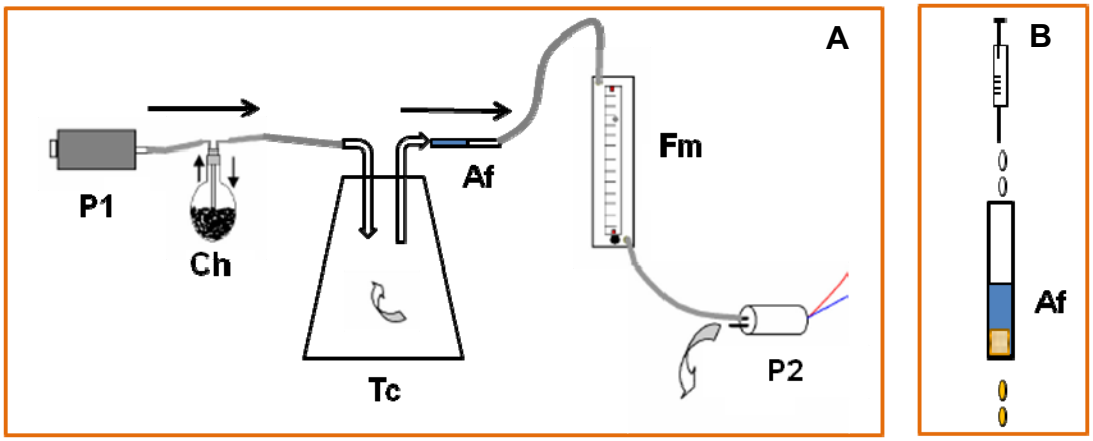


Fig. 8. A) Scheme of the dynamic headspace extraction to collect volatiles produced by different test organisms, placed in an air-tight trapping chamber (Tc). One pump (P1) pushes charcoal-filtered (Ch) air through the trapping chamber over the test samples; meanwhile a second pump (P2) purges the air accumulated with volatiles through an adsorbent filter (Af) at the outcome. The continuous air flow was checked with a flowmeter (FM). B) The collected volatiles were eluted from the adsorbent filter with an adequate solvent and stored in a freezer for subsequent GC-MS analysis.

The flow rate of the entering air was a little bit higher than the outgoing to generate a slight overpressure, thus avoiding that contaminated laboratory air enters into the system. An adequate flow rate of purged air is important to retain certain compounds on the adsorbent (Agelopoulos and Pickett, 1998). In the course of this project, different types of adsorbent filters, Porapak Q (Supelco, Bellefonte, PA, USA) and Super Q (Ars Inc., Gainesville, FL, USA) and variable sizes of headspace chambers were utilized depending on the studied insect or plant and the compounds looked for (Fig. 9). The volatiles were eluted from the adsorbent traps with HPLC-grade solvents, such as hexane and dichloromethane (DCM), and stored at -20ºC for subsequent analysis and bioassays.

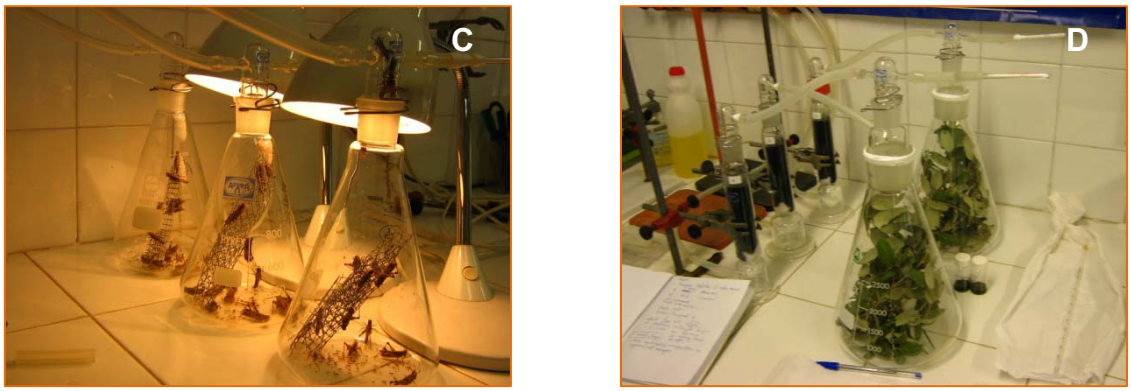


Fig. 9 A-D. Volatile collections by dynamic headspace extraction in different chambers of: A) adults of *C. florentinus*, B-C) adults and nymphs of *D. maroccanus* and D) of *Q. suber* branches.

Solid phase microextraction (SPME)

SPME is a solvent-free technique for the extraction of volatile organic compounds developed in the 1990s (Pawliszyn, 1997). The technique consists of a syringe containing a fiber coated with an extracting phase (a liquid polymer or a solid adsorbent). After extraction of the volatiles, the fiber is directly injected into a gas chromatograph (GC) or a gas chromatograph coupled to a mass spectrometer (GC-MS), where desorption of the odors takes place and analysis are carried out (Pawliszyn, 1999). For volatile collection insects were placed in a glass vial or small flask and the SPME fiber was introduced through a septum on the top of the flask (Fig. 10.). During a defined time (4-12 h), the produced odors were adsorbed on the fiber. To analyze cuticle hydrocarbons, the abdomen, thorax or the elytra were rubbed with the tip of the fiber before injecting into the GC-MS.

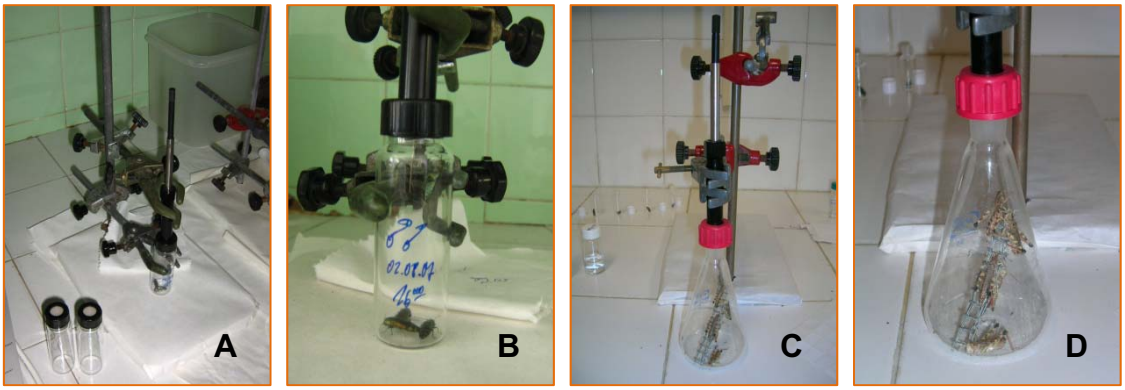


Fig. 10. SPME extraction of volatiles produced by A-B) adults of *C. florentinus* and C-D) Adults of *D. maroccanus*.

Structural characterization of chemicals

Structural characterization of the compounds in insect or plant headspace volatiles, body extracts or SPME extractions was carried out by GC and GC-MS. The chemicals were identified by comparison of their mass spectra, retention times and retention indexes with those of reference standards, and/or by comparison with those from commercial libraries or from a database (Adams, 2007). Retention indexes of each compound were calculated according to the following formula developed by van den Dool and Kratz (1963) (Fig. 11).

$$RI = 100\ i\ (X - S_n/S_{n+1} - S_n) + 100\ n$$

Fig. 11. Formula to calculate the retention index (RI) of unknown compounds with respect to the retention times of straight chain hydrocarbons on a HP-5MS column, as described by van den Dool and Kratz (1963), where: i = difference of carbons of reference alkanes; X = retention time in minutes of unknown compound; S_n = retention time in minutes of minor alkane (before unknown compound); S_{n+1} = retention time in minutes of major alkane (after unknown compound); n = numbers of carbons of minor alkane.

For determination of the RI, 1 µl containing 100 ng of a C_8-C_{25} hydrocarbon mixture in hexane is co-injected with the sample into the GC-MS system. The differences of retention time between the unknown compound and the minor and major reference alkanes permit to calculate the relative retention time (RI), which is characteristic of each compound for the specific GC column used.

F) ELECTROPHYSIOLOGY

In general, electrophysiology is the study of electrical flows and their transduction in biological systems, like nervous cells, tissues and neurons (Gullan and Cranston, 2004). This includes the measurement of voltage changes and action potentials inside (intercellular) and outside the cell (extracellular) (Kaissling and Thorson, 1980). For the evaluation of semiochemical activity in insects, in most cases extracellular recording techniques are applied directly to the antennal receptors, like in the present work.

Electroantennogram (EAG)

The origin of electrophysiological studies in insects was initiated in the 1950s when the German biologist Dietrich Schneider developed a technique to analyze the electrophysiological activity of some analytical fractions from glandular extracts of the silkmoth, *B. mori* in male antennae. By means of this first electroantennogram, he could activate synchronously a sufficient number of receptor cells to produce an electrical response to the tested odor source (Schneider, 1957). Since then, this technique is a basic part of the Chemical Ecology studies, and, in particular, in the investigation of the olfactory perception in insects and the structural characterization of pheromone components (Roelofs, 1984) and kairomones (Wadhams, 1992). Hence, the EAG allows the study of different kind of odor molecules that provoke bioelectrical responses in stimulated insect antennae. This response is the summation of negative receptor potentials activated by depolarizations of olfactory cells or the receptor membrane (Kaissling and Thorson, 1980; Lánsky, 2001). EAG amplitude is a measure of the intensity of the stimulus until its saturation and recovery (Schneider, 1969). The amplitude depends on several factors, such as nature of the stimulus, species, sex and age. In the present work electrophysiological activity of naturally derived extracts and synthetic compounds were tested in *Coroebus spp*. and in *D. maroccanus*. Thus, freshly excised antennae were cut on both ends and transferred to a microelectrode, connected via an IDAC-2 amplifier (signal acquisition) (x 100) and a stimulus controller (Syntech, Hilversum, The Netherlands) to a PC for visualization. A pedal activated the stimulus controller to generate the stimuli (puff) from an odor source deployed on a filter paper (Whatman) placed into a Pasteur pipette (Fig. 12). Acquisition and data analyses were performed by EAG-Pro software (Syntech). Depolarization responses can be elicited by either an attractant (e.g. a pheromone) or a repellent (e.g. inhibitor).

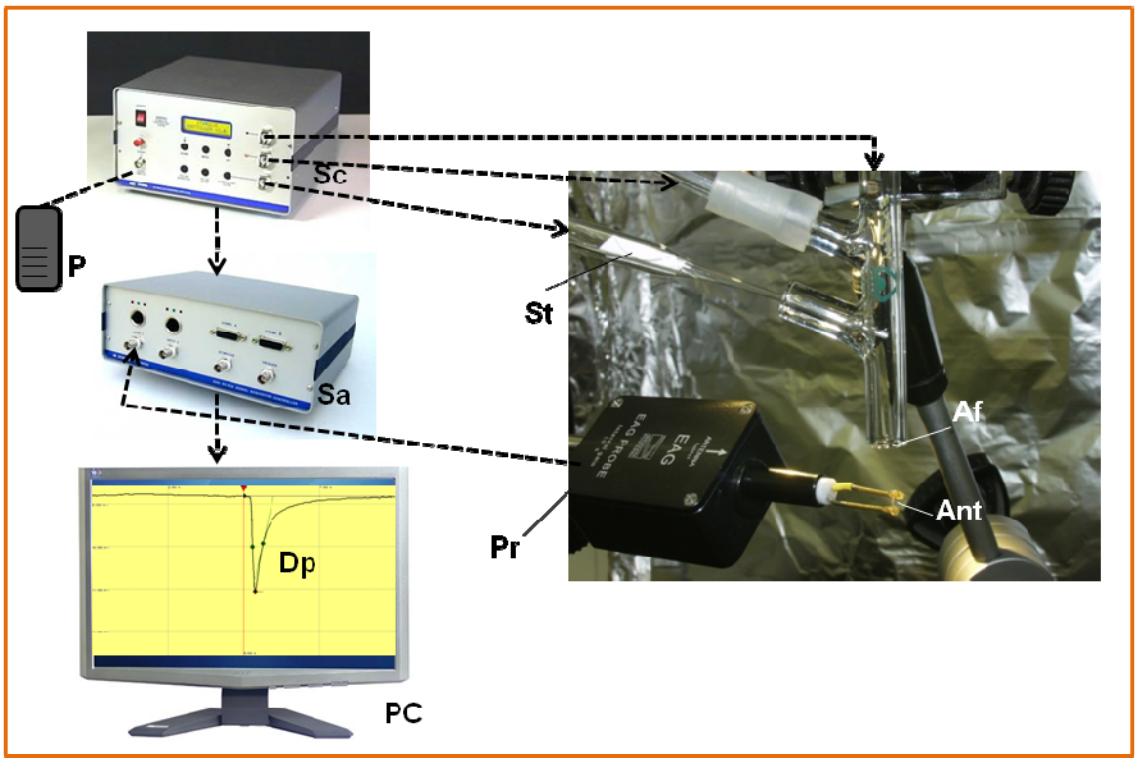


Fig. 12. Scheme of the EAG recording system used. Sc: Stimulus controller; Sa: Signal acquisition module (IDAC 2 amplifier); P: Pedal; St: Stimulus; Pr: Probe built-in with antennal microelectrode; Af: Air flow; Ant: Antenna; Dp: Depolarization; PC: Personal computer.

Gas chromatography coupled to electroantennographic detection (GC-EAD)

Twenty years after the first description of the EAG method a modified technique to evaluate the activity and identify single components of an odor mixture was established: an electroantennographic detector (EAD) coupled to a gas chromatograph (GC) (Moorhouse, et al., 1969; Arn, et al., 1975). Gas chromatography is a powerful technique to separate complex mixtures into individual components. In combination with an EAD it permits to know which single compounds as part of a natural extract or a synthetic blend elicit an electrophysiological response on the insect antenna. At the same time, it allows to exclude all compounds present in the odor mixture not active on the antennae. The retention time and mass spectra of the active GC peaks can lead to the final identification of the compound. In many cases, this elegant combination can help to identify a pheromone or kairomone, but there is no guarantee that a compound provoking antennal activity has also an impact on the insects' behavior.

In this work the insect antennae were prepared as described above and the same EAG equipment was used. GC analyses were performed on a Focus GC (Thermo Instruments, Barcelona, Spain) equipped with a flame ionization detector (FID) and a

HP-5MS fused silica capillary column (Agilent Technologies, Madrid, Spain) with helium as carrier gas (1-2 ml min^{-1}) and nitrogen as second make up gas. The column effluent was split 1:1 for simultaneous detection by the FID and EAD. For simultaneously recording and analysis of the amplified EAD and FID signals on a PC, a GC-EAD program (Syntech GC-EAD v4.4, Kirchzarten, Germany) was used. A scheme of a GC-EAD system is displayed in Figure 13.

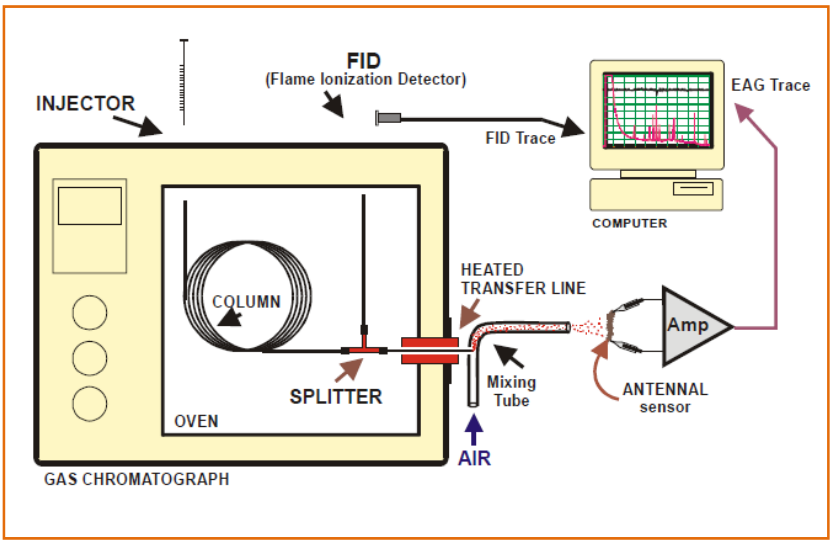


Fig. 13. Scheme of a GC-EAD system, according to the Syntech manual (Kirchzarten, Germany).

G) BEHAVIORAL ASSAYS

The behavioral assays are typically used in the laboratory to investigate the behavior (e.g. attraction or repulsion) of living organisms to different odor sources. They allow determination of the biological activity of certain extracts/compounds by measuring the behavior induced in test individuals in comparison to standard/control samples. In insect studies they are essential to confirm the effect of the identified compounds as a pheromone or kairomone.

In the present work a vertical posted Y-tube olfactometer was used (Fig. 14) to quantify the number of adult *C. florentinus* individuals that are attracted to natural extracts (conspecific insect and host plant volatiles) and synthetic compounds compared to a control. Numbers of responding insects were analyzed by the χ^2 *goodness-of-fit test*, using Yates correction for continuity (α = 0.05) (Zar, 1999).

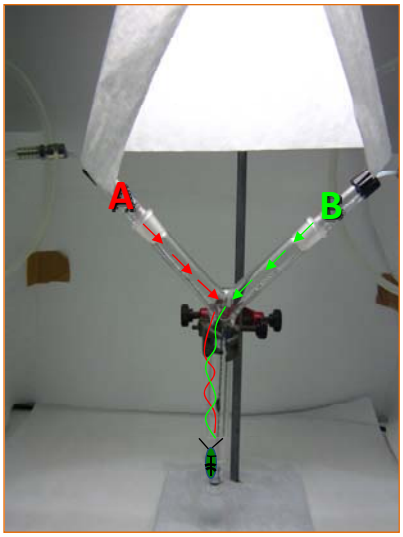
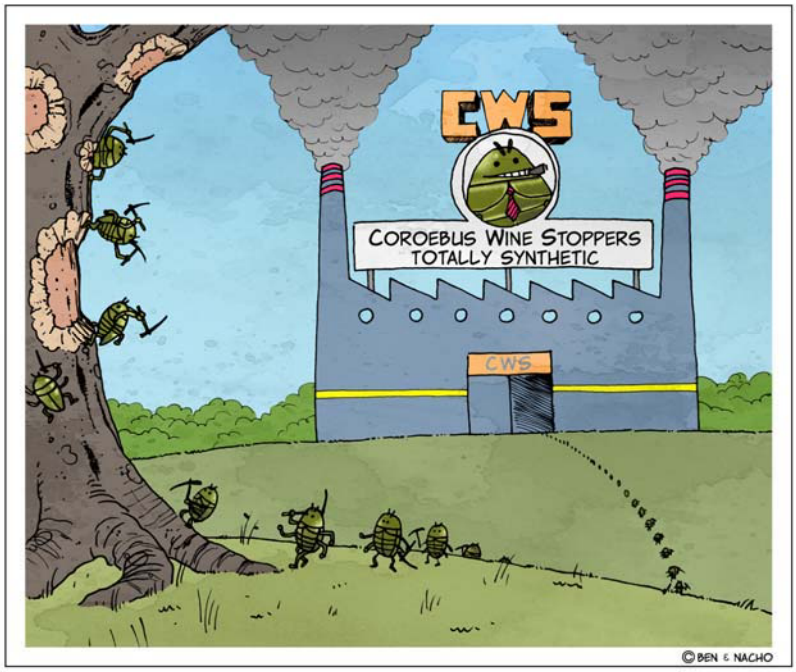


Fig. 14. Vertical posted Y-tube olfactometer used in laboratory bioassays to determine the attraction of *C. florentinus* adults to different odor sources (A) vs control (B).

BUPRESTIDAE (COLEOPTERA) – *COROEBUS SPP.*



'And wine [that] maketh glad the heart of man, [and] oil to make [his] face to shine, and bread [which] strengtheneth man's heart.'
Psalms 104: 15

CHAPTER 1

Electrophysiological and behavioral responses of the cork oak pest, *Coroebus florentinus* (Herbst, 1801) (Coleoptera: Buprestidae) to conspecific and host plant semiochemicals

1.1 PREFACE

In the past few decades, chemical signals and defenses produced by Coleoptera have been the subject of numerous studies that focus on occurrence, biosynthesis, and biological significance (Francke and Dettner, 2005). However, some coleopteran families have received relatively little attention. In particular, there have been only a few studies on the chemical ecology of Buprestidae, the jewel beetles.

In this project, the oak branch borer[1] (OBB) *Coroebus florentinus* (Herbst) (Coleoptera: Buprestidae), pest of the cork oak *Quercus suber* L., has been investigated for the first time. *Q. suber* is a valuable endemic plant species of the Mediterranean Region (Vogiatzakis, et al., 2005), with a worldwide surface of more than 2.5 million ha (Institute C.M.C. 1999, http://www.iprocor.org; CEFE 2005, http://earthtrends.wri.org), mainly distributed over Portugal and Spain (Fig 1.1) (Soria, et al., 1992; Borges, et al., 1997). The cork oak is a sclerophyllous evergreen tree species that is adapted to summer conditions of a 4-month dry period with little or no precipitation, high temperatures with maxima that can reach 35-40ºC, and high irradiance exceeding 2000 µmol m^{-2} s^{-1} (PAR) at midday (Faria, et al., 1996). Its great economic interest derives from an annual production of cork over 340.000 tons with a worth value of 1.5 billion $ (Table 1.1) (http://www.realcork.org). Cork is used in a variety of manufactured products, from construction materials to gaskets but most importantly as a stopper for premium wines. Wine stoppers are the most visible and most profitable of the products derived from cork with an estimated production of about 13 billion wine stoppers per year (http://www.corkqc.com). In spite of the social and economic impact of cork, nothing is known about the chemical ecology of one major cork pest of the Iberian Peninsula, *C. florentinus* (Soria and Ocete, 1993; Soria, et al., 1994; Evans, et al., 2007).

OBB adults are olive-green metallic colored with several black and bluish bands in the posterior part of a 12-16 mm long elliptic formed body (Bonnemaison, 1976; Montoya, 1989). Larvae, which are greater than adults, have the head inserted into the thorax and the prior part of the body expanded. The tail end, constrains pincers and pupae are white with dark eyes.

[1] The name oak branch borer (OBB) was introduced during this work to distinguish *C. florentinus* from its sister species, the oak flathead borer (OFB) *C. undatus*.

Table 1.1 Worldwide surface of the cork oak *Q. suber* L. and total amount of cork production in 2004[a]

Country	Forest Area Hectares	Worlds Forest Area [%]	Production Tons (000)	Total Production [%]
Portugal	736,000	33%	185	54%
Spain	500,000	22%	88	26%
Algeria	410,000	18%	20	6%
Morocco	340,000	15%	15	4%
France	100,000	4%	5	1%
Tunisia	99,000	4%	9	3%
Italy	90,000	4%	18	5%
TOTAL	**2,275,000**	**100%**	**340**	**100%**

[a] Data courtesy of APCOR (http://www.corkqc.com)

Fig .1.1 Map of distribution of the cork oak (*Q. suber* L.) forests (in black) in the Mediterranean region.

In June-July OBB females invade the tree by laying eggs in groups or separately inside the bark of young and healthy branches, preferably in those exposed to the sun. After eclosion, the endophyte larvae start feeding, constructing large galleries (up to 1 m). The larval feeding stops 1-3 years later after producing an inner ring that prevents circulation of the sap. Thus, the larva is protected of possible drowning provoked by the intense sap flow. After the pupal phase inside the branch, the phytophagous adults emerge around summer solstice feeding in groups on *Quercus spp.* foliage and living only 2-3 weeks for mating and egg-deposition.

The presence of the insect is not lethal to standing trees but its damage provokes dryness and yellow leaves, and finally wounds and death of branches and shoots

(Soria and Ocete, 1993; Soria, et al., 1994; Lombardero and Fernández de Ana Magán, 1996). Mature healthy trees are able to recover quite easily after the drop off of infested branches, but if repeated attacks occur it may result in the weakening of the tree paving the way for the attack of other pests (Dajoz, 2000; Recalde and San Martín, 2003). For example, the cork can be attacked by fungi, such as the saprophytic basidiomycete *Armillaria mellea*, which lives on the ground, decaying leaves, bark, wood, manure, etc. This fungus attacks the roots of the trees and kills their cortical tissue, growing up inside the trunks bark and causing its death. The chemical and physical changes in cork are shown by modifications in its mechanical, structural, and optical properties and, more importantly, the fungus in contact with the cork can provoke off-flavors in wine (Rocha, et al., 1996).

Increasing attacks by *Coroebus spp.* could be associated with cork oak decline in the Iberian Peninsula (Evans, et al., 2007)**.** Although in many areas of Spain the oak decline has been primarily linked to infection by the soil pathogen *Phytophthora cinnamoni* (Brasier, et al., 1993), later it has been assumed as the result of combined actions of several biotic and abiotic stress factors (Moraal and Hilszezanski, 2000). This includes unusually prolonged dry periods, such as *Coroebus spp.* attacks. Nevertheless, so far no effective control treatments have been established because larvae feed underneath the bark of the branches and, thus, it is quite hard to reach them. Therefore, direct control with insecticides is impractical because spraying of the chemicals would involve the whole tree. Moreover, it should be noted the serious risk for humans implied when any type of chemical insecticide is used, which could contaminate the cork and ultimately the food chain. In addition, knowledge of natural enemies of the insect is practically null (Solinas, 1974; Kenis and Hilszczanski, 2007).

We present herein for the first time initial studies directed to investigate the presence of a sex or aggregation pheromone and/or a foraging or oviposition kairomone of *C. florentinus* to eventually develop an environmentally-friendly approach to control the pest. To this aim we have examined the antennal and behavioral responses of males and females to volatiles emitted by adults of both sexes as well as body extracts. We have also collected and analyzed volatiles from the host plant and tested the activity in a binary choice Y-tube olfactometer and in electrophysiological tests.

1.2 METHODOLOGY

1.2.1 Chemicals

The reference compounds used in electrophysiological and olfactometer assays, nonanal (95%), decanal (99%), (*E*)-2-hexenal (98%), (*E*)-2-hexenol (96%), 1-hexanol (98%), (Z)-3-hexenyl acetate (98%), *n*-hexyl acetate (99%) and dodecyl acetate (97%) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Geranylacetone (96%, containing 40% of nerylacetone) was obtained from TCI Europe (Zwijndrecht, Belgium). *n*-Hexane (analytical purity >95% by GC) (J.T. Baker, Deventer, Holland) was used as solvent. Isopropyl dodecanoate was obtained by esterification of dodecanoic acid with isopropyl alcohol in refluxing sulfuric acid (Narasimhan, et al., 2006; Sanna, et al., 2009). The product was purified by column chromatography on SiO_2 eluting with hexane: Et_2O (95:5) as colorless oil (99% yield; 97% purity by GC). The product was characterized by ^{1}H / ^{13}C-NMR, IR and MS.

1.2.2 Insects

Specimens of living adults of *C. florentinus* (Fig. 1.2) were obtained directly from woods in Llagostera (41°49′45″N 2°53′36″E) and Romanyà de la Selva (41°51′0″N 2°59′6″E) (Girona province, Spain). At the end of May during the period 2005-2010 affected cork oak trees with OBB attacks (visible branches at the tree top with yellow and dry leaves) were identified. The branches (ca. 700 per year, approx. 30 cm long × 4 cm diameter) supposedly containing at least one pupae inside, were cut and set in cardboard boxes (80×40×40 cm^3). Every box was filled with 30-40 branches. Half of them were maintained at 5-9ºC and 50-70% RH for four weeks to retard emergency of the adults. In this way, it was possible to work with living insects for a longer period since the life expectancy of *C. florentinus* is only around 3 weeks. The other half of the branches was brought to the lab and placed in several sealed cardboard light-protected boxes (50×40×40 cm^3) provided with a removable glass container (11 cm long × 7 cm diameter) fixed in the middle of the front wall. The entering light through the glass attracted the emerging adults from inside the box. The containers were checked daily and the insects collected and sexed.

The distinct features to distinguish both sexes were a) the body size, females (1.5 cm long) are up to 20% (1.2-fold) larger than males, and b) the length of the antennae, male antennae (0.4 cm) are 2x longer than those of females (Fig. 1.2). Adults were kept separately by sex in square glass containers (15×15 cm^2) with a removable plastic lid with wire gauze on the top at 26±2ºC, 50±10% RH and 14:10 L:D photoperiod. Fresh *Q. suber* leaves (cork oak foliage) were provided every two days to feed the insects.

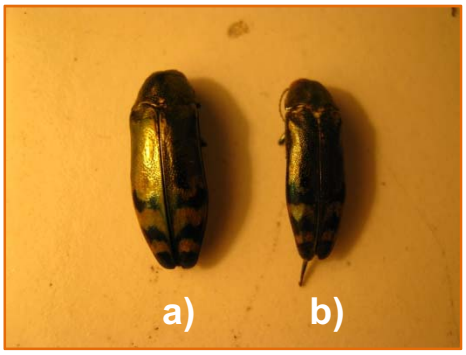


Fig .1.2. Adult a) female and b) male *of C. florentinus.*

One to two-week-old beetles were used in the bioassays when they were supposed "mature", as described for the emerald ash borer (EAB) *Agrilus planipennis* (Lelito, et al., 2009).

1.2.3 Scanning electron microscope (SEM)

Antennae of male and female *C. florentinus* adults (N=3) were clipped beneath the distal segment near the head, washed with gradient concentrations of ethanol and mounted on specimen stubs with adhesive tape. Under vacuum, antennae were coated with a gold solution three times for 1 min. Then, micrographs were taken with a Stereoscan S-360 (Leica) electron microscope at 15 kV (Servicios Científicos Técnicos, University of Barcelona, Spain).

1.2.4 Collection and analysis of volatiles – Insects

A charcoal-filtered air flow (350 ml min^{-1}) was passed through male (N=20) and female (N=20) adults, placed separately in a glass trapping chamber (15 cm long × 3 cm OD), for 24 h at room temperature. The volatiles were trapped with Super Q glass tubes (6 cm long × 6.4 mm OD) (Ars Inc., Gainesville, FL, USA) containing 30 mg of the adsorbent. Volatile extracts were obtained by washing the Super Q traps with 2 ml of analytical grade hexane. Each volatile extract contained 480 beetle/h equivalents (1 BHE = volatiles released by one adult for one hour) and was stored at -20ºC for subsequent chemical analysis and electrophysiological assays. For GC-MS analysis, 100 µl of the headspace volatile extracts were concentrated under a nitrogen stream to a volume of ca. 1 µl to which 1 µl of a 100 ng $µl^{-1}$ solution of dodecyl acetate were added as internal standard (IS). The complete volume of this mixture was injected in

splitless mode on a Thermo Finnigan Trace 2000 GC system coupled to a Trace MS quadrupole mass spectrometer (ThermoFisher Scientific, Madrid, Spain). Helium (1 ml min^{-1}) was the carrier gas and the column used was a HP-5MS (5% phenylmethylsiloxane; 30 m×0.25 mm ID×0.25 µm) (Agilent Technologies, Madrid, Spain) under the following chromatographic conditions: injection at 60ºC, held for 5 min and program of 5ºC min^{-1} to 280ºC, which was maintained for further 10 min. The column effluent was ionized by electron impact (EI) at 70 eV at a source temperature of 200ºC. Mass range was from 40 to 500 *m/z* at a scan time of 1 s and the solvent delay was 4.0 min. Separately 1 µl of a C_8-C_{25} hydrocarbon mixture in hexane (100 ng μl^{-1}), containing a series of odd- and even-numbered *n*-alkanes, was injected for calculating the retention indexes (RIs) of the detected compounds according to van den Dool and Kratz (1963). Compounds were identified by comparison of their MS and RIs with those of authentic standards and/or with those from a commercial library (NIST Registry of Mass Spectral Data, 2005) or from the database published by Adams (2007).

For abdominal extracts, the abdomens of 3 one-week old virgin males and females were excised beneath the thorax and placed in 5 ml glass vials containing 500 µl of hexane. After 3 h extraction at room temperature, the abdomens were removed and the extracts were stored at -20ºC for subsequent analysis and bioassays. GC-MS analyses were performed by injecting 1 µl of the extracts (without concentration) adding 100 ng of the IS, as described above.

1.2.5 Collection and analysis of volatiles - Host plant

Volatile collection of the host plant was carried out by placing 5-6 freshly cut branches (ca. 20 cm long, fresh weight ca. 35 g) into a 3 L Erlenmeyer flask. Charcoal-filtered compressed air (550 ml min^{-1}) was passed over the twigs and the volatiles adsorbed into a Porapak Q (150/175 mg, 50/80 mesh) cartridge (Supelco, Bellefonte, PA, USA). Collection (two replicates) lasted 24 h and the adsorbed products were eluted with 2 ml of hexane. To 1 µl of the extracts (without concentration) was added 1 µl of dodecyl acetate in hexane as IS (100 ng μl^{-1}), and the resulting mixtures were analyzed by injecting into the GC-MS system, as described above. Linear retention indexes were estimated by co-injection of a hydrocarbon mixture (C_8-C_{25}). The oven temperature was initially set at 50°C for 1 min, programmed at 3°C min^{-1} to 120°C, then at 5°C min^{-1} to 200°C, 10ºC min^{-1} to 260ºC and held at this temperature for 10 min more. Identification of compounds was carried out as described above (1.2.4). For quantification, peak

areas of the compounds were compared to that of the internal standard. Percentages of the different components were calculated relative to the most abundant compound.

Leaf extract preparation was done as follows: 5-6 leaves (ca. 500 mg) of fresh branches were cut into small pieces, placed into 5 ml glass vials and immersed in hexane (3 ml). After 3 h extraction, plant material was filtered through Whatman filter paper and the extracts were stored at -20ºC for subsequent analysis and bioassays. GC-MS analyses were performed as described above by injecting 1 µl of the unconcentrated samples containing 100 ng of the IS. Temperature program was the same as used for plant volatiles.

1.2.6 Electrophysiological assays – GC-EAD and EAG

Coupled gas chromatography-electroantennographic detection (GC-EAD) analyses were carried out on a Focus GC (Thermo Instruments, Barcelona, Spain), equipped with a FID detector and a HP-5MS fused silica capillary column (30 m×0.25 mm ID×0.25 µm) (Agilent Technologies, Madrid, Spain) with helium as carrier gas (1-2 ml min^{-1}) and nitrogen as a second make-up gas. The column effluent was split 1:1 for simultaneous detection by the FID and EAD. The transfer tube to the EAD preparation was heated to 230°C, and the GC conditions were the same as for the GC-MS analysis (see above). The outlet for the EAD was delivered to the insect antenna through an L-shaped glass tube (12 cm long × 6 mm ID) by a humidified airstream. For the antennal preparation, antennae of both sexes were excised, carefully cut on both ends, and the distal and proximal segments were placed in contact with two microelectrodes using a conducting gel (Spectra 360, Parker Lab. Inc., Hellendoorn, The Netherlands). The microelectrodes were connected to an IDAC-2 interface (Syntech, Kirchzarten, Germany) and the antennal and FID signals were amplified (100x), filtered (DC to 1 kHz) and recorded simultaneously by the GC-EAD v4.4 software (Syntech). Two microliters of the concentrated headspace extracts of *C. florentinus,* the unconcentrated host plant volatiles, and 1 µl of a synthetic 3-component blend (nonanal, decanal and geranylacetone) (100 ng $µl^{-1}$ each), detected in the headspace volatiles of adults were injected in splitless mode. Antennae of 5 males and 5 females were used and a compound was considered electrophysiologically active when it elicited antennal responses obviously different from background noise (Zhang, et al., 2001).

EAG dose–responses were done on OBB male and female antennae to the synthetic mixture of nonanal, decanal and geranylacetone. In addition, female

responses to the dose of 1 µg of five green leaf volatiles (GLVs) ((*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, (*Z*)-3-hexenyl acetate and *n*-hexyl acetate) detected in *Q. suber* volatiles were recorded. For EAG studies the same electrophysiological setup and antennal preparation as for GC-EAD analysis were used. The stimulation procedure was arranged as described by Acin and coworkers (2010). Synthetic compounds (100 ng, 1 µg and 10 µg) dissolved in hexane were deposited on a Whatman filter paper (2.5 cm diameter) and puffed over the antenna from the lowest to the highest concentration and vice versa. A solvent blank (10 µl of hexane) stimulation was done at the start of the experiment and in between two different and consecutive stimuli. The solvent was allowed to evaporate before the tests and each stimulations was followed by a minimum of 60 s purge period of air to ensure recovery of antennal receptors. Compounds were tested 3x on each antenna and a minimum of 10 antennae per sex were considered. The amplified signals were analyzed by the EAG Pro program (Syntech, Kirchzarten, Germany). Difference of the mean test responses (3 puffs) minus those of the blank determined the electrophysiological activity of the stimulus. The depolarization means were compared for significance using analysis of variance (*one-way ANOVA*) followed by *DMS post-hoc* tests ($P<0.05$) applying PASW 18 software (SPSS Inc., Chicago, IL, USA).

1.2.7 Laboratory behavioral bioassays

A modified vertically-posted two choice Y-tube glass olfactometer was used to test the olfactory response of OBB males and females to conspecific insect and host plant volatiles and synthetic compounds (Fig. 1.3). The olfactometer consisted of a main tube (10 cm long × 18 mm ID) with two 8 cm long arms separated by a 90º angle, inside which a Y-shaped iron wire was positioned to facilitate locomotion of the insects towards one of the ends of the olfactometer, similarly to the design of Sabelis and van de Baan (1983). Each arm was connected to a glass adaptor (4.5 cm long × 1.2 mm ID) containing the test and control stimulus (live insects or volatile/synthetic compounds adsorbed on a Whatman filter paper). Charcoal-filtered air (2.5 l min^{-1}), passing through the arms, brought the stimuli to the test insects placed at the entrance of the main tube. The system was emblazed by a 60 W white light bulb procuring a homogenous illumination around the olfactometer. Experiments were carried out with 1-2 week-old OBB adults from 10.00 am to 17:00 pm at room temperature (26±2ºC) and 50±10% RH. Test individuals were taken out from their containers 2 h before the tests and kept individually in plastic dishes (3 cm high × 5 cm diameter).

The beetles were placed individually at the base of the main arm and their behavior was observed for 5 min. Insects that walked upwind and reached at least the middle of one of the two short arms without returning to the intersection within 5 min was recorded as a positive response, whereas those that did not choose either arm for the same period were excluded for analysis. After 5 replicates of every treatment, the Y-tube and the iron wire were cleaned with alcohol or acetone and left to dry for 5 min. The relative position of the olfactometer arms were reversed when 10 individuals had been tested. Insects were recycled for testing but only 3 days after the previous experiment. Preliminary assays with no odor were performed to exclude the possible preference of the test insects for one of the two arms (positional effect of the setup). For statistical analysis, the number of insects responding to treatment and control stimuli were subjected to χ^2 *goodness-of-fit test*, using Yates correction for continuity (α = 0.05) (Zar, 1999). The null hypothesis was that the percentage of individuals choosing the odor treatment and the blank was equal to 50%. Details of the different odor treatments, sex of test insects, and number of replicates are shown in Table 1.2.

Fig.1.3. Dual choice Y-tube olfactometer for behavioral studies of *C. florentinus* adults.

Table 1.2 Details of behavioral experiments done on C. *florentinus* adults in a two-choice olfactometer

Exp. No.	Odor treatment[a]	Control	Sex	Replicates (N)
1	air	air	Adult ♂ + ♀	62
2	live females[a]	air	Adult ♀	30
3	live females[a]	air	Adult ♂	51
4	live males[a]	air	Adult ♀	74
5	live males[a]	air	Adult ♂	30
6	abdominal extract female[b]	solvent	Adult ♂	36
7	abdominal extract male[b]	solvent	Adult ♀	34
8	3-component blend (100 ng)[c]	solvent	Adult ♂	45
9	3-component blend (100 ng)[c]	solvent	Adult ♀	40
10	3-component blend (1 µg)[c]	solvent	Adult ♂	30
11	3-component blend (1 µg)[c]	solvent	Adult ♀	30
12	geranylacetone (100 ng)	solvent	Adult ♂	46
13	geranylacetone (100 ng)	solvent	Adult ♀	30
14	geranylacetone (1 µg)	solvent	Adult ♂	34
15	geranylacetone (1 µg)	solvent	Adult ♀	30
16	nonanal (100 ng)	solvent	Adult ♂	30
17	nonanal (100 ng)	solvent	Adult ♀	30
18	nonanal (1 µg)	solvent	Adult ♂	30
19	nonanal (1 µg)	solvent	Adult ♀	40
20	decanal (100 ng)	solvent	Adult ♂	41
21	decanal (100 ng)	3-comp.blend[c,d]	Adult ♂	30
22	decanal (100 ng)	nonanal[d]	Adult ♂	30
23	decanal (100 ng)	solvent	Adult ♀	35
24	decanal (1 µg)	solvent	Adult ♂	48
25	decanal (1 µg)	solvent	Adult ♀	40
26	leaf extract *Q. suber*[e]	solvent	Adult ♀	31
27	leaf extract *Q. suber*[e]	solvent	Adult ♂	46
28	volatiles *Q. suber*[f]	solvent	Adult ♀	30
29	volatiles *Q. suber*[f]	solvent	Adult ♂	30
30	5 GLVs (1 µg)[g]	solvent	Adult ♀	31
31	5 GLVs (1 µg)[g]	solvent	Adult ♂	31
32	(*E*)-2-hexenol (1 µg)	solvent	Adult ♀	31
33	(*E*)-2-hexenal (1 µg)	solvent	Adult ♀	30
34	1-hexanol (1 µg)	solvent	Adult ♀	30
35	(*Z*)-3-hexenyl acetate (1 µg)	solvent	Adult ♀	30
36	*n*-hexyl acetate (1 µg)	solvent	Adult ♀	30

[a] Live individuals were used as the odor source (N=3).
[b] Abdominal extracts from 3 individuals of each sex (100 µl).
[c] 3-component blend = geranylacetone, nonanal, decanal in 1:1:1 ratio
[d] 100 ng of each compound were used as odor treatment 2.
[e] 1000 µg of leaf extract was applied =100 µl (10 µg μl^{-1})
[f] Amount applied volatile extract=100 µl (18.25 µg μl^{-1} fresh weight branches).
[g] GLVs = (*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, (*Z*)-3-hexenyl acetate and *n*-hexyl acetate (1 µg each).

1.3 RESULTS

1.3.1 SEM studies of adult antennae

Morphological analysis of OBB antennae by SEM primarily showed that male and female antennae are almost identical in their structure but differ in size. Male antennae (4.5 mm long) are up to 2x longer than those of females. The antennae, which are situated between the compound eyes and curve down ventrally, consist of 11 segments called antennomers. The scape (longest part of the antenna), representing the segment most proximal to the head, is followed by the pedicel and nine flagellomeres (Figs. 1.4 A, E). These nine flagellomeres constitute what is commonly referred to as the “antennal flagellum”. The flagellomeres are similar in shape in both sexes but in males they are progressively expanded toward the tip of the antennae. In the middle part of the flagellum they are ca. 450 µm long (in females ca. 275 µm), whereas the last segment only measures ca. 360 µm.

The SEM technique allows identification of the different types of sensilla distributed along the antennae. Its morphology is based on the common terminology used by Zacharuk (1980) and Crook and coworkers (2008a). Both sexes possess four different types of sensilla: *s. chaetica*, *uniporous sensilla* and two types of *s. basiconica* (Figs. 1.4 B-D, F-H). Distributions vary with the sex with a higher number of *s. basiconica* in males than in females.

The relatively long *sensilla chaetica* (mechanoreceptors) measure ca. 175 µm (ranging between 130 and 320 µm) in males and ca. 130 µm (90-175 µm) in females. They are located along the entire length of the antennae evenly distributed around the circumferences of each flagellomere, and its number is nearly equal in both males and females the number (Figs. 1.4 B, F).

The *uniporous sensilla*, considered to contain the gustatory/taste receptors, are short (ca. 7-11 µm), smooth sided pegs and located in groups within a crater on the distal surface of the outermost flagellar subsegments (Figs. 1.4 B, C, F, G). This sensilla type exhibit a single, oval-shaped pore at the distal tip. Male antennae have significantly more of these sensilla on every flagellomere except on the tip when compared with females.

Sensilla basiconica are multiporous chemorecpetors responsible for olfaction. In both males and females the number of this type of sensilla increased toward the distal tip of the antenna (Figs. 1.4 C, D, G, H). Two different types of single-walled

multiporous sensilla are present, *s. basiconica* type I and type II (Fig. 1.4 D). Type 1 sensilla are situated along the edge of a crater filled with numerous *uniporous sensilla* at the distal part of the flagellomeres. They are longer than type 2 sensilla with a length of ca. 45 µm in males and ca. 18-20 µm in females. *S. basiconica* of type 2 are located within the depression round the *uniporous sensilla*. This type measures ca. 13 µm in males and ca. 4-7 µm in females. The number of both types differs depending on the flagellomeres considered, but we can observe significantly more sensilla in males than in females.

1.3.2 Volatile composition

A total of 28 compounds were identified in the headspace volatiles and abdominal extracts of OBB male and female virgin adults (Table 1.3). GC-MS analyses of the abdominal samples revealed the presence of several saturated acids (entries 7, 13 and 14) and one mono-unsaturated (entry 16), whereas in insect volatiles one alcohol (entry 5), several aldehydes (entries 1, 3, 4 and 6), ketones (entries 2 and 10), and two isopropyl carboxylates (entries 12 and 15) were detected. However, most of the identified compounds from both types of samples are saturated linear or branched hydrocarbons. The chain-length ranges from C_{14} to C_{30} with one or two methyl groups in the case of branched compounds. No qualitative differences were observed in the headspace composition between male and female volatiles and only the amount of some compounds may differ in some cases.

Regarding host plant odors, 32 compounds with a total amount of 143.5 ± 22.9 µg were identified from headspace volatiles of freshly cut branches and leaf extracts of *Q. suber*. Results are summarized in Table 1.4. Basically two groups of chemically different compounds can be noticed. On the one hand, saturated and monounsaturated six-carbon aldehydes, alcohols and esters, the so-called green leaf volatiles (GLVs). The second major group is composed of compounds that belong to the class of isoprenoids or terpenoids, whose carbon skeletons are multiple of characteristic C_5 units (McGarvey and Croteau, 1995). According to the number of C_5 units, they are subdivided into mono- (C_{10}) and sesquiterpenes (C_{15}) (Kesselmeier and Staudt, 1999). Quantification of compounds revealed that the highest amount emitted by 5-6 freshly cut *Q. suber* branches are the *trans*-configurated GLVs (*E*)-2-hexenol (33.4 ± 6.2 µg) and (*E*)-2-hexenal (31.9 ± 1.2 µg), followed by the unsaturated acetate (*Z*)-3-hexenyl acetate (24.2 ± 5.6 µg). The percentage of detected compounds relative to the most abundant compound, (*E*)-2-hexenol, is displayed in Table 1.4.

Fig.1.4. Scanning electron micrographs (SEM) of male (A-D) and female antennae (E-H) of *C. florentinus* adults (AF, antennal flagellum; S, scape; P, pedicel). (B, F) Middle flagellomere with apical depressions (crater) filled with numerous *uniporous sensilla* (U) and *s. chaetica* (SC) distributed around the segments. (C, G) Apical depression mainly filled with *uniporous sensilla* (U) and *s. basiconica* (SB) within and around the crater. D) Distal antennal flagellomer of a male antenna with numerous *s. basiconica* of both types (SB1 and SB2). H) *S. basiconica* type I (SB1) of a female *C. florentinus* antenna.

Table 1.3 Compounds detected in abdominal extracts[A] and volatiles[V] of males and females of *C. florentinus*, identified by comparison of their mass spectra (MS) and retention indexes (RI) with those of standards and/or reference databases (Adams, 2007; NIST, 2005)

Entry	Compound	MS/RI[a]	M_r[b]	RI[c]
1	benzaldehyde[V]	ST/ST	106	963
2	6-methyl-5-heptene-2-one[V]	LI/LI	126	988
3	octanal[V]	ST/ST	128	1005
4	nonanal[A, V]	ST/ST	142	1103
5	2-phenylethanol[A]	ST/ST	122	1112
6	decanal[V]	ST/ST	156	1207
7	nonanoic acid[A]	ST/ST	158	1282
8	isobornyl acetate[V]	ST/ST	196	1289
9	tetradecane[V] ($C_{14}H_{30}$)	ST/ST	198	1399
10	geranylacetone[V]	ST/ST	194	1454
11	hexadecane[V] ($C_{16}H_{34}$)	ST/ST	226	1599
12	isopropyl doedecanoate[V]	ST/ST	242	1635
13	tetradecanoic acid[A]	ST/ST	228	1767
14	hexadecanoic acid[A]	ST/ST	256	1985
15	isopropyl palmitate[V]	ST/ST	298	2025
16	henicosane[V] ($C_{21}H_{44}$)	ST/ST	296	2100
17	docosane[V] ($C_{22}H_{46}$)	ST/ST	310	2200
18	tricosane[A, V] ($C_{23}H_{48}$)	ST/ST	324	2300
19	11-methyltricosane[A, V] ($C_{24}H_{50}$)	LI/LI	338	2330
20	tetracosane[A, V] ($C_{24}H_{50}$)	ST/ST	338	2400
21	pentacosane[A, V] ($C_{25}H_{52}$)	ST/ST	352	2500
22	13-methylpentacosane[A, V] ($C_{26}H_{54}$)	LI/LI	366	2529
23	13-methylhexacosane[A, V] ($C_{27}H_{56}$)	LI/LI	380	2625
24	heptacosane[A, V] ($C_{27}H_{56}$)	ST/ST	380	2700
25	11,14-dimethylhexacosane[A, V] ($C_{28}H_{58}$)	LI/LI	394	2728
26	nonacosane[A, V] ($C_{29}H_{60}$)	ST/ST	408	2900
27	13-methylnonacosane[A, V] ($C_{30}H_{62}$)	LI/LI	422	2943
28	triacontane[A, V] ($C_{30}H_{62}$)	ST/ST	422	3000

[a] Type of identification by comparison of MS and RI with standards (ST) or literature data (LI).
[b] Relative molecular mass
[c] On a HP-5MS (30 m×0.25 mm I.D., 0.25 µm) fused silica capillary column.

1.3.3 GC-EAD recordings

Part of a representative GC-EAD profile of volatiles collected from OBB adults is shown in figure 1.5 A. Three EAD active compounds were revealed on male antennae stimulated with female volatiles. Male headspace samples with the same volatile composition showed similar activity.

Table 1.4 Compounds detected in volatiles of *Q. suber* branches, identified by comparison of their mass spectra (MS) and retention indexes (RI) with those of standards and/or reference databases (Adams, 2007; NIST, 2005), and percentage of chemicals relative to the most abundant compound (*E*)-2-hexenol.

Entry	Compound	Relative Ratio ±SE[a]	M_r[b]	BP[b]	RI[c]
	Green Leaf volatiles (GLV's)				
	Aldehydes				
1	hexanal	10.0±4.1	100	41	813
2	(*E*)-2-hexenal	98.3±14.6	98	41	856
	Alcohols				
3	(*Z*)-3-hexenol	33.0±2.4	100	67	859
4	(*E*)-2-hexenol	100	100	57	870
5	1-hexanol	59.2±16.3	102	56	872
	Esters				
6	(*Z*)-3-hexenyl acetate	71.9±3.6	142	43	1009
7	*n*-hexyl acetate	11.5±1.5	144	43	1014
8	(*E*)-2-hexenyl acetate	18.6±3.4	142	43	1016
9	(Z)-3-hexenyl isobutyrate	0.7±0.2	170	67	1143
10	(*E*)-2-hexenyl isobutyrate	1.3±0.2	170	71	1150
11	(*Z*)-3-hexenyl 2-methylbutyrate	5.7±2.0	184	67	1232
12	(*E*)-2-hexenyl 2-methylbutyrate	1.6±0.5	184	57	1238
	Monoterpenes (C10)				
13	*α*-thujene	0.5±0.0	136	93	925
14	*α*-pinene	2.0±0.5	136	93	932
15	camphene	0.1±0.0	136	93	946
16	sabinene	1.2±0.3	136	93	972
17	*β*-pinene	1.3±0.3	136	93	975
18	limonene	0.3±0.0	136	68	1028
19	cineol	0.5±0.0	154	81	1030
20	(*E*)-β-ocimene	0.9±0.3	136	93	1047
21	γ-terpinene	0.5±0.0	136	93	1057
22	linalool	1.9±0.3	154	71	1100
	Homoterpenes (C11)				
23	(3*E*)-4,7-dimethyl-1,3,7-nonatriene	8.9±2.0	150	69	1117
	Sesquiterpenes (C15)				
24	*α*-cubebene	0.3±0.0	204	105	1352
25	copaene	0.4±0.1	204	161	1377
	Others				
26	nonane	0.1±0.0	128	43	900
27	(*E,E*)-2,4-hexadienal	0.8±0.0	96	81	909
28	*n*-pentyl acetate	0.4±0.0	130	43	912
29	benzaldehyde	0.9±0.1	106	77	959
30	vinyl hexanoate	0.8.0.0	142	60	983
31	γ-hexalactone	0.9±0.0	114	85	1053
32	nonanal	0.8±0.2	142	41	1104

[a] Based on the relative areas on GC-MS.
[b] (M_r) Relative molecular mass; ($BP_{(m/z)}$) Base peak of mass spectra.
[c] On a HP-5MS (30 m×0.25 mm I.D., 0.25 μm) fused silica capillary column.

The EAD active compounds were identified as nonanal, decanal and geranylacetone by comparison of their mass spectra and GC retention indexes on a nonpolar capillary column (HP5-MS), with those of synthetic standards (Table 1.3). These same headspace volatiles only elicited slight responses in female antennae (results not shown). Activity of the identified compounds in male antennae was confirmed by testing a synthetic mixture (3-component blend) of these compounds (Fig. 1.5 B). Another active peak provoking an electrophysiological response on GC-EAD was nerylacetone, an isomer of geranylacetone present in the commercial sample of the latter. In both profiles decanal seemed to provoke stronger EAD responses in males than the other volatile compounds. In female antennae the highest EAD activity was displayed by geranylacetone (results not shown).

GC-EAD analyses of headspace volatiles of the host plant *Q. suber* indicated that the identified GLVs (*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, (*Z*)-3-hexenyl acetate and *n*-hexyl acetate are the major components eliciting EAD responses on both sexes of *C. florentinus* (Figs. 1.6 A, B). No antennal responses were detected to the minor terpenoid components at the natural dose of the extract. However, the homoterpene (3*E*)-4,7-dimethyl-1,3,7-nonatriene was EAD active on female antennae (entry 23 in Fig. 1.6). Other possible active compounds remain unknown.

1.3.4 EAG responses

Direct electroantennogram responses were also determined on male and female OBB antennae. Three synthetic compounds, nonanal, decanal and geranylacetone, derived from headspace samples of conspecific virgin adults and active in GC-EAD assays, were tested. All tested stimuli at doses of 100 ng, 1 µg and 10 µg elicited significant EAG responses (Table 1.5). In all tested stimuli the mean response threshold of male antennae appeared to be at least twice that of females. Dose-dependent responses were displayed in male and female antennae by the 3-component blend of the synthetic compounds and by geranylacetone alone, whereas the doses of nonanal and decanal did not provoke significant differences in the antennal response of females. In contrast, decanal showed a surprisingly high EAG response at the lowest dose, significantly higher than at the other doses tested.

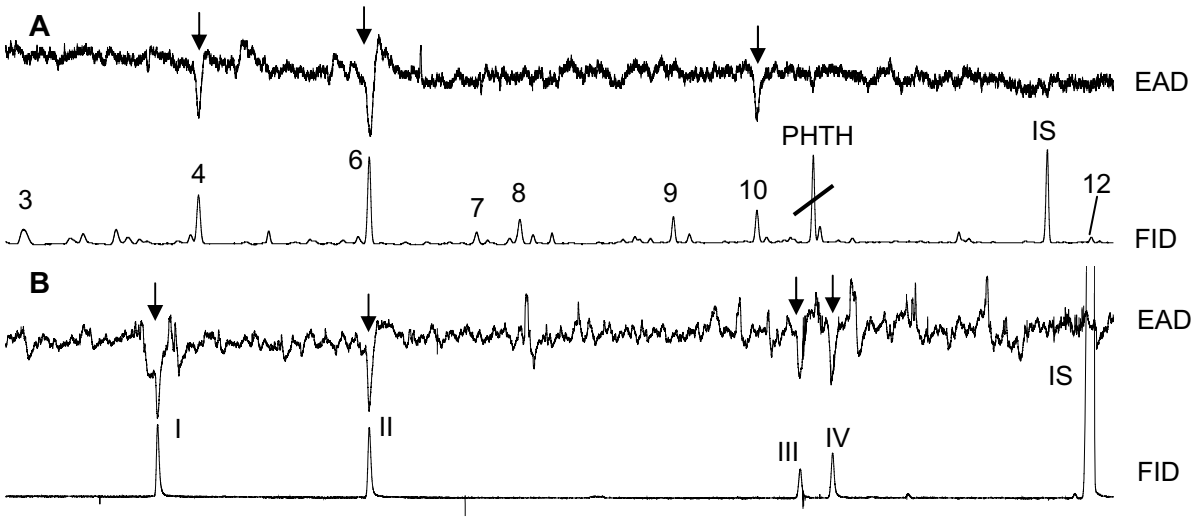


Fig. 1.5. Representative GC-EAD profile on male antennae of *C. florentinus* responding to (A) 100 µl of female volatiles collected during 24 h (compound numbers correspond to entries of Table 1.3.; IS = internal standard; PHTH: phthalate) and (B) a 3-component blend of synthetic compounds (100 ng) (I: nonanal; II: decanal; III: nerylacetone; IV: geranylacetone). Nerylacetone is an isomer of geranylacetone present in the commercial sample of the latter. Active compounds are marked by black arrows.

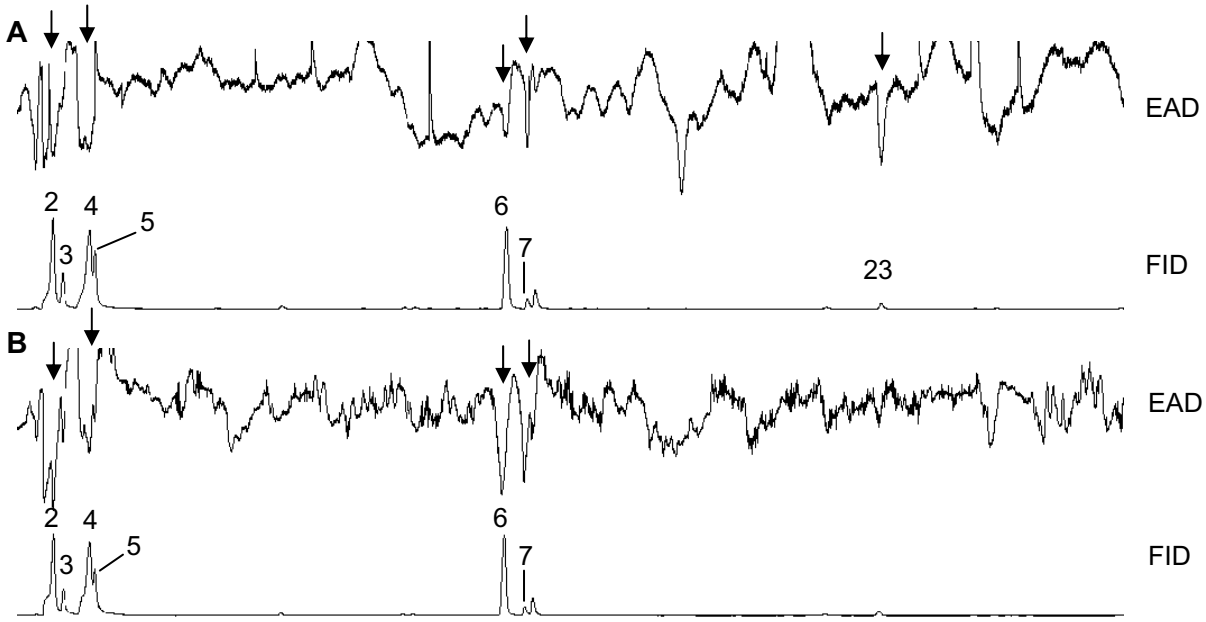


Fig. 1.6. Representative GC-EAD profile on (A) female and (B) male antennae of *C. florentinus* responding to *Q. suber* volatiles collected during 24 h. Active compounds are marked by black arrows. Compound numbers correspond to entries of Table 1.4: 2: (*E*)-2-hexenal; 3. (*Z*)-3-hexenol; 4: (*E*)-2-hexenol; 5: 1-hexanol; 6: (*Z*)-3-hexenylacetate; 7: *n*-hexyl acetate; 23: (3*E*)-4,7-dimethyl-1,3,7-nonatriene.

Table 1.5 Mean EAG responses ±S.E.M. (mV) of female and male antennae of *C. florentinus* to different doses of nonanal, decanal and geranylacetone separately and in a 3-component blend in 1:1:1 ratio[†,‡].

	FEMALES					MALES				
Compound	100 ng	1 µg	10 µg	F_{values}	*P*	100 ng	1 µg	10 µg	F_{values}	*P*
3-component blend	0.11±0.04 b,B	0.20±0.04 b,A	0.42±0.06 a,AB	9.694	***	0.21±0.04 c,C	0.38±0.14 b,A	1.03±0.11 a,A	18.760	***
Geranylacetone	0.15±0.03 b,AB	0.25±0.05 b,A	0.69±0.16 a,A	9.439	***	0.40±0.13 b,C	0.65±0.09 b,A	1.08±0.14 a,A	14.032	***
Nonanal	0.29±0.07 a,A	0.25±0.06 a,A	0.41±0.09 a,B	1.201	n.s.	0.89±0.12 a,B	0.57±0.14 a,A	0.68±0.13 a,B	1.379	n.s.
Decanal	0.32±0.07 a,A	0.18±0.05 a,A	0.35±0.04 a,B	2.409	n.s.	2.15±0.18 a,A	0.40±0.08 b,A	0.40±0.07 b,B	53.024	***
F_{values}	2.891	0.589	2.458			60.471	1.027	7.510		
P	*	n.s.	n.s.			***	n.s.	***		

[†] The 3-component blend contained the same amount of each compound as the individual components.

[‡] Asterisks indicate significant differences on ranked normalized data between treatments (doses within rows, compounds within columns) (One-way Anova, $*P<0.05$; $***P<0.001$, n.s. = $P>0.05$). Values followed by different letters, in low case within each row and in capital letter within each column, are significantly different at $P \leq 0.05$ (DMS *post-hoc* test). F_{values} = variance of group means.

On the other hand, activity of five GLVs, (*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, (*Z*)-3-hexenyl acetate and *n*-hexyl acetate, identified from *Q. suber* headspace volatiles and that elicited strong GC-EAD responses in female antennae, was studied at the dosage of 1 µg in females (Fig. 1.7). 1-Hexanol significantly elicited the strongest response compared to the aldehyde and the two acetates but not to (*E*)-2-hexenol. (*Z*)-3-hexenyl acetate and *n*-hexyl acetate displayed the smallest EAG activity of all tested compounds. Therefore, the relative activity of the GLVs tested compounds in female antennae of *C. florentinus* followed the order alcohols > aldehyde > acetates.

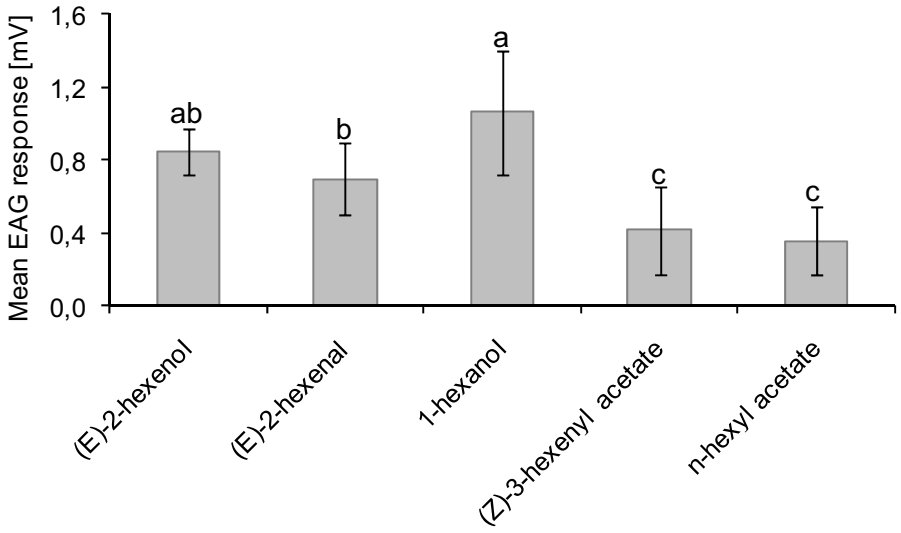


Fig. 1.7. Mean EAG responses (±S.E.M.) of female *C. florentinus* antennae (N=11) to five synthetic GLV´s [1µg each]: (*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, (*Z*)-3-hexenyl acetate and *n*-hexyl acetate. Bars with different letters indicate significant differences (*DMS post-hoc test*, $P \leq 0.05$).

1.3.5 Y-tube olfactometer bioassays - Live insects and abdominal extracts

A preliminary study with a dual choice Y-tube olfactometer showed that there was no preference in the responses of male and female OBB adults for one of the two arms to air only (χ^2=1.629, P>0.05), indicating a lack of positional effect of the experimental setup. Responsiveness of individuals in the bioassays was almost 100%. Results revealed that adult virgin males were significantly attracted to 3 live females (χ^2=7.098, P<0.01) and to abdominal extracts of 3 females (χ^2=5.472, P<0.02), but not to live males (χ^2=0,567, P>0.05) (Fig. 1.8). In contrast, adult virgin females showed no preference for insect volatiles or extracts derived from conspecific individuals (Fig. 1.8).

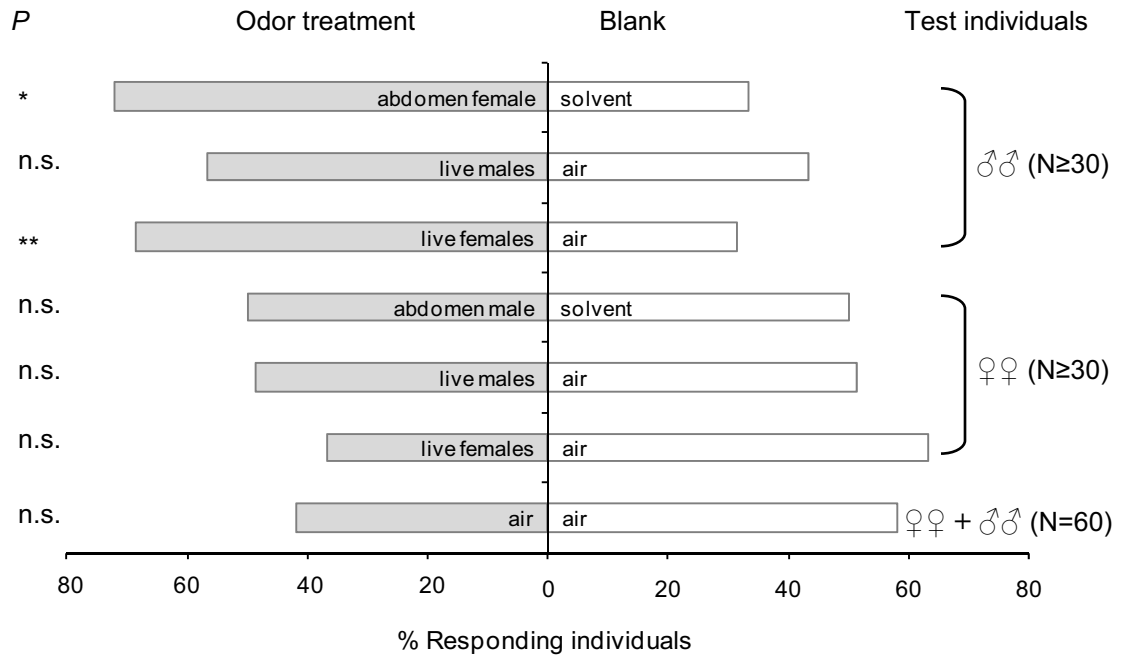


Fig. 1.8. Behavioral responses of virgin male and female OBB in a Y-tube olfactometer to volatiles of live individuals and abdominal extracts of both sexes (Experiments 1-7, see Table 1.2). Preferences for odors vs blank (solvent and air) were analyzed by χ^2 goodness-of-fit test with Yates correction for continuity (*P≤0.05; **P≤0.01; n.s. = P>0.05). The same amount of hexane (50 µl) was applied to treatment and control stimuli. N≥30 for each dual choice. To figure out side preferences (air vs. air) (N=60).

1.3.6 Y-tube olfactometer bioassays - Synthetic compounds

The behavioral response of OBB males to different doses (100 ng and 1 µg) of 3 synthetic compounds (nonanal, decanal and geranylacetone) and a blend of them, as in the headspace volatiles of conspecific insects, are presented in Figure 1.9. Test individuals were significantly attracted to both doses of the 3-component blend (χ^2_{100ng}=6.444, P<0.02; $\chi^2_{1\mu g}$=6.567, P<0.02) and to decanal at 100 ng (χ^2=3.935, P<0.05), while no preference was shown for the other odor treatments.

In contrast, male adults showed no preference between decanal and the 3-component blend (χ^2=0,567, P>0.05), but chose decanal over nonanal significantly (χ^2=4.833, P<0.05) (Fig. 1.10).

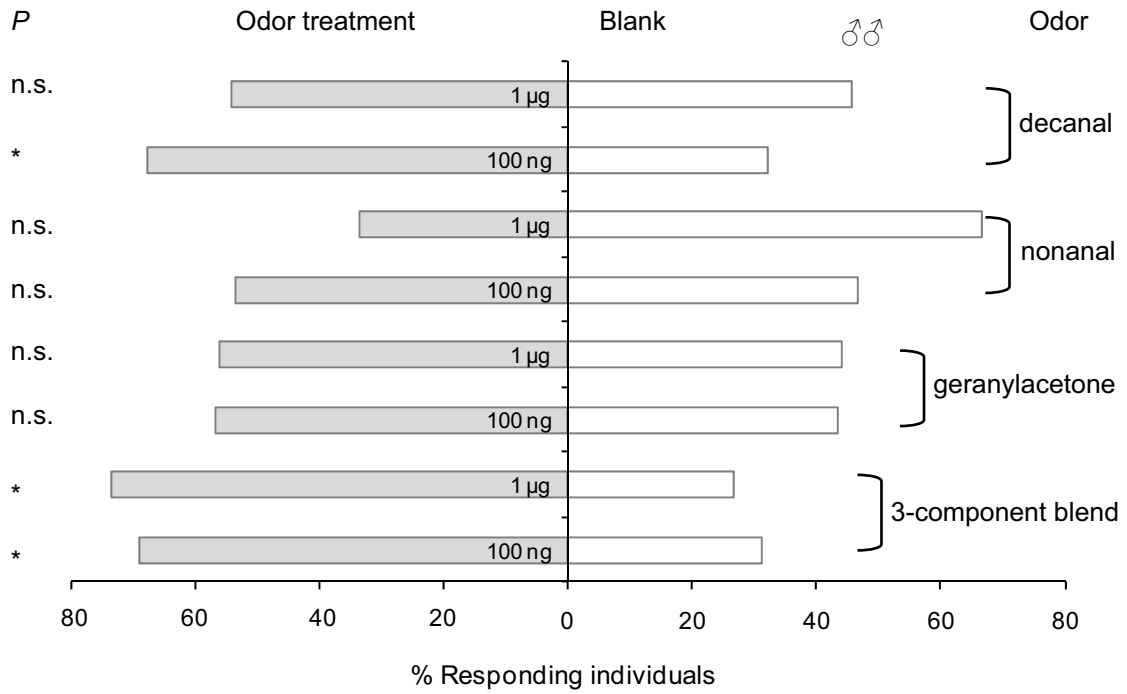


Fig. 1.9. Behavioral responses of virgin male OBB in a Y-tube olfactometer to nonanal, decanal and geranylacetone tested individually and in a 3-component blend at 100 ng and 1 µg doses (Experiments 8, 10, 12, 14, 16, 18, 20, 24, see Table 1.2). Preferences for odors dissolved in hexane versus blank (solvent) were analyzed by χ^2 goodness-of-fit test with Yates correction for continuity (*P≤0.05; n.s. = P>0.05). The same amount of hexane (10 µl) was applied to treatment and control stimuli. N≥30 for each dual choice.

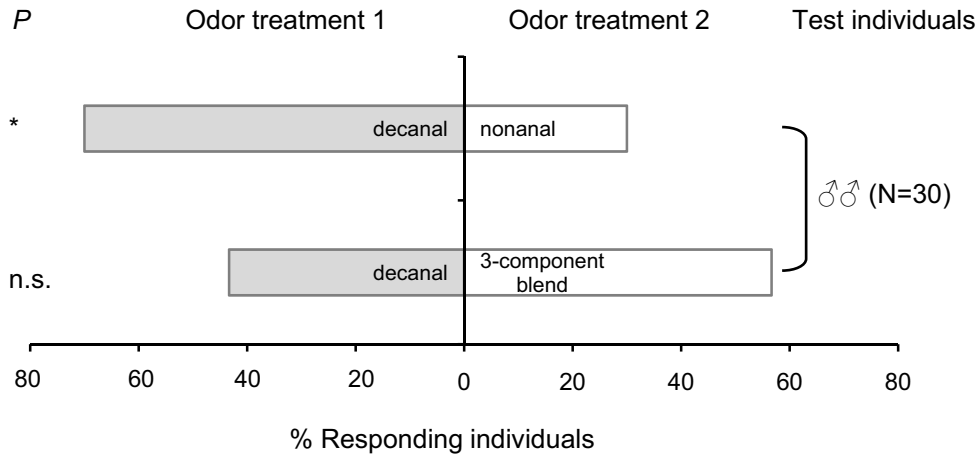


Fig. 1.10. Behavioral responses of virgin male OBB in a Y-tube olfactometer to nonanal, decanal and the 3-component blend (geranylacetone, nonanal, decanal) (100 ng each) derived from volatiles of *C. florentinus* males and females (Experiments 21 and 22, see Table 1.2). Preferences for decanal (odor treatment 1) versus the 3-component blend and nonanal (odor treatment 2) were analyzed by χ^2 goodness-of-fit test with Yates correction for continuity (*P≤0.05; n.s. = P>0.05). The same amount of hexane (10 µl) was applied to the different odor treatments. N=30 for each dual choice.

When OBB females were tested, they showed a significant preference for the 3-component blend (χ^2=4.925, *P*<0.05) and geranylacetone (χ^2=6.567, *P*<0.02) at the dose of 100 ng, and for nonanal at 1 µg dose (χ^2=4.925, *P*<0.05) (Fig. 1.11). No significant attraction was displayed toward the other odor treatments and doses.

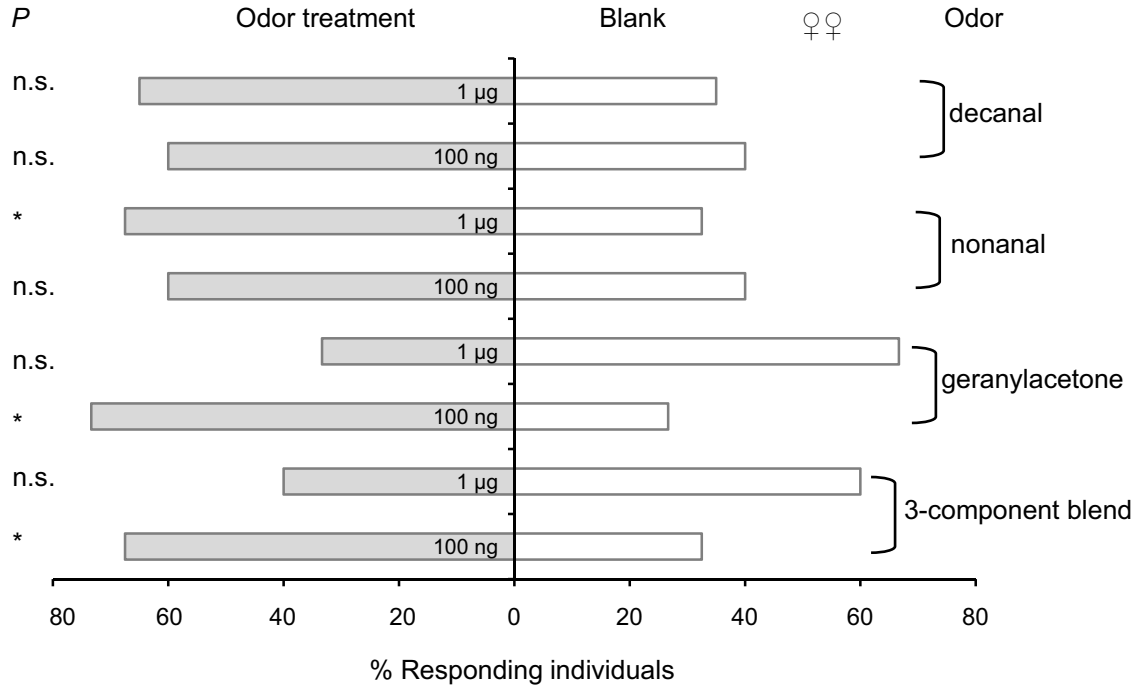


Fig. 1.11. Behavioral responses of virgin female OBB in a Y-tube olfactometer to nonanal, decanal and geranylacetone tested individually and in a 3-component blend at 100 ng and 1 µg doses (Experiments 9, 11, 13, 15, 17, 19, 23, 25, see Table 1.2). Preferences for odors dissolved in hexane versus blank (solvent) were analyzed by χ^2 goodness-of-fit test with Yates correction for continuity (*$P \leq 0.05$; n.s. = $P > 0.05$). The same amount of hexane (10 µl) was applied to treatment and control stimuli. N≥30 for each dual choice.

1.3.7 Y-tube olfactometer bioassays - Host plant volatiles and GLVs

Attraction of male and female OBB adults in the Y-tube olfactometer to headspace volatiles and leaf extracts of *Q. suber* and to a mixture of five synthetic GLVs revealed different results. Thus, whereas females were significantly attracted to all offered host plant odors ($\chi^2_{\text{leaf extract}}$=3.935, *P*<0.05; $\chi^2_{\text{volatiles}}$=4.833, *P*<0.05, χ^2_{GLVs}=5.484, *P*<0.02) males showed no significant preferences for any of them (Fig. 1.12).

On the other hand, results of the attractiveness of the GLVs (*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, (*Z*)-3-hexenyl acetate and *n*-hexyl acetate at the dose of 1 µg

showed that female were significantly attracted to the saturated and unsaturated alcohols ($\chi^2_{1\text{-hexanol}}$=6.567, P<0.02; $\chi^2_{(E)\text{-2-hexenol}}$=3.935, P<0.05) and to (Z)-3-hexenyl acetate (χ^2=4.833, P<0.05), while no preference was demonstrated for the other two compounds (Fig. 1.13).

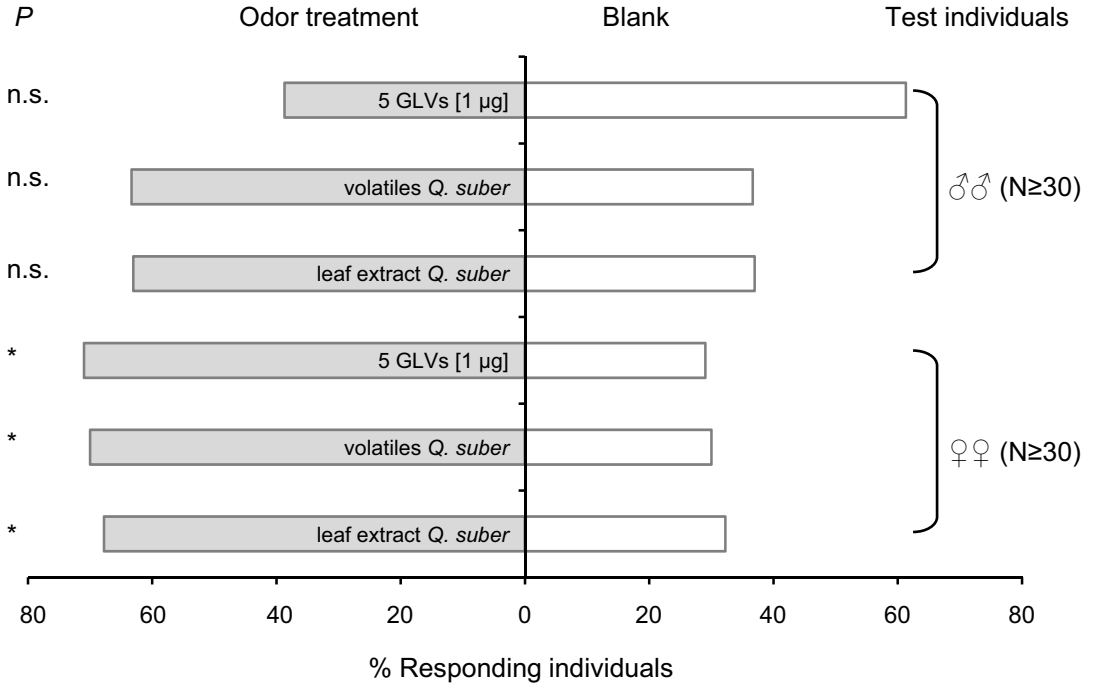


Fig. 1.12. Behavioral responses of virgin male and female OBB in a Y-tube olfactometer to a mixture of 5 synthetic GLVs at 1 µg dose and odors derived from volatiles and leaf extract of the host plant *Q. suber* (Experiments 26-31, see Table 1.2). Preferences for odors dissolved in hexane versus blank (solvent) were analyzed χ^2 goodness-of-fit test with Yates correction for continuity (*P≤0.05; n.s. = P>0.05). The same amount of hexane was applied to treatment and control stimuli. N≥30 for each dual choice.

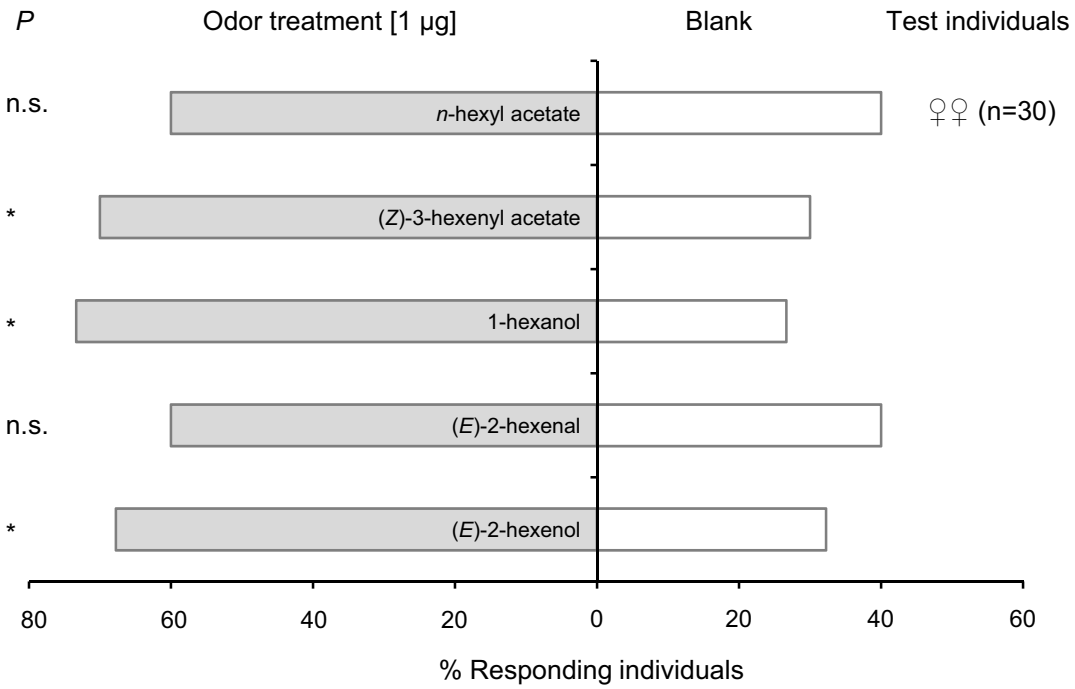


Fig. 1.13. Behavioral responses of virgin female OBB in a Y-tube olfactometer to 5 synthetic GLVs (1 µg each) present in volatiles of the host plant *Q. suber* (Experiments 32-36, see Table 1.2). Preferences for odors dissolved in hexane versus blank (solvent) were analyzed by $\chi 2$ goodness-of-fit test with Yates correction for continuity (*$P \leq 0.05$; n.s. = $P > 0.05$). The same amount of hexane (10 µl) was applied to treatment and control stimuli. N=30 for each dual choice.

1.4 DISCUSSION

Chemical ecology, behavior and physiology of beetles, particularly of pests have been largely investigated in the last decades because of its great economic impact. This comprises research on pheromones and their functions, emitted by a great variety of beetle species, such as bark and ambrosia beetles (Scolytidae), wood boring beetles (Cerambycidae), flat bark beetles (Cucujidae) and grain beetles (Silvanidae) (Hardie and Minks, 1999). In contrast, other Coleopteran families like Buprestidae have not been the subject of such variety of investigations. Laboratory studies have been carried out on the emerald ashborer (EAB), *A. plannipenis* (Rodriguez-Saona, et al., 2006; Bartelt, et al., 2007; De Groot, et al., 2008; Lelito, et al., 2007; 2009; Silk, et al., 2009), as well as some field experiments, capturing different jewel beetles (Montgomery and Wargo, 1983; Chenier and Philogene, 1989; Martikainen, et al., 2001; McIntosh, et al., 2001). In this line, very little is known about the bioecology of other buprestid species, including the oak branch borer (OBB), *C. florentinus*, pest of the cork oak *Q. suber* in the Iberian Peninsula and subject of this work. This chapter represents the first scientific study carried out on this pest species. Due to the fact that *C. florentinus* together with its sister species *C. undatus* provoke great damage in cork trees by reducing the production of high quality cork (Soria and Ocete, 1993; Soria, et al., 1994; Evans, et al., 2007) and no control measurements have been established so-far, we have investigated the chemical communication of the OBB. The final aim of the investigation is to find a pheromone and/or a kairomone that could be applied in the integrated pest management of this pest to reduce the population size and minimize the damage. This is important not only for preventing the direct damage on cork oaks, but also to inhibit spreading of the insect. Most wood-boring insects have a strong risk of being introduced, spread and established into new regions, as shown by the recent introductions of EAB, an Asian species in the USA (Haack, et al., 2002; McCullough and Roberts, 2002).

First of all, scanning electron micrographs of the antennae were made to examine the morphological structure of the sensilla for the identification of possible pheromone perceiving organs. We also analyzed the volatile composition and extracts of OBB adults and volatiles from *Q. suber* branches. Furthermore, we have determined the electrophysiological and behavioral responses of OBB males and females to the identified compounds.

The SEM analysis revealed that the antennae of *C. florentinus* male and female are nearly identical in its structure, but sexual dimorphism abounds with respect to the

size and number of sensilla. By comparison with the structure of already described beetle sensilla (Crook, et al., 2008a; Merivee, et al., 1999; 2000; 2002), *uniporous sensilla*, *s. chaetica* and two subtypes of *s. basiconica* were identified. In addition, it was observed that male antennae are up to 2x longer and contain more olfactory sensilla of both basiconic types than female antennae. This could indicate a possible stronger olfactory perception of males for certain types of odors. Generally, it is assumed that bigger antennal superficies correspond to a higher number of receptors, and, as consequence the insect is more sensitive to pheromone or other odor molecules (Davies, 1988). By comparison with antennae of the EAB, it was apparent that the morphology, disposition and types of sensilla of the OBB and the EAB are quite similar. Contact chemical cues are described to play an important role in mate recognition in the EAB (Lelito, et al., 2009; Silk, et al., 2009). Furthermore, Crook and coworkers (2008a) postulated that the presence of a greater number of gustatory *uniporous sensilla* in male antennae is possibly linked to mate recognition. Males of the OBB, likewise, dispose of noticeable more *uniporous sensilla* than females. Therefore, we suggest that short range, contact cues could also be important for mate recognition in this species, particularly by males. Corresponding bioassays to confirm these findings are in process. The findings that the OBB contain *uniporous sensilla* and several *s. basiconica* are in agreement with former studies showing that these sensilla are the dominant sensory apparatus in the family of Buprestidae (Volkovitsh, 2001). Flagellar pits or rather the depressions that comprise among others *uniporous sensilla* are very common among buprestid species, and they have been used for systematic and classification of the group (Bellamy, 1985). Additionally, it has been shown that mate location in jewel beetles can be facilitated by host selection, followed by visual, tactile, and possibly auditory cues rather than by using pheromones over any distance (Carlson and Knight, 1969; Gwynne and Rentz, 1983).

In the headspace volatiles of virgin adults of the OBB a total of 28 chemically different compounds were identified, but no sex differences were observed in the composition. This seems to be contradictory with the results obtained from behavioral bioassays in which virgin males were attracted to conspecific females, which could indicate the possible presence of a female-produced sex pheromone. Although no qualitative differences were found in the composition of male and female volatiles, the results obtained in the Y-tube olfactometer showed that possible female specific compounds attracting males are present in the volatile bouquet. Maybe these compounds are produced in too small amounts, which were not detectable with the utilized methods but perceived by males. For example, in *C. undatus*, using different

types of SPME fibers (not utilized in *C. florentinus*), traces of two cuticular methyl-branched hydrocarbons were found in females, but absent in male samples (Chapter 2). Its function remains unclear but studies on the EAB also revealed the presence of two female-specific methyl-branched hydrocarbons assumed as contact pheromone components (Lelito, et al., 2009; Silk, et al., 2009). Therefore, SPME analyses and a modified volatile collection on OBB are planned to detect differences in the volatile composition and possible sex-specific volatile compounds. Surprisingly, in EAB evidence for a volatile long-range sex pheromone remains inconclusive as well (Bartelt, et al., 2007).

Nevertheless, several of the detected and identified compounds have been described as pheromones or part of the pheromone system in other species. The C_9- and C_{10}-aldehydes nonanal and decanal and geranylacetone are known to be part of the aggregation pheromone of the common bed bug *Cimex lectularius* (Siljander, et al., 2008), whereas geranylacetone alone was found in other beetles performing pheromonal activity, as well as in male beetles of the genus *Nicrophorus* (Coleoptera: Silphidae) (Haberer, et al., 2008). Besides, GC-MS analysis of headspace volatiles from adults of the cerambycid beetle *Hedypathes betulinus* showed three male-specific compounds, one of them identified as geranylacetone (Fonseca, et al., 2010). To know the impact these compounds may have in the communication of the OBB electrophysiological and behavioral assays were performed. These compounds, i.e. nonanal, decanal and geranylacetone individually, or as a synthetic 3-component blend, elicited strong GC-EAD responses in male antennae. The highest activity was shown by decanal. In contrast, in females only slight responses were observed (nearly not detectable), the strongest activity being displayed by geranylacetone. These results were confirmed when different doses of the three compounds were tested in EAG, the males responding 7x stronger than females. Therefore, the three compounds, particularly decanal, could play a crucial role in the odor system of adult males, whereas in females this possible role might be assigned to geranylacetone. It should be noted that these compounds are emitted also by a variety of insects and that their electrophysiological activities were noticed in a number of studies before. For instance, nonanal and decanal, detected in the airborne volatiles of females of the scarab beetle *Hoplia equina*, elicited antennal GC-EAD responses in conspecific males (Zhang, et al., 2003). Also Dickens (2006) showed that potato volatiles including nonanal were active in GC-EAD experiments on the antennae of the Colorado potato beetle, *Leptinotarsa decemlineata*. In addition, in headspace volatiles of honeybees, nonanal and decanal

were reported to provoke antennal responses (GC-EAD) by the small hive beetle *Aethina tumida* (Torto, et al., 2005).

The assumption that nonanal, decanal and geranylacetone could influence the behavior of the OBB is strengthened by the findings obtained in behavioral bioassays with a vertical posted Y-tube olfactometer. Males are significantly attracted to a mixture of the three active compounds and in particular to decanal alone, indicating that decanal is probably the bioactive compound of the mixture that causes attraction. Besides, in a contrast-bioassay offering two odor treatments males significantly favored decanal over nonanal. Maybe there is an effect of the chain length favoring the longer chain (C_{10}) over the shorter one (C_9), which has to be verified by testing additional compounds (aldehydes) composed of more or less carbons. In the Y-tube olfactometer, females, in turn, highlighted preferences for the mixture and geranylacetone alone. In summary, these results could indicate sex differences in the odor perception of volatiles produced by males and females. Since the active compounds are present in headspace volatiles of both sexes, the function as sex-pheromones can be excluded but rather they could mediate aggregation in adult individuals. For that reason, further investigations are necessary and aggregation bioassays with the active compounds and adults of *C. florentinus* will be implemented.

In addition to the just discussed 3 components of the OBB headspace volatiles, two more compounds were identified as isopropyl dodecanoate and palmitate. These compounds are known as a class of insect pheromones (Francke et al., 1979), but up to know it is unknown if they play a role in the chemical communication of *C. florentinus*. These chemicals displayed no electrophysiological activity in GC-EAD and furthermore, isopropyl dodecanoate did neither attract nor repel adults in dual choice olfactometer bioassays.

The major group of all identified compounds from OBB volatiles (headspace and abdominal extracts is composed by saturated linear and branched hydrocarbons. In several studies it has been outlined that they are the source of the greatest number of insect pheromones together with terpenoids and fatty acids (Morgan, 2010). The signal functions of hydrocarbons include serving as close range and contact pheromones, as described before, but also as sex attractants and aphrodisiacs, anti-aphrodisiacs, caste and kin recognition cues, aggregation pheromones and kairomones (Howard and Blomquist, 2005).

In the headspace volatiles of the host plant *Q. suber*, the major part of the identified compounds was composed by saturated and monounsaturated six-carbon

aldehydes, alcohols and esters. These so-called 'green leaf volatiles' (GLVs) are typically found in volatiles of green plants and can be produced by all tissues (Hatanaka, et al., 1987; Takabayashi, et al., 1996). Open stomata or leaf cuticle very often release small amounts of GLVs (Hatanaka, et al., 1995), but the production increase drastically after stress or after herbivore damage (Paré and Tumlinson, 1996). Most of the other volatile organic compounds (VOCs) emitted by freshly cut *Q. suber* branches are characterized by mono-, homo- and sesquiterpenes. These results are in accordance with the findings made by Staudt and coworkers (2004), but opposed to former assumptions, that only the Holm oak *Q. ilex* is a monoterpene emitter, not *Q. suber* (Kesselmeier and Staudt, 1999; Loreto, 2002). This inconsistency could be the result of geographic variability in the overall capacity of *Quercus* species to emit volatiles, which may be related to past genetic isolation of populations, adaptations to local growth conditions, or hybridization between emitting and non-emitting oak species (Manos, et al., 1999; Belahbib, et al., 2001). Our results point out that the cork oaks from Catalonia belong to the monoterpene emitting clade.

In GC-EAD bioassays of headspace volatiles of *Q. suber,* the GLVs (*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, (*Z*)-3-hexenyl acetate and *n*-hexyl acetate elicited strong electrophysiological responses in male and female antennae. The homoterpene (3*E*)-4,7-dimethyl-1,3,7-nonatriene provoked clear GC-EAD responses only in females. In behavioral bioassays, virgin females and not males preferred natural and synthetic host plant volatiles, particularly (*E*)-2-hexenol, 1-hexanol and (*Z*)-3-hexenyl acetate, the most abundant compounds from *Q. suber* volatiles. In EAG, female antennae responses to the five GLVs differed significantly, and (*E*)-2-hexenol and 1-hexanol showed the strongest activity. Thus, we suggest that the two C_6-alcohols and maybe (*Z*)-3-hexenyl acetate might play a role as foraging or sexual kairomone in the OBB. Furthermore, our results suggest that females have stronger need than males to find a proper place for mating and to locate a suitable host for its ovipostion.

In prior investigations has been demonstrated that GLVs can function as host attractants, pheromone synergists, or sexual kairomones for a number of coleopteran herbivorous. These results could help to shed light on the chemical ecology and behavior of *C. florentinus*. For example, it has been outlined that GLVs in combination with toluquinone or a female-produced volatile compound act as sex attractant, mediating the mate finding of males of *Melolontha melolontha* and *M. hippocastani* (Ruther, et al., 2000; Reinecke, et al., 2002a). Electrophysiological experiments using typical GLVs showed that some of these compounds elicited electrophysiological responses in male antennae of *M. melolontha*. Interestingly and with regard to this

work, green leaf alcohols, such as (*Z*)-3-hexen-1-ol, (*E*)-2-hexen-1-ol and 1-hexanol attracted individuals of *M. melolontha*, whereas the corresponding aldehydes and acetates were behaviorally inactive. This discovery was called alcoholism in cockchafers (Reinecke et al., 2002b). As mentioned above in the OBB the strongest electrophysiological and behavioral responses were also provoked by C_6-alcohols. Moreover, Rodriguez-Saona and coworkers (2006) have shown that adult virgin female *A. planipennis* were attracted to volatiles from damaged host plants (*Fraxinus mandshurica*) in olfactometer bioassays, while males did not respond significantly to the same volatiles. In contrast, several of the identified compounds from ash foliage (inclusive GLVs) provoked strong GC-EAD responses also in males (De Groot, et al., 2008). These results are in agreement with the findings we have made in *C. florentinus.* It is hypothesized that females may use induced volatiles in long-range host finding, while their role for males is unclear. If attraction of females to these volatiles in an olfactometer is confirmed in field experiments, host plant volatiles may find practical application in detection and monitoring of OBB populations.

In field experiments different types of traps with three lure treatments and a blank (unbaited) control were placed in a *Coroebus spp.* infested cork tree forest (see Chapter 2). The following lures were tested: a commercially available bark beetle attractant (Pherotech), a mixture of nonanal, decanal and geranylacetone detected in male and female OBB volatiles and, a mixture of the five GLVs detected in *Q. suber* volatiles and active in behavioral and electrophysiological bioasssays. Surprisingly, not a single individual of *C. florentinus* was captured. In contrast a remarkably high number of *C. undatus* females, its sister species; was trapped with host plant volatile lures and the 3-component blend. Perhaps the position and height of the traps affected the catches. They were placed at 1.5-2.0 m above the ground to cover the infested trunks from where *C. undatus* adults emerge. In contrast, OBB adults generally emerge from the branches, located much higher than 2.0 m. Thus, in future experiments traps with the same attractants should have to be hanged in the crown. In a similar manner, significantly more EAB individuals were caught on traps placed in the mid-canopy of ash trees (13 m) in comparison to those placed at ground level (Francese, et al., 2008).

In summary, although apparently virgin adults of both sexes of *C. florentinus* emit the same volatile organic compounds, males are significantly attracted to conspecific live females and body extracts, but not to males. This may imply that a possible sex pheromone produced by females is involved. On the other hand, the blend of nonanal, decanal and geranylacetone, which are electrophysiologically active and attract individuals of both sexes in olfactometer bioassays, may be part of a possible

aggregation pheromone. In addition, GLVs and other host plant odors induce strong electrophysiological and behavioral responses in females, which may indicate that these compounds are involved in a possible foraging andor oviposition kairomone.

CHAPTER 2

Analytical studies for characterization of pheromones in *Coroebus undatus* (Fabricius) (Coleoptera: Buprestidae). Field tests

2.1 PREFACE

Wood boring and bark beetles are amongst the most significant forest pests worldwide and represent a major threat to the survival of trees and whole ecosystems biosecurity (Rudinsky, 1962; Lieutier, et al., 2004; Brockerhoff, et al., 2006b). This group of beetles comprises several taxa; one of these are the jewel beetles (Buprestidae) (Brockerhoff, et al., 2006a).

The oak flathead borer, *Coroebus undatus* (Fabricius) (Coleoptera: Buprestidae) (later referred to as OFB) represents one of the most hazardous insect pests of the cork oak, *Quercus suber*, an endemic tree of the occidental Mediterranean (Soria, et al., 1992; 1994; Suñer and Abós, 1994). The cork oak is the only producer of cork and therefore of enormous importance for numerous industries. Cork has a variety of applications, such as sealing, isolation materials, but most prominently in the wine stopper production (Chapter 1, Introduction).

Adult individuals of the OFB are characterized by a 10-16 mm long elliptic body of green-brownish color with several transversal green-metallic zig-zag bands in the posterior part and visual differences between sexes are spare (Cobos, 1986; Romanyk and Cadahia, 1992). In contrast to its sister species *Coroebus florentinus*, females lay eggs individually or in small groups in bark flaps and fissures of the trunk from June to July (Evans, et al., 2007). Ten to 20 days later the hatched larvae perforate successive layers of the cork, penetrate into the bark and construct large galleries around the trunk (1.8 m long and 3-4 mm wide) (Fig. 2.1) (Romanyk and Cadahia, 1992). The galleries are filled with larvae frass that downgrade the quality (Gonzalez-Adrados, et al., 2000) and incapacitate cork for wine stopper production, so that utility of cork is reduced for trituration only. Furthermore, *C. undatus* infestation provokes yellow patches and decay in the proximity of the galleries that last in the inner cork (Bernal and Cardillo, 2004). Although the OFB does not usually kill the host, the described wounding in the cork provoked by larval feeding and frass leads to important economic losses in the amount and quality of the cork harvested. For example, the value of the cork from an infested tree can be reduced 10 fold. In Spain, it was recorded that the production of

Fig. 2.1. Galleries on the trunk of *Q. suber* provoked by *C. undatus* larvae.

good quality cork caused by *C. undatus* infestation decreased from 80 to 30 % of its total production over the last years. Furthermore, recent data showed that only in Catalonia, where around 3.500 ha of cork oak are harvested every year, the economic loss is around 4.320.000 €/year (personal communication). In Andalucia in turn, this value is estimated to reach 8.500.000 €/year, whereas the region of Extremadura suffers losses of around 5.000.000 € annually due to the induced OFB damage. Thus, there is a strong economic interest in controlling this severe pest, and to protect cork oak trees from subsequent infestations.

From other wood boring and bark beetles it is known that early detection of new establishments is critical for an effective implementation of pest management and eradication efforts (Myers, et al., 2000). Thus, it is important to maintain surveillance programs that involve inspections of forests and trees (Carter, 1989). Additionally, special designed traps baited with specific attractants, like host volatiles and sex or aggregation pheromones suitable for a range of high-risk pest beetles, such as Scolytinae and Cerambycidae, are used for monitoring and in some cases mass trapping (Brockerhoff, et al., 2006a). Also, in the buprestid, *Agrilus planipennis* (emerald ash borer, EAB) this approach has been applied (Francese, et al., 2005, 2008; Crook, et al., 2008c; Grant, et al., 2010).

Generally, time of development of larvae and pupae of the OFB takes two years, although in some cases individuals may complete their life cycle in one year (Romanyk and Cadahia, 1992). Because of their long life cycle, their low density populations and the natural protection offered by the bark, no effective control treatments have been established so far. In addition, no natural enemies have been described until now (Kenis and Hilszczanski, 2007). Furthermore, the application of chemical control measures is constrained due to the risk of cork contamination with unknown health consequences for the consumer, and the vulnerability period is restricted to the moment of emergence, mating or oviposition.

The present work is the first study on the biology of *C. undatus* with special emphasis on its chemical communication. To capture live individuals at the moment of emergence, traps of transparent mosquito net covering the trunks have been designed. Live adult beetles were immediately transferred to the lab and their volatile emission, behavior and morphology of antennae (SEM) were analyzed. Moreover, a first attempt to set up an integrative pest management program for the OFB has been started by attracting adults in the field with the objective to accomplish a pilot surveillance approach. The aim of this project is to maximize trap catches for monitoring and/or

mass trapping. For this purpose, the effectiveness of different trap parameters (shape, positioning, semiochemical lures) for capturing OFB beetles were tested. Optimization of these parameters has demonstrated considerable effects on the attraction of the EAB (Francese, et al., 2005, 2008; Crook, et al., 2008c; Lelito, et al., 2008).

2.2 METHODOLOGY

2.2.1 Chemicals

The reference compounds used in trapping experiments in the field to capture *C. undatus* adults, that is nonanal (95%), decanal (99%), (*E*)-2-hexenal (98%), (*E*)-2-hexenol (96%), 1-hexanol (98%), (*Z*)-3-hexenyl acetate (98%) and hexyl acetate (99%) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Geranylacetone (96%, containing 40% of nerylacetone) was obtained from TCI Europe (Zwijndrecht, Belgium). Absolute ethanol (99.5% by GC) (Panreac, Barcelona, Spain) was used as solvent for the lures.

2.2.2 Insects

Since rearing of the insect in the laboratory proved to be unsuccessful in spite of different semi-synthetic artificial diets used, specimens of living adults of the OFB were collected directly from the woods of Santa Coloma de Farners (41°51′53″N 2°39′51″E, Girona province, Spain). To this aim, 90 *Q. suber* trees with signs of severe infestations, such as fresh exudation and dryness, were selected and traps of transparent mosquito net were placed around the trunks to catch recently emerged young adults (Fig. 2.2).

2.2.3 Trap design and fixation – Capture of live adults

The cylindrically shaped drapery covered the whole perimeter of the trunk within the height of 90 cm. Foam bands (10 cm wide) at the upper and lower end of the net were fixed to the trunks with wire to avoid escape of the emerged beetles.

However, traps were not completely tightened to the trunks in order to accommodate the insects' movement and to facilitate their collection. Traps were checked every week for emerged adults, collected and transferred immediately to the laboratory for subsequent analyses.

Fig. 2.2. Transparent mosquito net traps to capture *C. undatus* adults after emergence. By courtesy of J. M. Riba.

A beneficial side effect of this trapping was to gain a better knowledge about the emergence time and flight period of the OFB. In the laboratory adult beetles were kept separately by sex in squared glass containers (15×15 cm^2) with a removable plastic lid with wire gauze on the top at 26±2ºC, 50±10% RH and 14:10 L:D photoperiod. Due to the fact that size and shape of males and females are nearly identical, individuals were maintained separated for post-mortem analyses of the genitalia for determination of the sex. Therefore, the abdomen was opened with precision forceps to confirm the presence or not of a male aedago. Fresh *Q. suber* leaves (cork oak foliage) were provided every two days to feed the insects.

2.2.4 SPME extraction and GC-MS analysis

Male and female OFB adults (N=3 each) were separated from the rest of insects and food-deprived for 24 h. For extraction of cuticle hydrocarbons, the abdomen of test individuals were rubbed with the fiber of different SPME for a ca. 30 s. Fibers of different polarities, PDMS (polydimethylsiloxane, 100 µm), Carbowax (70 µm) and Carboxen (50 µm) were purchased from Supelco (Bellefonte, PA, USA). For GC-MS analysis the fiber was inserted into the injection port of a Thermo Finnigan Trace 2000 GC system coupled to a Trace MS quadropule mass spectrometer (ThermoFisher Scientific, Madrid, Spain) for 5 min. Helium (1 ml min^{-1}) was the carrier gas and the column used was a 30 m×0.25 mm ID×0.25 µm HP-5MS (5% phenylmethylsiloxane) (Agilent Technologies, Madrid, Spain) under the following chromatographic conditions: injection in splitless mode (5 min) at 40ºC, held for 5 min and program of 10ºC min^{-1} to 280ºC which was maintained for a further 10 min. The column effluent was ionized by electron impact (EI) at 70 eV at a source temperature of 200ºC. Mass range was from 40 to 500 *m/z*. To determine the retention indexes (RI) of the compounds emerging from the column, 1 µl of a 100 ng $µl^{-1}$ C_8-C_{25} hydrocarbon mixture in hexane was also injected in the same conditions. The RI of each compound was calculated according to van den Dool and Kratz (1963). Compounds were identified by comparison of their mass spectra and retention indexes with those of authentic standards and/or from a commercial library (NIST Registry of Mass Spectral Data, v1.7, 1999) or from the database published by Adams (2007).

2.2.5 Scanning electron microscope (SEM)

Isolated antennae of male and female *C. undatus* adults (N=3) were clipped beneath the distal segment near the head, cleaned with gradient concentrations of ethanol and

mounted on specimen stubs with adhesive tape. The antennae were coated under vacuum with a gold solution three times for 1 min. Then, micrographs were taken with a Stereoscan S-360 (Leica) electron microscope at 15 kV (Servicios Científicos Técnicos, University of Barcelona, Spain).

2.2.6 Volatile analysis of Q. suber *branches*

Volatiles of five freshly cut *Q. suber* branches were collected and analyzed as described in Chapter 1 (section 1.2.5). The identified compounds were quantified by comparison of their GC areas relative to that of an internal standard (dodecyl acetate 100 ng μl^{-1}). Percentages are refered to the most abundant compounds.

2.2.7 Field trapping – Trap placement

Trapping experiments, aimed at testing different trap designs, attractants and position of traps were conducted in 2006-2010 in two private cork oak stands in Arbúcies (41°49′4″N 2°31′1″E) and Santa Coloma de Farners (41°51′53″N 2°39′51″E) (Girona province, Spain) (Fig. 2.3). These habitats have had heavy OFB infestations over several years, and therefore appeared to be ideal sites to initiate the OFB surveillance program. At the end of June, prior to adult emergence, the traps, containing the lures and separated 50 m from each other, were distributed at random over the woods.

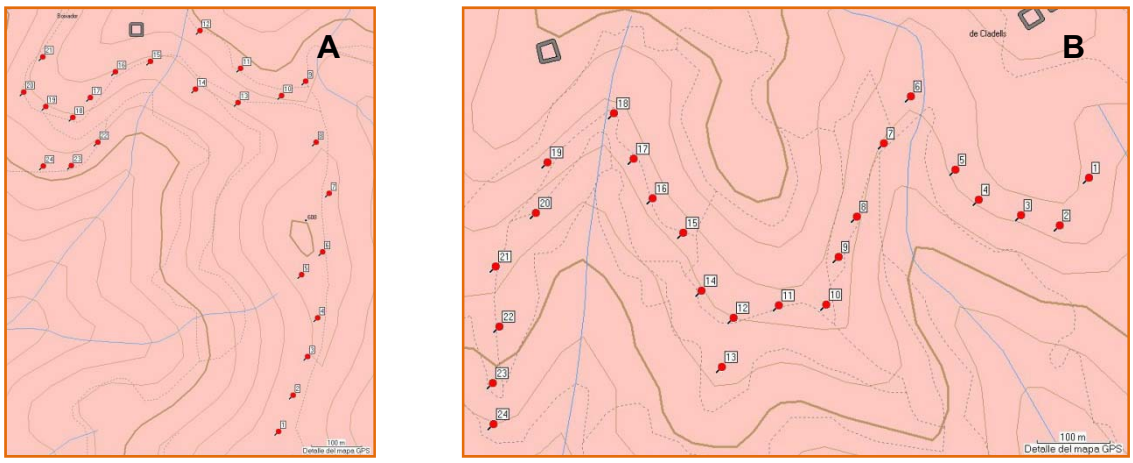


Fig. 2.3. Location of purple-colored traps in both study sites to capture *C. undatus* adults. Twenty four traps separated 50 m each were placed in (A) Arbùcies and (B) Santa Coloma de Farners.

Trap placement was done with the help of Dr. J.M. Riba and personal from Forestal Catalana (Generalitat de Catalunya, Spain) with the expressed permit of the owner of the forest. The traps were hanged in trees located in open talus of the tracks. A total of 48 traps (24 per zone) were placed in the 2010 campaign, the first year of analyzable data and shown in the present work (Table 2.1). Traps were checked weekly and the collected beetles were sent to our laboratory for sexing. For statistical analysis, the number of total catches per trap and mean of captures per trap and revision in both study sites were compared for significance using analysis of variance (*one-way ANOVA*) followed by *DMS post-hoc* tests ($P<0.05$).

2.2.8 Trap color, design and hanging height

The experience of the first years (unpublished data) and recent studies on the EAB (Francese, et al., 2005; Crook, et al., 2009; Crook and Mastro, 2010) led to the conclusion that purple-colored traps were most promising to attract OFB adults. Thus, the three types of traps tested in this work, (see before) were painted in purple (Fig. 2.4).

a) **Eight-unit Lindgren-type funnel trap** (PheroTech, Victoria, BC, Canada) is a trap typically used to catch bark beetles and wood borers (Brockerhoff, et al., 2006a) (Fig. 2.4 A). They were placed in both study sites (N=8 in zone A, N=12 in zone B) hanged from branches at 1.5-2.0 m above the ground. Former experiences had shown that traps placement near the crown failed to capture OFB individuals (personal observation). The attractant lures were clipped in the middle of the funnel and an insecticide pellet (Vaporsa®) was placed at the bottom of the trap to kill the insects caught.

b) **Prism traps**, described by Francese and coworkers (2008), were slightly modified (Fig. 2.4 B) and placed in both study sites (N=8 in zone A, N=12 in zone B). Each trap consisted of one panel of purple-painted corrugated polypropylene (100×53 cm^2; 0.40 cm in thickness), folded crosswise to obtain a 3-faced prism (33×53 cm^2 each side). The surface of every side was coated with insect trapping glue (Tanglefoot) to stick the attracted individuals. The whole trap was attached to a wooden pole using two metallic angle brackets at the height of 1.5-2.0 m from the ground. The lure was placed in the centre of the trap, suspended from the upper

angle bracket and fixed by a plastic clip. Each side of the prism possessed six holes in the middle, arranged linearly from top to bottom, to facilitate lure emission.

c) **Single Panel trap** is a modified model of the Prism trap. They were placed exclusively in zone A (N=8). The trap consisted of a single purple corrugated plastic panel (33×53 cm^2) (Fig. 2.4 C). The surface was coated on both sides with Tanglefoot, as described above, and the panel was also attached to a wooden pole with two metallic angle brackets at 1.5-2.0 m from the ground. Semiochemical lures were clamped between the two metallic angle brackets in front of the panel.

Fig. 2.4. Purple-coloured trap types to capture *C. undatus* adults. (A) Eight-unit Lindgren-type funnel trap; (B) Prism trap and (C) Panel trap coated with Tanglefoot.

2.2.9 Semiochemical lures

In the trapping experiments, white semipermeable flexible tubes (55 cm long × 0.6 cm OD) (Thermo Fisher Scientific, Barnant C-flex, Madrid, Spain) were used as dispensers for semiochemical emission. They were filled with different doses of possible attractants, dissolved in 2.4 ml of absolute ethanol (Panreac, Barcelona, Spain). Lures included: 1) ethanol alone as control (N=12 in zone A; N=8 in zone B); 2) a commercially available general attractant for bark beetles and wood borers (PheroTech, Victoria, BC, Canada) (N=12 in zone A); 3) a three component blend of nonanal, decanal and geranylacetone (10 mg each), as volatiles produced by adult males and females of *C. florentinus* that have demonstrated activity in behavioral and electrophysiological tests (N=8 in zone B); 4) a synthetic mixture of the five most abundant green leaf volatiles (GLVs) identified from volatiles of *Q. suber* branches (N=8 in zone B). These compounds are (*E*)-2-hexenal (93 mg), (*E*)-2-hexenol (100 mg), 1-hexanol (51 mg), (Z)-3-hexenyl acetate (66 mg) and *n*-hexyl acetate (11 mg).

The quantity of each compound corresponded to the natural occurrence in the headspace volatiles relative to the major component (*E*)-2-hexenol (Fig. 2.8). All lures were stored in a freezer until used and replaced every 3 weeks.

Table 2.1 Details of Field trapping experiments (trap types and attractants)

Study sites[a]	Attractant / Trap type	Control	Pherotech[b]	3-component blend[c]	GLV mixture[d]	Σ
Zone A	Lindgren	4	4			8
	Prism	4	4			8
	Single Panel	4	4			8
Zone B	Lindgren	4		4	4	12
	Prism	4		4	4	12
	Σ	20	12	8	8	

[a] Zone A: Arbúcies; zone B: Santa Coloma de Farners
[b] Commercially available attractant for wood-boring and bark beetles.
[c] Three component blend of synthetic compounds (nonanal, decanal and geranylacetone).
[d] GLV mixture consisting of 5 compounds: (*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, (Z)-3-hexenyl acetate and *n*-hexyl acetate.

2.3 RESULTS

2.3.1 Analytical studies of abdominal volatiles of C. undatus

From mid-June to the end of July only 12 live adults were collected from 90 transparent mosquito net traps in Santa Coloma de Farners. Among the adults, we identified 4 males and 8 females. By SPME rubbing of the abdomen and subsequent GC-MS analysis we have identified a total of 18 female and 16 male saturated linear or branched hydrocarbons (Fig. 2.5, Table 2.2).

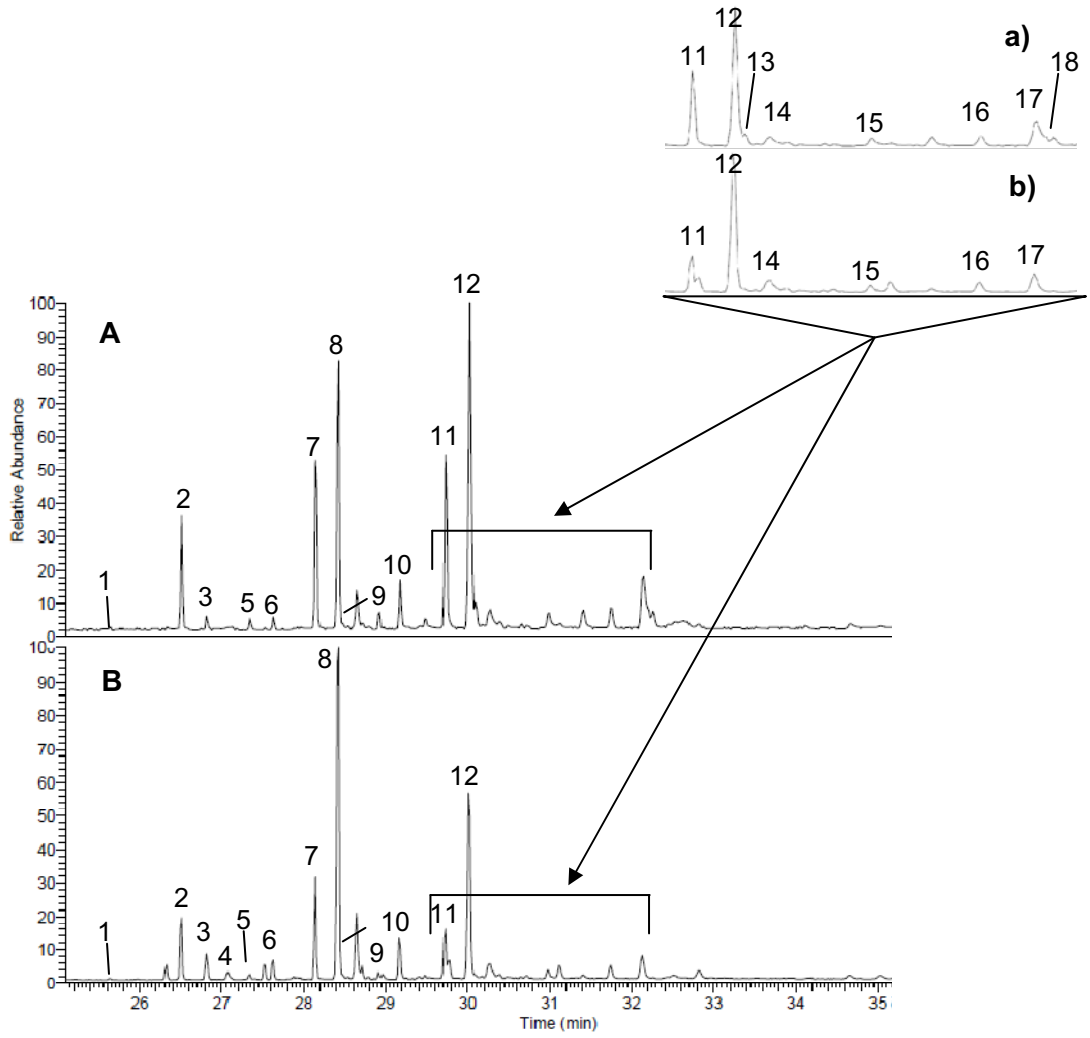


Fig. 2.5. Representative GC-MS profiles of 10-14-day old (A) female and (B) male *C. undatus* abdominal extracts, obtained by rubbing the abdomen with the tip of a SPME absorption fiber (Carbowax, 70 µm). Numbers on peaks (1-18) refer to identified compounds listed in Table 2.2.

The detected and in part identified hydrocarbons (Table 2.2) are long-chained (>C_{20}) and the branched compounds possess one or two methyl groups. The most abundant hydrocarbons in males and females are 13-methylpentacosane (entry 8) and 11,14-dimethylhexacosane (entry 12) followed by the saturated linear hydrocarbons n-pentacosane (entry 7) and n-heptacosane (entry 11). Two of the identified tentatively dimethyl-branched hydrocarbons, 7,20-diMe-C_{26} and 7,22-diMe-C_{28} (entries 13 and 18) are only present in female extracts, although they were detected only in very small amounts. Besides, two more unknown branched hydrocarbons (entries 14 and 15) were found, still to identify. No further significant qualitative differences in abdominal hydrocarbon composition were observed between sexes.

Table 2.2 Detected hydrocarbons (HC) in abdominal SPME extracts of males and females of *C. undatus*

Entry	Compound	Number of carbons	Sex[a]	M_r[b]	RT[c]
1	docosane	22	M/FEM	310	25.63
2	tricosane	23	M/FEM	324	26.51
3	11-methyltricosane	24	M/FEM	338	26.81
4	9,12-dimethyldocosane	24	M/FEM	338	27.06
5	tetracosane	24	M/FEM	338	27.33
6	10,13-dimethyltricosane	25	M/FEM	352	27.61
7	pentacosane	25	M/FEM	352	28.14
8	13-methylpentacosane	26	M/FEM	366	28.41
9	hexacosane	26	M/FEM	366	28.90
10	13-methylhexacosane	27	M/FEM	380	29.16
11	heptacosane	27	M/FEM	380	29.73
12	11,14-dimethylhexacosane	28	M/FEM	394	30.01
13	7,20-dimethylhexacosane (proposal)	28	FEM	394	30.09
14	unknown branched HC	28	M/FEM	394	30.26
15	unknown branched HC	28	M/FEM	394	30.98
16	nonacosane	29	M/FEM	408	31.73
17	13-methylnonacosane	30	M/FEM	422	32.11
18	7,22-dimethyloctacosane (proposal)	30	FEM	422	32.24

[a] Sex of *C. undatus* adults in which compounds were detected: M = males; FEM = females.
[b] Relative molecular mass
[c] On a HP-5MS (30 m×0.25 mm I.D., 0.25 µm) fused silica capillary column.

2.3.2 SEM studies of adult antennae

The morphology of antennae and the typology and distribution of antennal sensilla of the OFB was studied using scanning electron microscopy (SEM). Male and female antennae consist of the scape, pedicel and 9 flagellomeres (Figs. 2.6 A, E). They are situated between the compound eyes and curve down ventrally. No sexual dimorphism in form and structure was observed. Length of the generally widened flagellomeres decreased slightly towards the tip, while the scape and pedicel are nearly round in their cross section. In both sexes four types of sensilla were distinguished: *uniporous sensilla* (gustatory and taste), *sensilla chaetica* (mechanoreceptors) and two types of *sensilla basiconica* (olfactory).

On the distal surface of the nine flagellomeres were oval-shaped craters, where *uniporous sensilla* were inserted as described in Chapter 1 (Figs. 2.6 B, C, F-H). These sensilla are short sided pegs ca. 10 µm long and arranged in groups of more than 100 sensilla.

S.chaetica are relatively long, in contrast to the other types, and they are located on every segment of the entire antennae, including scape and pedicel (Figs. 2.6 B, F, G). These sensilla are usually ca. 80 µm long, ranging between 50 and 100 µm, and were found in more or less equal numbers on both male and female antennae.

The multiporous *s. basiconica* are found along the edge of the depressions (type 1) (Figs. 2.6 D, G) and within the crater (type 2) (Figs. 2.6 C, H) round the *uniporous sensilla*. Males dispose of more *s. basiconica* type 1 than females, whereas the number of *s. basiconica* type 2 is nearly identical in both sexes. *S. basiconica* type 1 are ca. 20 µm (range 17-23 µm) long, whereas type 2 is a little bit shorter (ca. 15 µm).

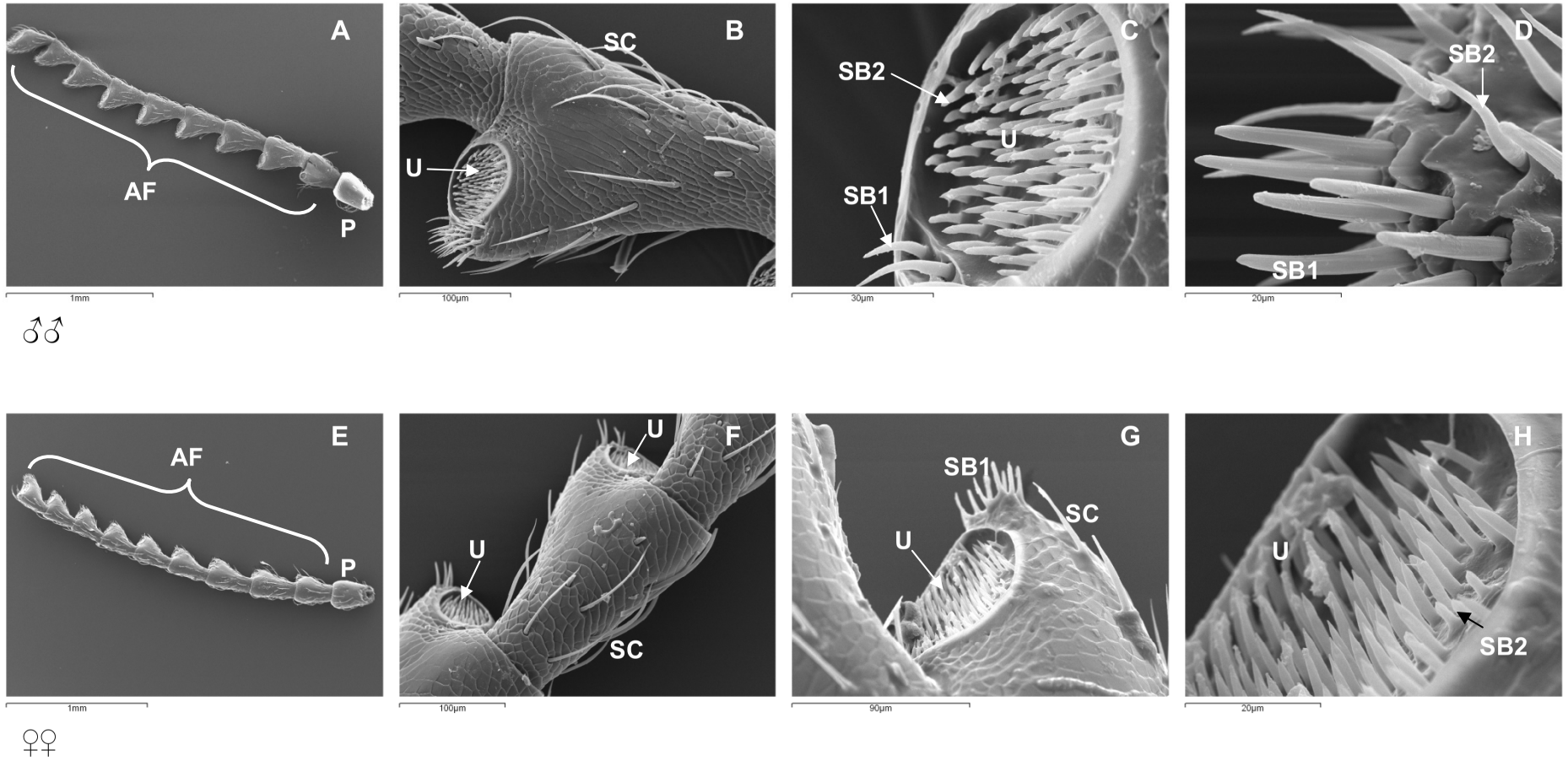


Fig. 2.6. Scanning electron micrographs (SEM) of (A) male and (E) female antennae of *C. undatus* adults (AF, antennal flagellum; P, pedicel). (B, F) Middle flagellomeres with apical depressions (crater) filled with numerous *uniporous sensilla* (U) and *s. chaetica* (SC) distributed around the flagellomeres. (C, G, H) Apical depression mainly filled with *uniporous sensilla* (U) and *s. basiconica* (SB1+SB2) within and around the crater. (D) Distal antennal flagellomer of a male antenna with numerous *s. basiconica* of types SB1 and SB2.

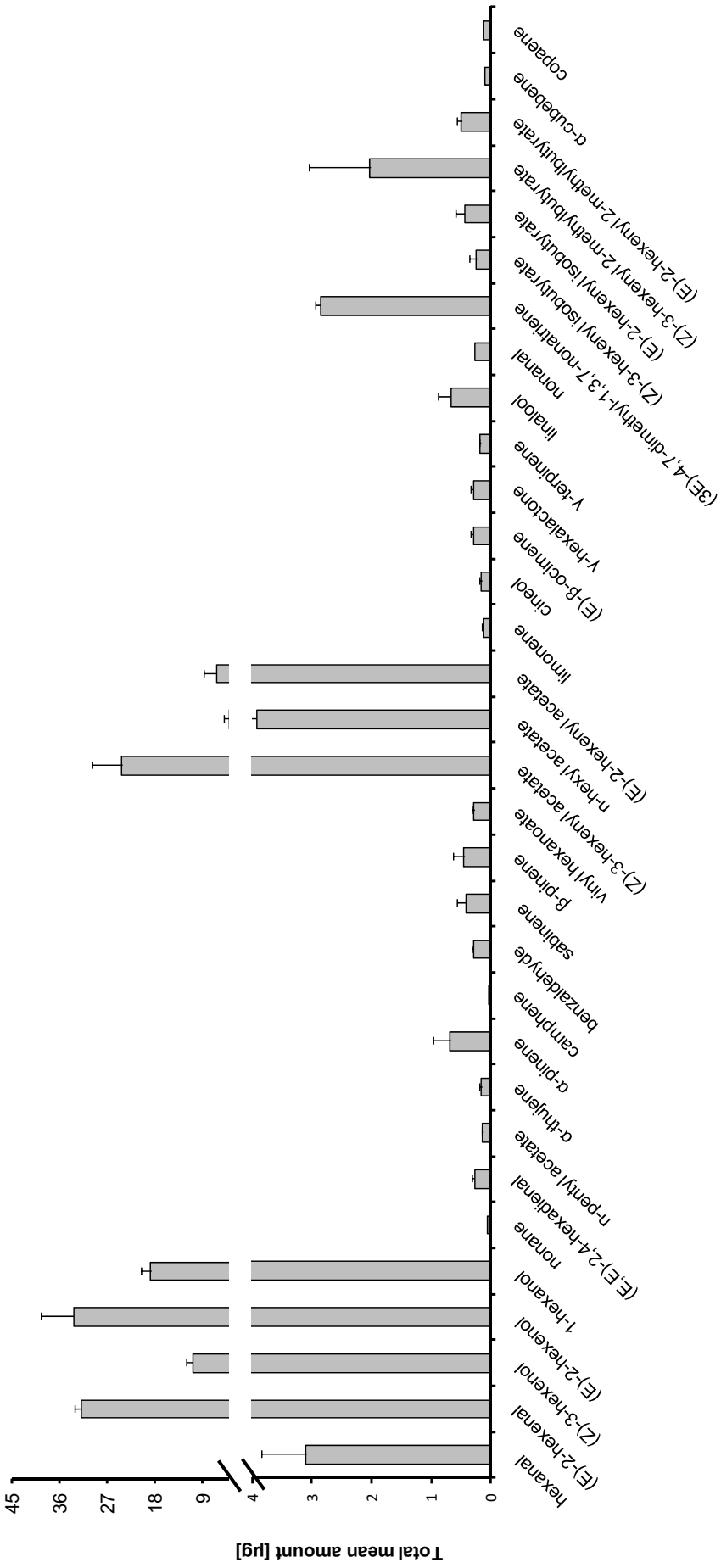


Fig. 2.7. Total mean amount (±S.E.M.) of 32 compounds identified from headspace volatiles emitted by freshly cut *Q. suber* branches collected during 24 h (N=2).

2.3.3 Identification and quantification of host plant volatiles of Q. suber

In the headspace samples of freshly cut *Q. suber* branches a total of 32 compounds were identified, in agreement with the results described in Chapter 1 (Table 1.3). The major group of volatiles was composed by GLVs that also represented the compounds with the highest amounts, followed by monoterpenes.

Quantification of *Q. suber* volatiles can be found in Figure 2.7. The highest quantities emitted during 24 h by 5-6 branches with a fresh weight of 36.5 ± 2.12 g correspond to the aldehyde (*E*)-2-hexenal [31.90 µg], the alcohols (*E*)-2-hexenol [33.34 µg] and 1-hexanol [18.77 µg] and the acetates (*Z*)-3-hexenyl acetate [24.22 µg] and *n*-hexyl acetate [3.93 µg]. Percentages of the natural composition of these five compounds in relationship to the most abundant compound (*E*)-2-hexenol are represented in Figure 2.8.

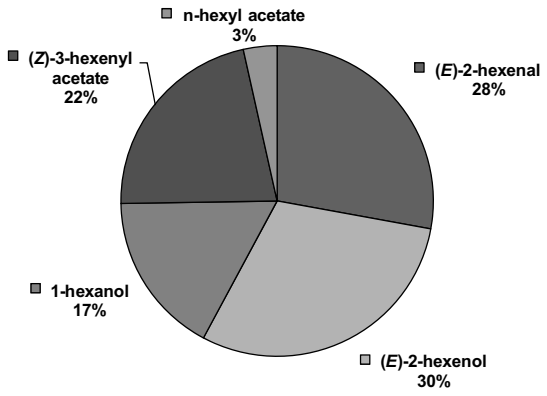


Fig. 2.8. Percentage of the five most abundant GLVs emitted by freshly cut *Q. suber* branches collected during 24 h by the dynamic headspace method.

2.3.4 Field trapping of C. undatus – Study sites

During the years 2006-2009 the first attempts were made to know the biology, emergence and especially the flight period of OFB adults by field collections and direct observations. In addition, communications and citations of museum collections were consulted. The results indicated that the main flight period of this beetle starts in the first week of July lasting 2-3 weeks. Therefore, in 2010 trapping experiments in two cork oak stands in Catalonia (Spain) with different types of purple-colored traps and

three kinds of semiochemical lures were designed to cover the lifetime of adult individuals from emergence to mating.

In the course of the OFB surveillance campaign a total of 228 adults of *C. undatus* were captured in 48 traps located in the zones A (Arbúcies), and B (Santa Coloma de Farners) (24 each). In comparison to preceding years, the total number of trapped insects and the number of insects per trap increased significantly more than 3x compared to the most successful year (2008) (Table 2.3).

Table 2.3 *C. undatus* adults captured per trap during the period 2006-2010

Year	2006	2007	2008	2009	2010
Captured *C. undatus*	2	6	61	20	228
Number of traps	6	6	40	40	48
Insects/trap	**0.3**	**1.0**	**1.5**	**0.5**	**4.8**

Examination of traps started on July 6th. The flight period of adult insects resulted to be different in both sites (Fig. 2.9). Thus, whereas in Santa Coloma de Farners most insects were captured on the first day of inspection (45 individuals), in Arbúcies the curve of insects captured reached its maximum (29 individuals) one week later (Fig. 2.9). In Arbúcies a total of 89 individuals were captured and 139 in Santa Coloma de Farners (total 228 adults).

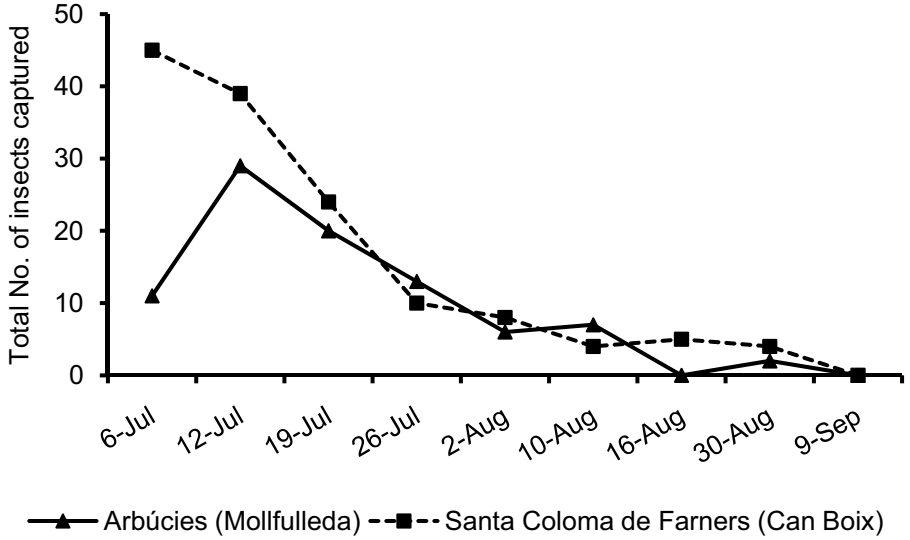


Fig. 2.9. Total number of *C. undatus* adults captured per day of revision in both trapping zones (A and B) from July to September.

2.3.5 Trap design

In both sites 3 different types of purple-colored traps were tested. In zone A (Arbúcies) were placed 8 traps of the types Lindgren, Prisma and Single Panel, whereas in zone B (Santa Coloma de Farners) only Lindgren and Prism traps (12 each) were installed. The total numbers of insects caught in both sites were 6 in Lindgren, 50 in Prism and 33 in Panel for zone A and 18 in Lindgren and 121 in Prism for zone B (Fig. 2.10). In both zones, therefore, more insects were attracted to Prism traps (zone A: 50 ind.; zone B: 121 ind.) than to the eight-unit funnel traps (Lindgren). Single Panel traps used, exclusively in zone A, caught less insects than Prism traps but more than Lindgren traps.

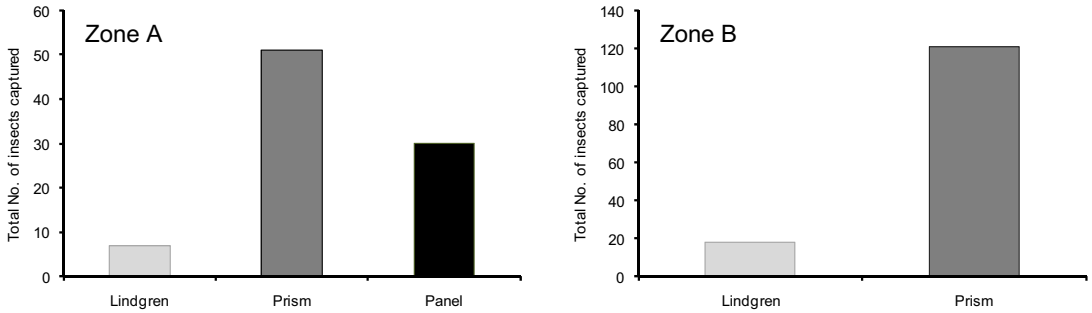


Fig. 2.10. Total number of *C. undatus* adults captured with Lindgren, Prism and Single Panel traps in Arbùcies (zone A) and Santa Coloma de Farners (zone B).

These results can be seen in more detail by examining the total number of catches with the 3 trap types per revision day (Figs. 2.11; 2.12). In Arbúcies most insects were captured with Prism traps during the first 4 weeks of inspection (6^{th}-26^{th} July), while the last 4 weeks (2^{nd} to 30^{th} August) no differences were observed among traps (Fig. 2.12). In turn, in Santa Coloma de Farners during the complete study more individuals were attracted to Prism traps than to Lindgren traps (Fig. 2.12).

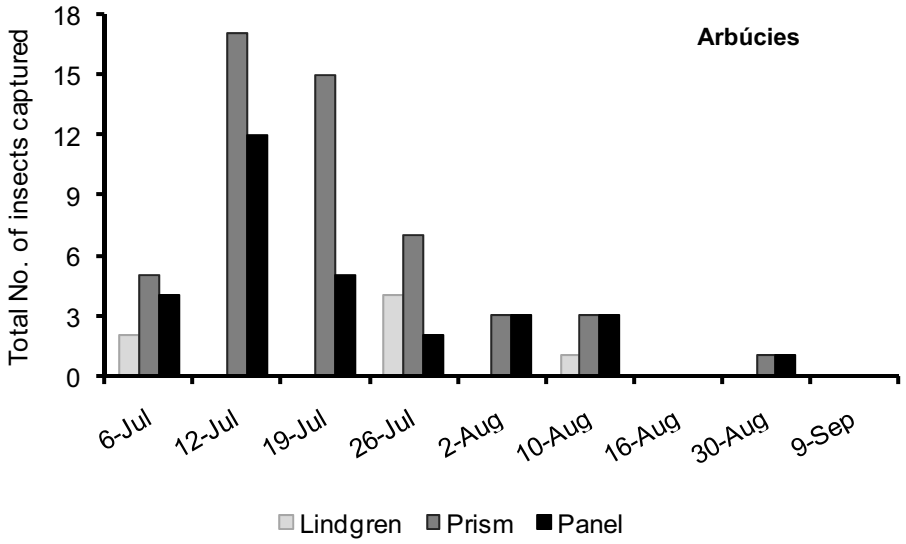


Fig. 2.11. Total number of *C. undatus* adults captured per revision day with Lindgren, Prism and Single Panel traps in Arbùcies (zone A).

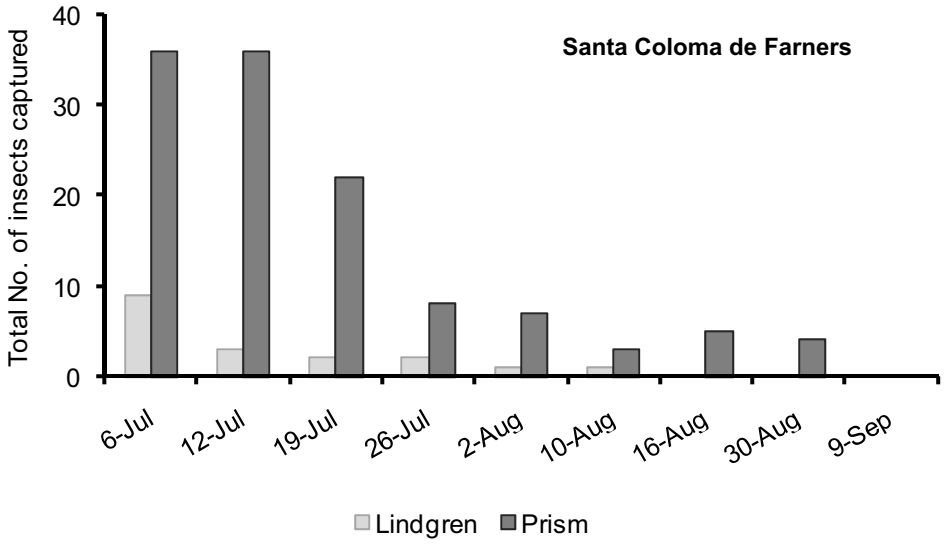


Fig. 2.12. Total number of *C. undatus* adults captured per revision day with Lindgren and Prism traps in Santa Coloma de Farners (zone B).

Statistical analysis of the mean of total captures per trap type (I) and the mean of insects per trap type and per revision day (II) showed that

(I) significant differences are observed among the catches per trap using the three trap types by the *DMS post-hoc test* in the following order: Prism traps (8.55 ± 0.84

ind/trap) > Single Panel traps (4.13 ± 1.34 ind/trap) > Lindgren traps (1.20 ± 0.22 ind/trap) (Fig. 2.13).

(II) on the first two revision days (6th and 12th July) and on the last one (30th August) significantly more OFB individuals were attracted to Prism traps compared to the other two types by the *DMS post-hoc test*. On the other days clear preferences towards the Prism traps are shown, although not statistically significant (Fig. 2.14).

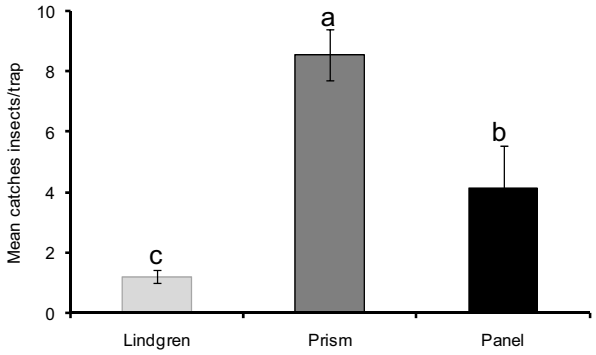


Fig. 2.13. Mean of total captches (±S.E.M.) per trap of *C. undatus* adults in both study sites using Lindgren, Prism and Single Panel traps. Bars with different letters indicate significant differences among treatments (*DMS post-hoc test*, *P ≤ 0.05*).

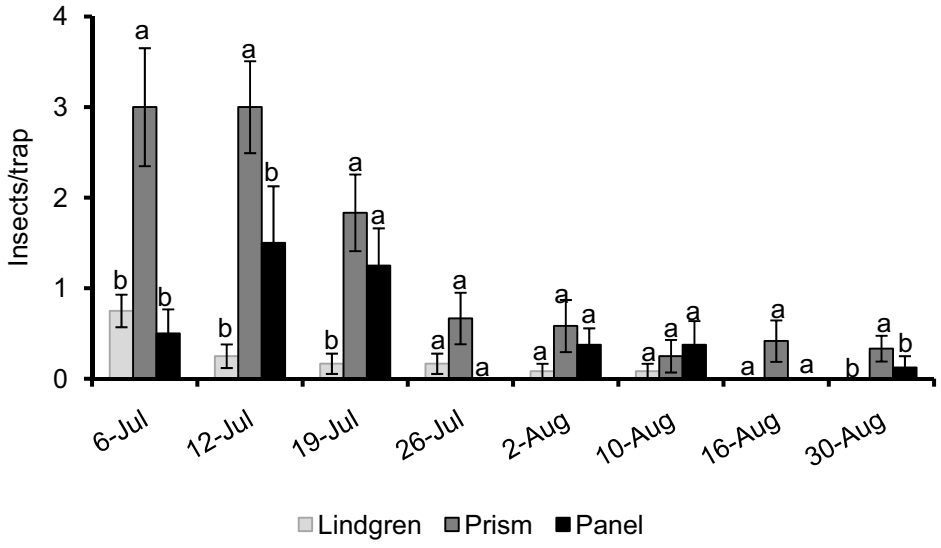


Fig. 2.14. Mean of catches (±S.E.M.) per trap and revision day of *C. undatus* adults in both study sites using Lindgren, Prism and Single Panel traps from July to September. Bars with different letters indicate significant differences among treatments (*DMS post-hoc test*, *P ≤ 0.05*).

2.3.6 Semiochemical lures

In zone A a total of 40 insects were caught with Pherotech lures and 49 with ethanol (Fig. 2.15). In zone B, 52 insects were caught with the GLV mixture, 47 with the 3-component blend and 40 with ethanol (Fig. 2.15). There were no significant differences in the number of trapped insects with Pherotech (attractant) or ethanol (control) in a time-span of 95 days (Fig. 2.16).

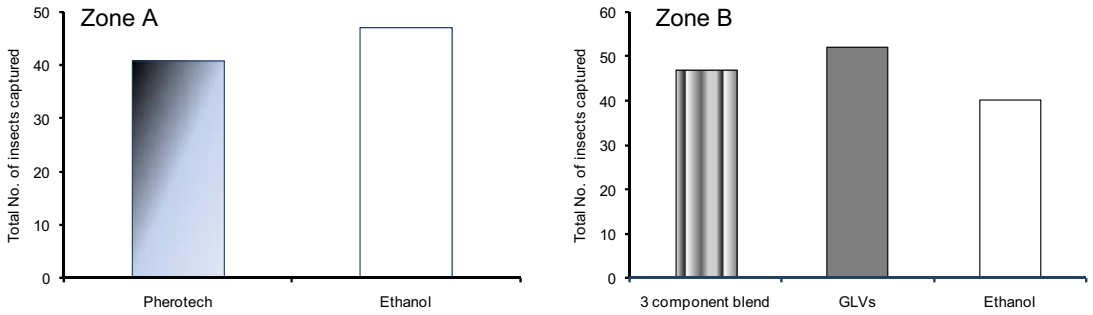


Fig. 2.15. Total number of *C. undatus* adults captured with traps baited with Pherotech lures, 3-component blend and mixture of GLVs as attractants and ethanol as control in Arbúcies (zone A) and Santa Coloma de Farners (zone B).

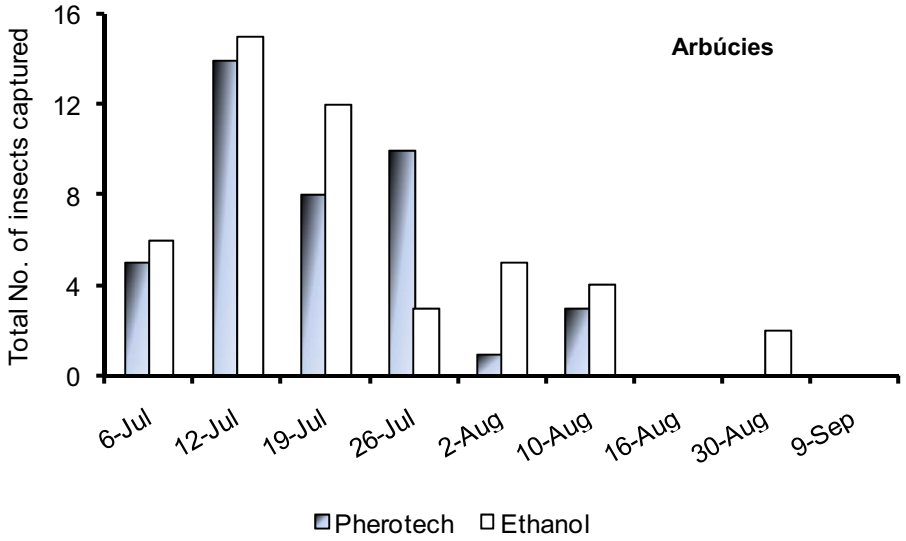


Fig. 2.16. Total number of *C. undatus* adults captured per revision day with Pherotech lures vs ethanol as control in Arbúcies.

In zone B (Santa Coloma de Farners) three different semiochemical lures were applied to Lindgren and Single Panel traps: the 3-component blend nonanal, decanal and geranylacetone, the mixture of the 5 GLVs (*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, (Z)-3-hexenyl acetate and *n*-hexyl acetate and ethanol as control. Except the first revision, in which more insects were caught by the 3-component blend and the GLV mixture, the remaining revisions did not show any preference for any of the semiochemicals tested (Fig. 2.17).

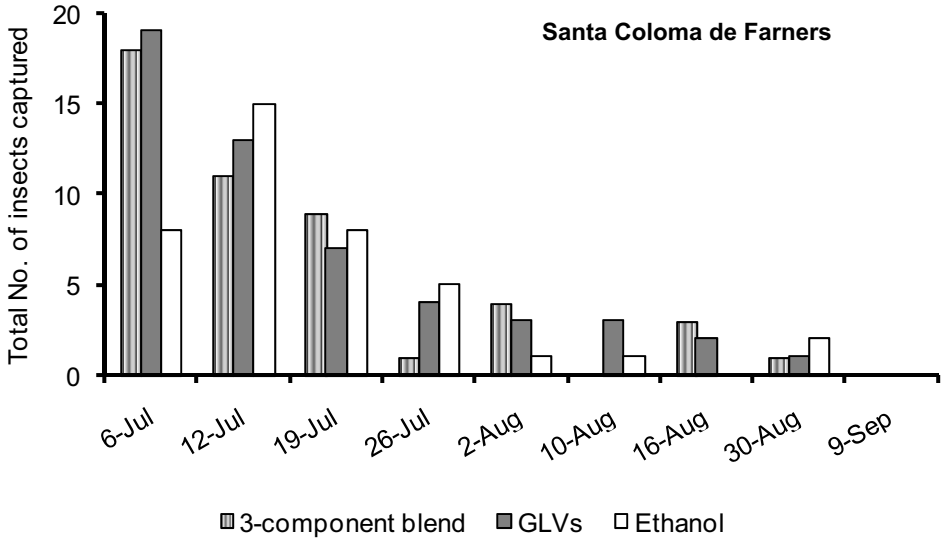


Fig. 2.17. Total number of *C. undatus* adults captured per revision day testing three attractants: 3-component blend, the mixture of the five most abundant GLVs of *Q. suber* and ethanol as control in Santa Coloma de Farners.

For statistical purposes, data of the two zones were combined to get the mean of total captures per trap type (only Lindgren and Prism traps) and attractant (3-component blend, GLVs, ethanol, Pherotech). The mean of captures was analyzed comparing the attractant effect of the semiochemical lures and ethanol alone with the effect of trap design using *DMS post-hoc test* (Fig. 2.18).

Significantly more insects per traps were captured with Prism traps. Between the semiochemicals and ethanol in Lindgren traps no differences were observed. In turn, with Prism traps the 3-component blend (10.25 ± 2.36 ind/trap) and the GLV mixture (11.50 ± 1.04 ind/trap) significantly caught more OFB individuals than Pherotech lures (7.25 ± 1.03 ind/trap) and ethanol (6.88 ± 1.32 ind/trap)

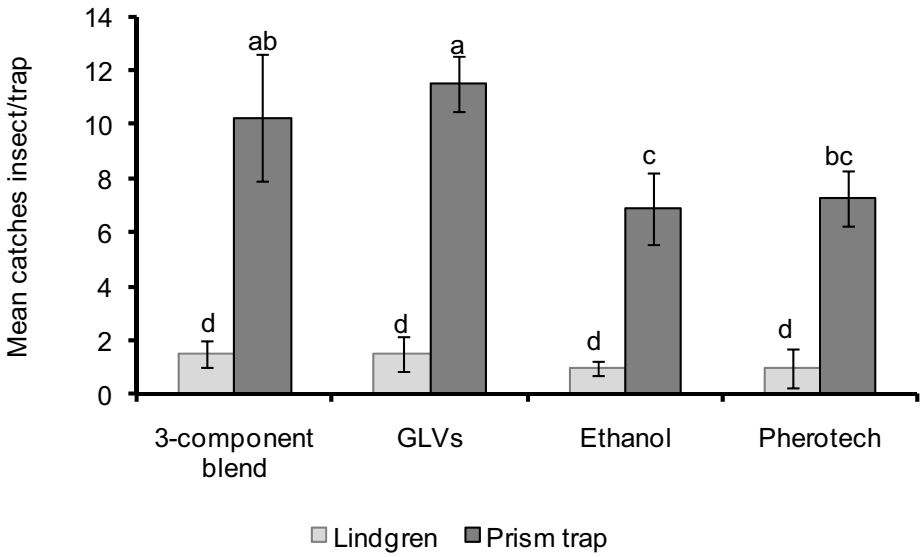


Fig. 2.18. Mean of total catches (±S.E.M.) per trap of *C. undatus* adults in both experimented sites with Lindgren and Prism traps combined with lures charged with 3-component blend, mixture of five GLVs, ethanol as control and Pherotech lures. Bars with different letters indicate significant differences between the treatments (*DMS post-hoc* test, $P \leq 0.05$).

2.4 DISCUSSION

The oak flat-head borer (OFB), *C. undatus* is considered the primary pest of the cork oak, *Q. suber* in the Mediterranean region. The economic damage provoked by larvae with respect to the cork production is very high. However, to date little is known about the biology, behavior and chemistry of this pest. An important factor which could explain the lack of knowledge about this insect derives from its difficult of disposing of live adults. Thus, even in a heavily infested cork oak stand (Santa Coloma de Farners, Girona, Spain) with an extensive trapping scheme using 90 transparent mosquito net traps, only 12 live individuals could be collected during 45 days. These results confirm personal observations of prior years that, in general, cork oak trees are infested by a maximum of 1-3 larvae. In addition, it is not clear yet, whether their life cycle takes one, two or more years (Romanyk and Cadahia, 1992). Therefore, we cannot exclude the possibility that from a tree attacked by several larvae only one adult emerges per year. Another explanation for low capture rates could be that more frequent revisions than once a week are needed to exclude that the insects escape from the traps by biting holes in the mosquito net. Additionally, the selection of oak trees might not have been ideal. We cannot exclude that despite visible infestation signs, the trees had low infestation rates for our trapping experiments. Nevertheless, since rearing of the insect in the laboratory proved to be unsuccessful in spite of different semi-synthetic artificial diets used in several attempts at the University of Lleida and Sevilla, availableness of OFB adults was restricted to these field catches. Despite the low sample volume of trapped adults, we were able to conduct a thorough analysis of the abdominal volatiles (cuticle hydrocarbons) and obtained preliminary results on the antennal morphology and structure.

The GC-MS analysis of abdominal volatiles extracted by SPME provided evidence that most components of the detected and in part identified hydrocarbon blend (18 compounds with a chain-length ≥ C_{20}) are similar in both sexes, except two so far unknown dimethyl-branched hydrocarbons, tentatively identified as 7,20-dimethylhexacosane and 7,22-dimethyloctacosane, which were exclusively produced by mature females (>10 days). Further work is needed to determine the complete structure and the correct position of the methyl groups of these compounds and to ascertain their real significance in the extracts. The waxy layer from the cuticle plays an important role in the biology of insects because it prevents insects from desiccation and may also provide patterns for mimicry or camouflage. Furthermore, the cuticle hydrocarbons may contain species-specific olfactory contact cues used for mate

recognition (Gibbs, 1998a, b; Howard and Blomquist, 2005). The first evidence of mate recognition in beetles mediated by contact cues from the waxy layer was found in the Cerambycidae, for example in *Xylotrechus colonus*, whose males were attracted to conspecific females after antennal contact with the cuticle of the female (Wang and Millar, 2000; Ginzel, et al., 2003a, c; Ginzel and Hanks, 2003b). Also, in the EAB *A. planipennis* (Coleoptera: Buprestidae), methyl-branched hydrocarbons (3-me-C_{23}, 9-me-C_{25}) were found on the cuticle of sexually mature females that facilitate mate recognition and elicit copulatory behavior in males (Lelito, et al., 2009; Silk, et al., 2009).

The findings of female-specific compounds in *C. undatus* point out to the hypothesis that a contact pheromone represented by the two detected dimethyl-branched hydrocarbons, 7,20-diMe-C_{26} and 7,22-diMe-C_{28} could also be present in this buprestid species. In addition, the chemical structure of both compounds seems to be quite similar to the described contact pheromones of the EAB. This discovery improves our understanding of the chemical ecology of the OFB and may have potential applications for management of this pest species. Whether or not contact chemoreception plays a crucial role in the communication of the OFB, we feel that it is worthwhile to pursue this study in future behavioral bioassays.

Scanning electron microscopy (SEM) analysis of the antennal structure and morphology of male and female revealed that antennae of OFB adults are of the same size and possess *uniporous sensilla*, *sensilla chaetica* and two subtypes of *sensilla basiconica*, like in the sister species *C. florentinus*. The possible function of these sensilla is based on what is already known in the sensilla of other beetles. In several studies it has been shown that *s. basiconica* types 1 and 2 are very common on the antennal flagellum of other beetles (Merivee, et al., 1999, 2000, 2002; Crook, et al., 2008a). In cigarette and ground beetles, for example, it was demonstrated that the wall of these sensilla is perforated by numerous tiny pores, like in *Coroebus spp.*, and that the lumen of the peg is filled with branched dendrites derived from two neurons (Okada, et al., 1992; Kim and Yamasaki, 1996). The number of pores and branched dendrites are considered to be evidence that the *sensilla basiconica* function as olfactory receptors (Altner and Prillinger, 1980; Zacharuk, 1980). Males of the OFB seem to have noticeably more sensilla of this type and exhibit more *uniporous sensilla* (gustatory/taste) on the most distal flagellomeres than females. In contrast, there is no obvious sexual dimorphism with respect to the presence of *s. chaetica* or their distribution.

In the EAB the occurrence of abundant *uniporous sensilla* on their antennae suggests that short range, contact cues are of great importance for male recognition. As cited above, a hypothetical contact chemical cue has been identified in field and laboratory assays by Lelito and coworkers (2007). Male *A. planipennis* spent significantly more time attempting to copulate with dead females than males or females, where hydrocarbons were removed by solvent wash. It can be assumed that OFB males, due to their great number of *uniporous sensilla* for taste, could use the hydrocarbons produced by females as contact chemical cues for mate recognition. However, which kind of olfactory stimuli the *s. basiconica* and which stimuli the *uniporous sensilla* respond to still needs to be clarified by electrophysiological and behavioral experiments. Detailed knowledge on the function of antennal sensilla in the OFB may give essential additional information about the role of olfaction in these beetles and could help to develop a biorational pest control.

The key to an effective pest management program for the OFB is a survey program equipped with tools for detecting and delimiting populations. In field trapping experiments in two heavily infested cork oak stands, the effect/attractiveness of different trap colors, designs, placement and a selection of host plant volatiles and compounds identified from *C. florentinus* adults were tested. Several field studies on the EAB demonstrated that adults significantly prefer purple or blue traps over red, green, yellow, black or white traps (Francese, et al., 2005, 2008; Lelito, et al., 2008). Studies of the retinal sensitivity of the EAB for the wavelength spectrum from 300 to 700 nm revealed that dark-adapted compound eyes respond to UV (340 nm), violet/purple (420-430 nm), blue (460 nm) and green (540-560 nm) (Crook, et al., 2009).

The number of OFB individuals captured during the summer of 2010, the most successful campaign so far was more than 3x higher than in previuos years, indicating a general improvement on trap design, color and attractants. Differences in the flight-period of adults observed during weekly revisions of traps between Arbúcies (zone A) and Santa Coloma (zone B) are supposedly the result of climatic conditions, latitude and altitude (Soria, 1990). In zone B, the highest number of trap catches, indicating a maximum flight of insects, occurred one week earlier than in zone A. Santa Coloma de Farners is located at an altitude of 380-440 m above sea level with a south orientation, whereas Arbúcies is orientated south-east at 520-590 m. The earlier emergence of the OFB in Santa Coloma might be caused by increased temperatures compared to zone A. Another explanation would be that food competition in zone A is higher than in zone B, which could provoke pressure in the recently emerged insects to find as fast as

possible the most suitable hosts for feeding, mating and/or ovipostion. Differences in the total number of captured individuals between the two zones could depend on the insects' population dynamics, different infestation levels and the fact that in previous years no trapping experiments were carried out in Santa Coloma de Farners. Also, orientation of the trapping site and trap position can have a strong influence on the screening behavior of OFB. In *Agrilus*, more larval galleries were found on the south and west facing sides of host trees than on north and east sides, suggesting a preference for ovipositing on the sunnier sides of trees (Timms, et al., 2006).

Significant differences in total captures and captures per revision day occurred between the three purple trap types in both zones. Most OFB catches were obtained on sticky Prism traps compared to sticky Single Panel and Lindgren traps. Single Panel traps captured significantly more adult beetles than Lindgren traps, indicating that Lindgren funnel traps, generally used for capturing a variety of wood boring and bark beetles are less appropriate for field trapping of *C. undatus*. There is a remote possibility that individuals attracted by Lindgren traps failed to fall onto the insecticide pellet area at the bottom of the trap, and escaped from it. The other two trap types, which were coated with glue, probably provided a better protection against possible escape of beetles. The reason for significant differences between Prism and Single-panel traps could be the trap surface. Three-sided Prism traps have a larger surface than the other trap types. Surprisingly, all captured *C. undatus* individuals were females, although this result is in agreement with that of Crook and coworkers (2009) in which purple traps captured more EAB females than males, especially in the canopy of the tree. The OFB females, presumably uses visual cues (purple) for host location and/or mate finding, as reported for other Buprestidae (Lelito, et al., 2008).

In addition to trap color, shape and position, the effect of different attractants commercially available (Pherotech), GLVs from host the plant, and headspace volatiles of the sister species *C. florentinus* (3-component blend) were tested. Although ethanol (control) was described as attractant kairomone for some large wood boring beetles, (Chenier and Philogene, 1989; Miller, 2006) and the buprestid beetle *Agrilus nilineatus* (Montgomery and Wargo, 1983), we had to choose ethanol as control lure because the other attractants were dissolved in ethanol. In Arbúcies (zone A) no differences were observed in the number of catches elicited by Pherotech lures and ethanol in none of the revisions. It is apparent that caught adults were not able to discriminate between the attractants and instead they were rather attracted by the color. In the second study site in Santa Coloma de Farners (zone B) no preferences for one of the semiochemical lures on Lindgren traps were observed. In contrast, Prism traps baited with the different

attractants showed significant differences in individual catches. The insects were significantly attracted to the GLV mixture in comparison to ethanol and Pherotech lures and the three component blend captured significantly more beetles than control traps. These results raise the question of a possible kairomonal effect of host plant volatiles that might initiate foraging and/or host location behavior in the OFB. Host plant GLVs tested in Y-tube olfactometer assays elicited also a positive response in *C. florentinus* females (Chapter 1).

In previous studies, several GLVs emitted by foliage of green ash (*Fraxinus pennsylvanica* Marshall) and white ash (*F. Americana* L.) trees and sesquiterpenes derived from the bark of stressed green ash trees have attracted EAB adults (Crook, et al., 2008c; De Groot, et al., 2008; Grant, et al., 2010; Crook and Mastro, 2010). In another study it was shown that the European cockchafer *Melolontha melolontha* is highly attracted to a mixture of GLVs, including 1-hexanol, (*E*)-2-hexenol and (*Z*)-3-hexenol, mimicking the bouquet of mechanically damaged leaves (Ruther, et al., 2000; Reinecke, et al., 2002a, b). In summary, green leaf volatiles are characteristic emissions of deciduous trees and levels are elevated by various stress factors, such as damage by insect feeding (Ruther, et al., 2000; Zhang and Schlyter, 2004; Rodriguez-Saona, et al., 2006). Male OFB feeding on cork oak foliage could also elicit a higher emission of GLVs that may attract females to its host and/or mate.

The attraction to the three component blend, comprising nonanal, decanal and geranylacetone, needs to be discussed and re-investigated in the future, as these compounds were detected in headspace volatiles from the sister species *C. florentinus*. Thus, volatile collections of *C. undatus* need to be conducted to confirm the presence of these volatile compounds. Nonetheless, it is possible that individuals were captured because nonanal, decanal and geranylacetone are ubiquitous compounds produced by several insect species and plants that have demonstrated their activity in laboratory and field experiments (Zhang, et al., 2003; Dickens, 2006; Haberer, et al., 2008; Siljander, et al., 2008). Nonanal, for example, is an aliphatic aldehyde found in at least 20 essential oils, including rose and citrus oils and the oil of several species of pine (de Groot and Poland, 2003). Furthermore, it has been shown that in the Scolytidae nonanal can function as attractant, disruptant or neither (Huber and Borden, 2001; Huber, et al., 2001).

Altogether, this work provides the first data on the morphology and chemistry of the OFB, *C. undatus*, and furthermore it represents an important step towards understanding the functioning of its chemical communication. Additionally, field

trapping experiments revealed the infestation levels of oak trees and the flight period of beetles (by monitoring). These data are essential for the initiation of an IPM program (by mass trapping) and may be used in the future.

ACRIDIDAE (ORTHOPTERA) – *LOCUST PEST SPECIES*

HOW LOCUSTS INITIATE AGGREGATION BEHAVIOUR.

'At his command came locusts, hoppers past all number, they consumed every green thing in the land, consumed all the produce of the soil.'
Psalms 105: 34–5

CHAPTER 3

Sex differences in the volatile emission of the Moroccan locust, *Dociostaurus maroccanus* (Orthoptera: Acrididae): Identification and electrophysiological responses

3.1 PREFACE

The Moroccan locust, *Dociostaurus maroccanus*, Thunberg 1815 (Orthoptera: Acrididae) is a polyphagous pest species of crops and pastures (El Ghadraoui, et al., 2002), which is predominantly found in semi-arid steppe or semi-desert areas (between approximately 28 and 49ºN) with abundant ephemeral vegetation (Uvarov, 1957).

Gravid females of this univoltine (one generation per year) species lay 2-4 egg pods, containing an average of 30 eggs per pod that are bound together by a specific froth, approximately one month after adult appearance (from May onwards). Generally, oviposition sites are characterized by mosaic vegetation and the egg pods are laid in rocky ground, just below the surface or up to 4 cm deep in firm soil (compact virgin soil is necessary). Contingent upon weather and ecological conditions, females oviposit in groups which can lead to an extremely high egg pod density (Martín-Bernal et al.; 1993). *D. maroccanus* displays an egg diapause in winter and depending on latitude and altitude hatching occurs from February to April (Quesada-Moraga and Santiago-Alvarez, 1999; 2001b; Santiago-Alvarez, et al., 2003). The nymph stage consists of five hopper instars (development takes from 25 to 40 days), before individuals start fledging in April and copulating 2-10 days later (Coca-Abia, personal communication). From the 2nd instar, hoppers show gregarious behavior and start marching together. During outbreaks, hopper bands destroy almost completely the vegetation of the hatching places. After that, they move towards cultivated plains and damage the sowings they find on their route. Adults show swarm activity with up to several thousand individuals per swarm, which can fly between 50 and 75 km during one season (max. 250 km). Increase in locust numbers usually occurs when temperatures exceed average and rainfall is below normal (Martín-Bernal et al.; 1993). For development of eggs humidity is of great importance, but too much rain can provoke their death by drowning (personal observation).

D. maroccanus can shift from a solitarious to a gregarious phase. In the desert locust, *Schistocerca gregaria*, this transformation is the result of a drastically increase in local population density, which depends on climatical and environmental circumstances (Pener, 1991; Pener and Yerushalmi, 1998; Bouaïchi and Simpson, 2003; Simpson and Sword, 2007). But compared to the desert locust, the Moroccan locust does not display the apparent changes of morphological, physiological and behavioral characteristics that have been described (Uvarov, 1966; Byers, 1991; Simpson, et al., 2005). In general, solitary adults of *D. maroccanus* are smaller than

gregarious ones with longer femur and elytra. The body color is brighter with black spots on the hind femora and the elytra are more marked. Morphological differences between solitary and gregarious phases also seem to vary geographically (Latchininsky, 1993). Usually, body length in adults varies from 16.5 to 28.5 mm (male) and 20.5 to 38.0 mm (female) (Fig. 3.1).

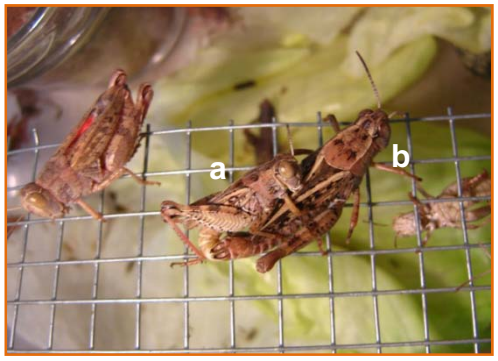


Fig. 3.1. Copulating (a) male and (b) female adults of *D. maroccanus*.

A striking characteristic of the Moroccan locust is its close association with human agricultural activities, displayed by creation of suitable habitats for the pest, such as deforestation, scrub destruction and excessive grazing by domestic animals. The reduction of available grasslands (due to expansion of croplands and overgrazing) leads to fragmentation of the upper soil horizon and provide the necessary conditions for locust concentration and gregarization, abetting subsequent outbreaks (Latchininsky, 1991; 1993).

Feeding by hopper bands and/or adults, in swarms of numerous individuals, result in high economic damage to many countries of the Mediterranean Basin, including South of France, North Africa and the Middle East (Uvarov, 1977; Latchinisky, 1998; Santiago-Alvarez, et al., 2003). Historical records from countries beyond Europe (e.g. Uzbekistan, Kazakhstan) showed that Moroccan locust outbreaks occurred until 1990s (Latchinisky, 1998). In contrast in Europe, most of the important outbreaks of *D. maroccanus* took place during the 1920s–1940s as direct result of the devastating effects of wars (Mendizábal, 1943; Moreno-Marquez, 1944), except in Spain, in which e.g. in Extremadura outbreaks of this pest persisted through the 1980s and 1990s, demanding broad-scale chemical control interventions (del Moral de la Vega, J., 1986; Arias, et al., 1993). In 1992, almost one-third of the surveyed area (113 600 ha) was infested by the Moroccan locust and the costs of applied chemicals for treatment of 68000 ha exceeded US$ 953.000. Estimation of total losses to grassland enfolded more than 5.000.000 US$ (Arias, et al., 1993). Nowadays, the threat by *D. maroccanus* continues in Spain, particularly in Andalucía, Aragón, Castilla-León, Castilla-La Mancha, Extremadura and Madrid, and treatment of infested zones with insecticides is increasing dramatically. In Aragón, chemical control of the Moroccan locust pest has been risen from 8.300 ha treated area in 1993 to 22.000 ha in 2003.

Chemical treatments of insect pests, especially of those with very high population densities such as locusts, are efficient at short notice, but also very aggressive to the environment provoking contamination of soil, water and air. In addition, they represent a strong risk on the health of potential consumers of crops (see General Introduction). The massive application of insecticides and its environmental impact evoked by chemical applications to control the Moroccan locust could produce the extinction of numerous species and thereby an irreversible loss of biodiversity. Therefore, it is essential to elaborate alternative control strategies that permit a selective handling of the pest and ensure a sustainable development of agricultural crops. Thus, for example, in Afghanistan mechanical control measures used by generations of farmers in the past to protect their crops were reintroduced in 1996 (Stride, et al., 2003). This involved monitoring of egg beds in the spring to predict egg hatch and timely mobilization of communities to create trenches in front of the advancing bands of hoppers. Between 1996 and 1999, about 30.000 ha of locusts were cleared by these measures saving US$ 300.000 in insecticide bill. In Spain, several studies were started to control *D. maroccanus* with natural enemies such as natural predators (Cañizo, 1943; 1956; Hernández-Crespo and Santiago-Álvarez, 1997a), microorganisms (Streett and Henry, 1990; Quesada-Moraga and Santiago-Alvarez, 2001a) or fungi (Hernández-Crespo and Santiago-Álvarez, 1997b; Collar-Urquijo, et al., 2002), but without any promising results up to now (Enserink, 2004).

With respect to the utilization of semiochemicals and especially pheromones in IPM programs, very little is known about the chemical communication in Acrididae. Most studies were focused on the behavior, physiology and pheromone system of the desert locust, *S. gregaria*, with special emphasis on the major male-specific pheromone, phenylacetonitrile (PAN) (reviewed in Hassanali and Torto, 1999; Ferenz and Seidelmann, 2003; Hassanali, et al., 2005). The desert locust is one of the most important insect pests of agricultural crops. In this species it has been found that olfactory cues play an important role in behavioral contexts, such as host plant location, oviposition and reproduction (Blaney and Simmons, 1990) (see also Chapter 4). Even though *S. gregaria* is a related species of the Moroccan locust, which also displays a more or less “innocuous” solitary but a very harmful gregarious phase, nothing is known about the chemical ecology of *D. maroccanus*. The lack of biorational methods and the certainty that chemical signals play an important role in the species-specific communication of Acrididae, especially in desert locust, both in nymphs and adults in solitary and gregarious phase, led us to investigate for the first time the possible presence of pheromones and their role in the chemical communication of the Moroccan

locust. To determine possible sources of pheromones, volatiles of male and female adults and nymphs of both sexes were collected and extracts of feces, egg froth and eggs were analyzed by GC-MS (EI and CI) and GC-EAD. Furthermore, the morphology and structure of the insect antennae and its sensilla were studied by SEM. In ovipostion bioassays we have tested if natural egg froth extracts or synthetic compounds identified from the Moroccan locust have any influence on the ovipositon behavior of gravid females. Female *S. gregaria* have been shown to deposit their egg pods in sites where other females are ovipositing and compounds identified from egg froth are assumed to elicit this aggregation (Uvarov, 1977; Saini, et al., 1995; Rai, et al., 1997). Similar behavior has been observed in the Moroccan locust and it is known that new generations of females return to suitable oviposition sites of previous years. This brought us to hypothesize that female produced egg froth could attract or provoke conspecific individuals to oviposit in the same sites. Summarized, this work represents the first attempt to discover biological active compounds that could be utilized in the biological control of the Moroccan locust.

3.2 METHODOLOGY

3.2.1 Chemicals

Phenylacetonitrile (PAN) (98%) was purchased from Fluka (Sigma-Aldrich Inc., St Louis, MO, USA). Veratrole (99%), acetophenone (98%), (*E*)-2-decenal (95%), 2-hexadecanone (98%), 1-hexadecanol (95%), 2-octadecanone (97%) and dodecyl acetate (97%) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). (*E,E*)-2,4-heptadienal (88%), (*E*)-3-octen-2-one (98%), (*E*)-2-octenal (94%) and (*E,E*)-2,4-decadienal (85%) were purchased from SAFC (KOSHER, Sigma-Aldrich Inc., St Louis, MO, USA). The aldehydes tetracosanal, hexacosanal and octacosanal were synthezised by oxidation of the corresponding alcohols using Dess-Martin periodinane (Lancaster, Mülheim/Main, Germany). *n*-Hexane (>95% by GC) (J.T. Baker, Deventer, Holland) and dichloromethane (DCM, >99.9%) (Fluka, Sigma-Aldrich Inc., St Louis, MO, USA) were used as solvents.

3.2.2 Insects

Since rearing of the insect in the laboratory proved to be unsuccessful in spite of several studies conducted on embryonic development and breeding systems (Quesada-Moraga and Santiago-Alvarez, 2000; 2001b), Moroccan locusts were caught directly in the field. During the campaigns 2006-2010 specimens of living nymphs and adults of *D. maroccanus* were obtained from non-treated semi-arid areas in Aragón (Alhama, 41°17′46.9206″N, 0°53′38.4678″O*,* Calatayud, Spain). From May to August colleagues from the CITA (Centro de Investigación y Tecnología Agroalimentaria, Zaragoza, Spain) collected several hundred individuals using butterfly nets directly from the field. They were placed in cardboard boxes, furnished with wire gauze in the front, and transferred to the laboratory. After separation of nymphs and adults and different sexes (males and females) part of the insects were sent to our laboratory for analyses. Adults and nymphs were kept separately under crowded conditions (50-100) in aluminum cages (50×50×50 cm^3) with wire gauze on lateral sides and on the back. Bottom and top were made of aluminum sheath (Fig. 3.2 A), while the top disposed of a trap-door for manipulation. The cages, emblazed by 60 W white light bulbs placed at the top for homogenous illumination, stood in a well-ventilated room at 26±2ºC, 50±10% RH and 14:10 L:D photoperiod. Fresh alfalfa, grass and wheat were provided twice a day to feed the insects.

Fig. 3.2. (A) Rearing and (B) experimental cages for gregarious *D. maroccanus*.

3.2.3 Experimental cages

The effect of natural egg pod and froth extracts of the Moroccan locust on the oviposition behavior of conspecific females was tested in plastic-fronted cages (45×45×45 cm^3) with wire gauze on lateral sides and a 60 W white light bulb on the top. The back and bottom of the cages were made of wooden sheath (Fig. 3.2 B). For oviposition experiments with gravid females, two glass oviposition cups (10 cm high × 5 cm ID) were placed in opposite corners into a false floor at 32.5 cm distance in diagonal. The oviposition cups were filled with sterilized and later moistened sand (15% water), similar to the oviposition experiments previously described for the desert locust (Saini, et al., 1995; Bashir, et al., 2000).

3.2.4 Scanning electron microscope (SEM)

For SEM analysis, antennae of male and female *D. maroccanus* adults (N=3) were prepared as described in Chapters 1 (1.2.3) and 2 (2.2.4). Micrographs were taken with a Stereoscan S-360 (Leica) electron microscope at 15 kV (Servicios Científicos Técnicos, University of Barcelona, Spain).

3.2.5 Collection and analysis of volatiles

Volatiles were collected by aeration of adults and nymphs that were placed separately in a dynamic headspace system. Charcoal-filtered air (flow 500 ml min^{-1}) was passed over 7 males and females in 2 different glass trapping chambers (22.5 cm long × 4 cm OD), for 4, 8 and 12 h at room temperature. The air (enriched with insect odors) was conveyed through a Super Q glass tube (6 cm long × 6.4 mm OD) (Ars Inc.,

Gainesville, FL, USA) at the outcome of the glass chamber. These glass collectors enclosed 30 mg of adsorbent to trap the emitted volatiles. Elution of trapped volatiles was done by washing the adsorbent with 200 µl HPLC grade DCM. Extracts were stored at -20ºC for subsequent chemical analysis and electrophysiological assays. Furthermore, to obtain information about development and maturation time of the Moroccan locust repeated volatile collections were done on males and females 1, 3, 4, 6, 7, 12, 21, 25, 26, 35, 39, 42 and 46 days from fledging.

GC-MS analysis were performed on a Thermo Finnigan Trace 2000 GC system coupled to a Trace MS quadropule mass spectrometer (ThermoFisher Scientific, Madrid, Spain) by injecting 1 µl of the headspace volatile extracts combined with 1 µl of a 100 ng μl^{-1} solution of dodecyl acetate as internal standard in splitless mode under electron impact (EI, 70 eV) conditions. For determination of retention indexes of the detected compounds 1 µl of a C_8-C_{25} hydrocarbon mixture at 100 ng μl^{-1} was also injected. The column was a 30 m×0.25 mm ID×0.25 µm HP-5MS (5% phenylmethylsiloxane) (Agilent Technologies, Madrid, Spain) with helium (1 ml min^{-1}) as carrier gas. Injections were done under the following chromatographic conditions: injection temperature at 40ºC, held for 5 min and programmed of 10ºC min^{-1} to 280ºC which was maintained for further 10 min. Mass range was from 40 to 500 *m/z* at a scan time of 1.0 s. Compounds were identified by comparison of their mass spectra and retention indexes with those of authentic standards and/or by comparison with those from a commercial library (NIST Registry of Mass Spectral Data, 2005) or from the database published by Adams (2007). Retention indexes of each compound were calculated according to van den Dool and Kratz (1963). Quantification of identified compounds was done by comparison of their areas with that of the internal standard (dodecyl acetate). The amount was calculated per individual and hour of release.

Volatiles of mature adults were also collected using a SPME assembly with a PDMS fiber (polydimethylsiloxane, 100 µm) (Supelco, Bellafonte, PA, USA). Five males and females were placed separately in Erlenmeyer flasks (100 ml) containing a braided wire for accommodation and closed by a screw cup with septum. The fiber was punctured through the septum to absorb the emitted volatiles for 12 h. The system was illuminated by a 60 W white bulb (L:D 14:10) and three replicates were conducted per each sex. The volatiles were analyzed by injecting the fiber into the injection port of the above GC-MS system, as described in Chapter 2 (2.2.4).

3.2.6 Preparation of extracts of body parts, egg pods, froth and feces

In several studies on the volatile emission of desert locust possible production sites of pheromones were described. These include wings and legs of mature males (Seidelmann, et al., 2003), egg froth of gravid females (Saini, et al., 1995; Rai, et al., 1997; McCaffery, et al., 1998) and feces of nymphs and adults (Obeng-Ofori, et al., 1994a; Torto, et al., 1996). Accordingly, different body extracts of the Moroccan locust were prepared. For body part extracts, legs and wings of 3 nymphs and 6-days old adults of both sexes (dazed by cooling) were excised close to the thorax. In addition, extracts of the femur, tibia and tarsus of three 44-days old mature males and females were prepared. The body parts were immersed in 1 ml of hexane in 5 ml glass vials. After 3 h of extraction at room temperature, wings and legs were removed and the extracts were stored at -20ºC for subsequent analysis. In a similar way extracts of adult feces and eggs and froth plugs of gravid females were prepared. Freshly excreted froth (150 – 350 mg), eggs (20-30) from an entire egg pod, and ca. 100 mg of fresh feces were extracted in 0.5 - 1 ml of hexane for 3 h. Solid rests or particles were filtered through cotton that was previously washed with hexane. The cotton was placed inside a Pasteur pipette. The extracts were stored at -20°C until use and for all extracts a minimum of three replicates were considered. For GC-MS analyses 1 µl of the extracts together with 1 µl of the internal standard was injected. For statistical comparison between amounts of male-specific compounds derived from body part extracts and headspace volatiles, the *Student's t-test* was applied.

3.2.7 Electrophysiological assays – GC-EAD and EAG

Coupled gas chromatography-electroantennographic detection (GC-EAD) analyses were carried out on a Focus GC (Thermo Instruments, Barcelona, Spain), equipped with a FID detector. Antennal responses of male and female adults to headspace volatiles of males were recorded. For that 2 µl of the headspace extracts of *D. maroccanus* were injected in splitless mode. A minimum of 5 individuals for each extract was tested using the same electrophysiological setup and antennal preparation, as described in Chapter 1 (1.2.6). Antennae were excised close to the head, carefully cut on both ends and the distal and proximal segments were placed in contact with the microelectrodes using a conducting gel (Spectra 360, Parker Lab. Inc., Hellendoorn, The Netherlands).

In electroantennogram (EAG) assays we determined the activity of natural extracts and synthetic compounds on adult Moroccan locust antennae of both sexes. The electrophysiological setup and antennal preparation was the same as in GC-EAD analysis, (see Chapter 1). Different doses of natural and synthetic extracts (Table 3.1) dissolved in hexane were deposited over a stepped Whatman filter paper (2.5 cm diameter).

Table 3.1. Volume of natural extracts and doses of synthetic compounds used in EAG analysis in male and female antennae of *D. maroccanus* adults.

Stimulus I	**Stimulus II**	**Volume**	**Dose**	**Antennae**
Natural extracts	Synthetics	[µl]	[µg]	[N]
Headspace volatiles		50[a,b]		10
Femur (male)		100[c]		10
Egg froth		20[d]		8
Feces		20[e]		8
	PAN		1; 10; 50	8-10
	acetophenone		1; 10; 50	8-10
	veratrole		1; 10; 50	8-10
	2-hexadecanone		10	8
	2-octadecanone		10	8
	1-hexadecanol		10	8
	2-pentadeylcyclopentanone		10	10
	(*E,E*)-2,4-heptadienal		10	13
	(*E*)-3-octen-2-one		10	9
	(*E*)-2-octenal		10	8
	(*E*)-2-decenal		10	10
	(*E,E*)-2,4-decadienal		10	13

[a] Volatiles dissolved in 200 µl hexane; collected from 7 males and females during 12 h.
[b] Volatiles dissolved in 1 ml hexane; collected from 7 males and females during 12 h.
[c] Femur of 3 adult males dissolved in 1 ml hexane.
[d] Freshly secreted egg plugs (100 mg ml^{-1}).
[e] Feces of adult males (0.19 mg μl^{-1}) and females (0.25 mg μl^{-1}).

After solvent evaporation, the filter paper was introduced into a Pasteur pipette and the antennae were stimulated by air puffs passing through the extracts. Dose-dependent responses to test the synthetic compounds PAN, veratrole and acetophenone were recorded. These compounds are part of the volatile system of *S. gregaria* and they have been described to have pheromonal activity in the desert locust (Hassanali and Torto, 1999; Hassanali, et al., 2005). In all experiments, hexane was used as control. Only antennal responses to stimuli higher than to the control were considered for analysis. Depolarization means were compared for significance using

analysis of variance (*one-way ANOVA*) followed by *DMS* and/or *Tukey's HSD post-hoc* test (P<0.05) applying PASW 18 software (SPSS Inc., Chicago, IL, USA).

3.2.8 Oviposition bioassays

For oviposition bioassays 3 odor treatments were set up to test the effect on the egg laying behavior of gravid Moroccan locust females. Odor sources were the following: i) a five component mixture of compounds detected in egg froth of *D. maroccanus* females, ii) a four component mixture of compounds derived from *S. gregaria,* and iii) an extract of freshly secreted egg froth of *D. maroccanus* females. In addition a control cage was prepared with no odor stimuli (iv). All compound blends were dissolved in hexane. Mixture of treatment (i) consisted of (*E,E*)-2,4-heptadienal, (*E*)-3-octen-2-one, (*E*)-2-octenal, (*E*)-2-decenal and (*E,E*)-2,4-decadienal (1 µg μl^{-1} each). Mixture of treatment (ii) consisted of PAN, acetophenone, veratrole and benzaldehyde (1 µg μl^{-1} each). For (iii) 350 mg of fresh froth plugs were extracted in 1 ml solvent.

To start with, 40 mature adult males and 40 females were placed in the four experimental cages. In each case two oviposition cups (number 2 and 3 in Figure 3.4) filled with moistened sand were positioned in opposite corners of the cages.

In each experimental assay 50 µl of the extracts and mixtures were applied to the moistened sand of one of the two cups and the same volume of solvent was applied to the second cup as control. Every week oviposition cups were checked for laid egg pods by sieving the sand. New moistened sand was added to the cups and fresh extracts were applied. Cups were rotated clockwise to the following corner (position 1-4) to exclude possible preferences of the test insects for one of the corners (positional effect of the setup). Locusts were fed with fresh alfalfa, grass and wheat daily. When necessary, dead individuals were replaced by new ones of the same age and gender. The whole experiment lasted eight weeks and was repeated in the campaigns 2009 and 2010.

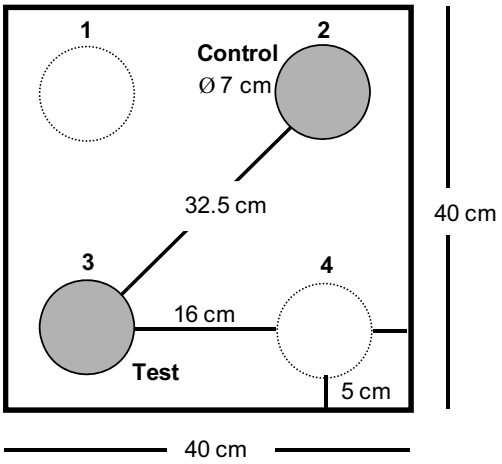


Fig. 3.3. Experimental set up for oviposition bioassays. Position of oviposition cups (grey cycle).

3.3 RESULTS

3.3.1 SEM studies of adult antennae

The morphology of antennae and the fine structure and distribution of various types of antennal sensilla in male and female crowd-reared (gregarious) insects of the Moroccan locust were investigated by scanning electron microscopy. The antennae in both sexes are filiform and consist of 24 segments. Throughout its length (ca. 8 mm) the antennae represent an even diameter (210 – 280 µm) and the antennal surface is covered by cuticular plates that surround the sensilla (Figs. 3.4 A, B). Four different types of sensilla (identical in both sexes) were identified: *s. basiconica*, *s. trichodea*, *s. coeloconica* and *s. chaetica* (Figs. 3.4 A, B). Distribution of sensilla types depend on the antennal segment, being lower in the segments most proximal to the head.

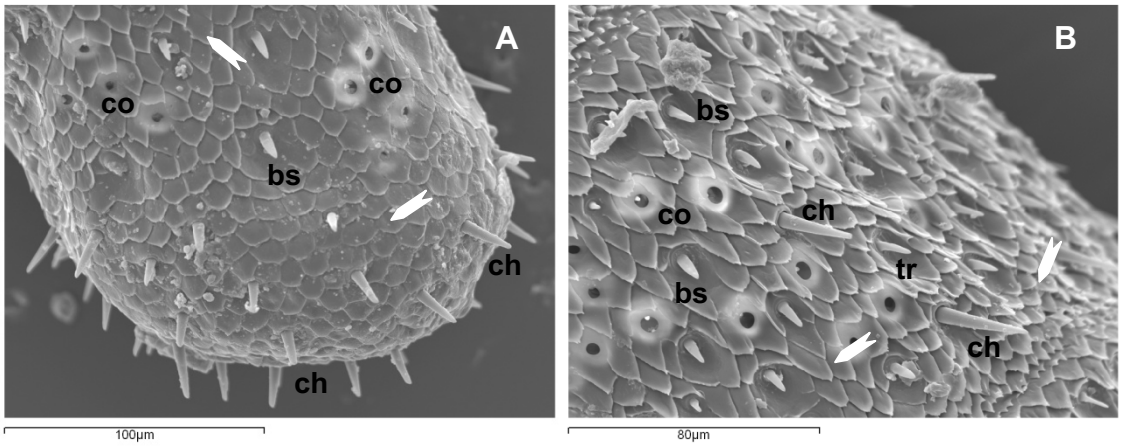


Fig. 3.4. Various sensillar types on the terminal 1st segment (A), and the 4th (B) on one antenna of adult *D. maroccanus*. The tip of the terminal segment is endowed with more *sensilla chaetica* (*ch*) than any other sensillar type, whereas segments proximal to segment 1 are dominated by olfactory sensilla: *sensilla basiconica* (*bs*), *coeloconica* (*co s.*), and *trichodea* (*tr s.*). The antennal surface is covered by cuticular plates (white arrows).

The *basiconic sensilla*, are hairs set in shallow depressions of the antennal cuticle, which are the most abundant sensilla type of this locust. The sensillum length measures between 9 and 11 µm and the basal diameter is about 5 µm. The surface of the hair possesses a high density of pores (23-29 μm^{-2}) with a diameter of ca. 60 nm (Figs. 3.5 A, B).

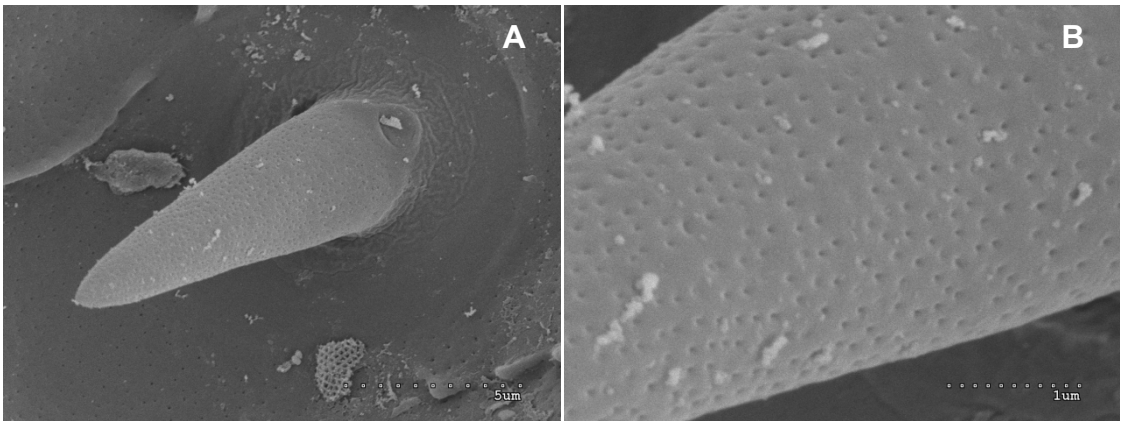


Fig. 3.5. (A) Olfactory *sensillum basiconicum*. (B) Scanning electron micrograph displaying a massive cuticular pore system.

The second most abundant type are the *sensilla coeloconica.* They are relatively short (ca.1.6 µm) and housed in spherical cuticular pits with an opening of approx. 5 µm in diameter (Fig. 3.6). This sensilla type is characterized by longitudinal ridges, a non-porous wall and a beaded tip with a terminal molting pore.

The *sensilla trichodea* are distributed all over antennal segments but less abundant than the other three types. They are about 9 µm long and have a more slander shape and a smaller basal diameter (ca. 3 µm) than *s. basiconica* (Fig. 3.7). The hair wall possesses a moderate density of pores between 11 and 14 μm^{-2}.

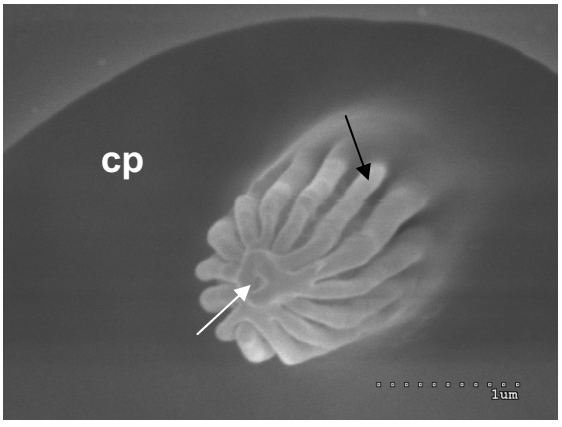


Fig. 3.6. Olfactory *sensillum coeloconicum*. SEM micrographs displaying *s. coeloconica* situated in the cuticular pit (cp); sensillum displays a beaded tip, longitudinal ridges (black arrow) and a terminal molting pore (white arrow).

The *sensilla chaetica* are the longest of all identified types on the antennae of *D. maroccanus,* measuring ca. 23 µm. The basal diameter of the sensillum is about 4.5 µm and it has a flexible socket suggesting a mechanoreceptor. The sensillar surface

has longitudinal cuticular grooves (Fig. 3.8 A) and a terminal pore at the tip of the sensillum (Fig. 3.8 B). There are no other pores on the sensillar wall. The highest number of this type of sensillum was shown on the terminal segment, and their distribution declined substantially on segment two to remaine constant in direction to the base of the antenna.

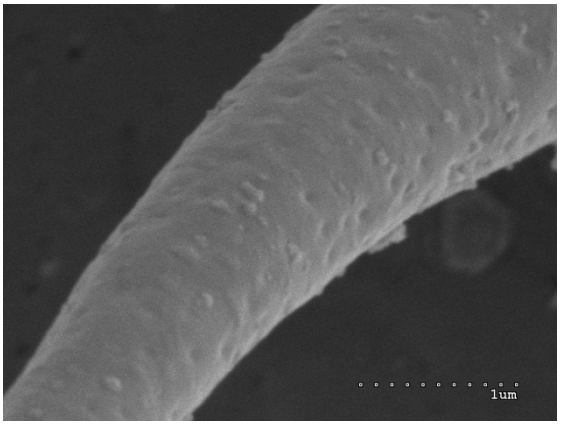


Fig. 3.7. Olfactory *sensillum trichodeum*. Scanning micrograph displaying relatively few cuticular pores compared with a *s. basiconicum*.

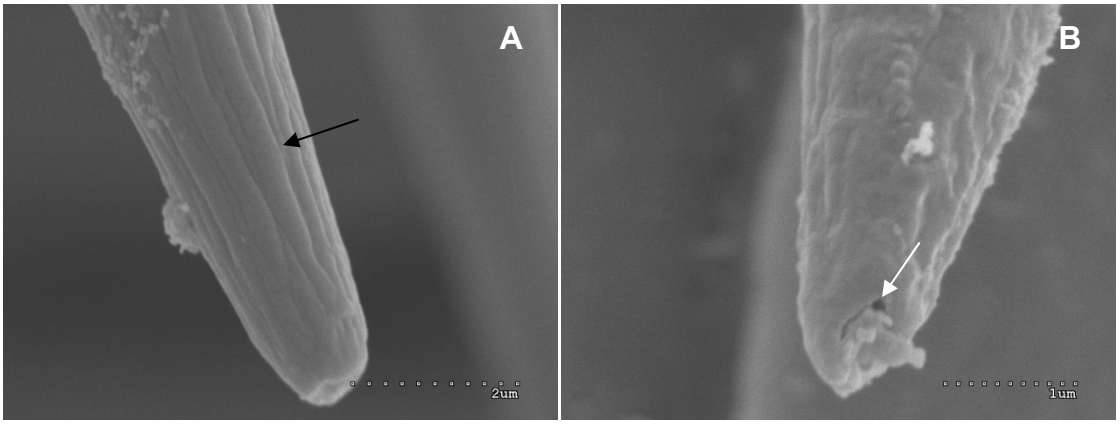


Fig. 3.8. Mechanoreceptor *sensillum chaeticum*. Scanning micrographs displaying (A) non-porous longitudinal ridges (black arrow) with a terminal pore (B) (white arrow).

3.3.2 Identification of compounds from volatiles of both sexes and natural extracts from mature females

A total of 58 compounds of different chemical classes were detected in all extracts of headspace volatile samples and body parts of adults and nymphs of the Moroccan locust (Table 3.2). These encompass acids, alcohols, aldehydes, hydrocarbons, ketones and two so far unidentified compounds. Qualitative differences in volatile composition were observed between stages and sexes.

Table 3.2 Detected compounds in headspace volatiles and natural extracts of male and female *D. maroccanus* adults, identified by comparison of their mass spectra and retention indexes with those of authentic standards or compiled in commercial libraries[a].

	Acids	Alcohols	Aldehydes	Hydrocarbons	Ketones	Others
Saturated	hexanoic acid	1-tetradecanol (C_{14})	nonanal (C_9)	tricosane (C_{23})	2-pentadecanone (C_{15})	not identified (01BP84)
	octanoic acid	1-hexadecanol (C_{16})	Decanal (C_{10})	tetracosane (C_{24})	2-hexadecanone (C_{16})	not identified (02BP84)
	dodecanoic acid	1-hexacosanol (C_{26})	Dodecanal (C_{12})	pentacosane (C_{25})	2-octadecanone (C_{18})	2-pentylfuran
	tetradecanoic acid	1-octacosanol (C_{28})	Tetradecanal (C_{14})	hexacosane (C_{26})	2-nonadecanone (C_{19})	5-ethyl-2(5H)-furanone
	pentadecanoic acid		Pentadecanal (C_{15})	heptacosane (C_{27})	3-eicosanone (C_{20})	indol
	hexadecanoic acid		Hexadecanal (C_{16})	octacosane (C_{28})		
			Tetracosanal (C_{24})	nonacosane (C_{29})		
			Hexacosanal (C_{26})	triacontane (C_{30})		
			Octacosanal (C_{28})	hentriacontane (C_{31})		
				dotriacontane (C_{32})		
				tritriacontane (C_{33})		
				tetratriacontane (C_{34})		
Unsaturated	oleic acid		(*E*)-2-octenal		(*E*)-3-octen-2-one	
			(*E,E*)-2,4-heptadienal		(*E,E*)-3,5-octadien-2-one	
			(*E*)-2-decenal			
			(*E,Z*)-2,4-decadienal			
			(*E,E*)-2,4-decadienal	C_{29}:2		
			(*E*)-2-undecenal	C_{31}:1		
Esterified	ethyl hexadecanoate					
	methyl hexadecanoate					
Branched				13-meC_{25}		
				11,13-diMeC_{26}		
				13-meC_{29}		
				unknown diMeC_{27}		

[a] Detected in: Female volatiles | Volatiles both sexes | Volatiles (male) | Egg froth | Feces and froth | Eggs

a) Headspace volatile extracts of male and female adults and nymphs

In the headspace samples nine compounds were detected present in male adult volatiles and absent in female volatiles (Fig. 3.9). By comparison of their mass spectra, retention times and retention indexes with those of authentic standards 7 of the male-specific compounds were identified as tetradecanal, 1-tetradecanol, pentadecanal, hexadecanal, 2-hexadecanone, 1-hexadecanol and 2-octadecanone (peaks 1-7). The two unidentified compounds 01BP84 (peak 8) and 02BP84 (peak 9) with molecular mass m/z 294 are isomers, since they present almost identical mass spectra, and have the same base peak at m/z 84 (Fig. 3.10). The most abundant male-specific compounds are hexadecanal (peak 4; Fig. 3.10) and 02BP84. In volatiles of male 5th instar nymphs traces of tetradecanal and hexadecanal were also found. The aldehydes nonanal, decanal and dodecanal are present in volatile emissions of both sexes and ages (Table 3.2).

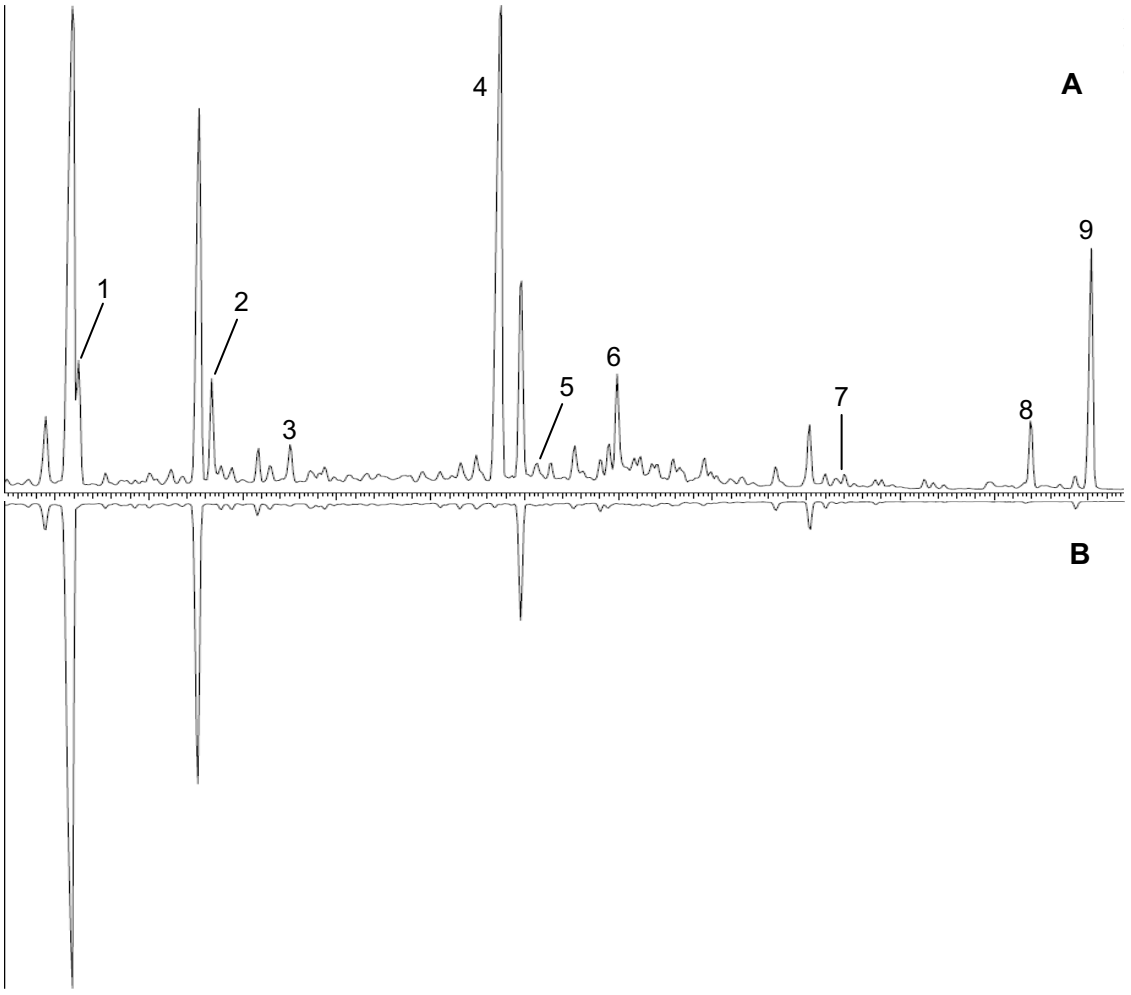


Fig. 3.9. Representative GC-MS profiles of headspace volatiles of seven mature adult males (A) and females (B) of *D. maroccanus*. Numbers on peaks (1-9) refer to detected compounds only present in males: 1) tetradecanal, 2) 1-tetradecanol, 3) pentadecanal, 4) hexadecanal, 5) 2-hexadecanone, 6) 1-hexadecanol, 7) 2-octadecanone, 8) 01BP84 and 9) 02BP84.

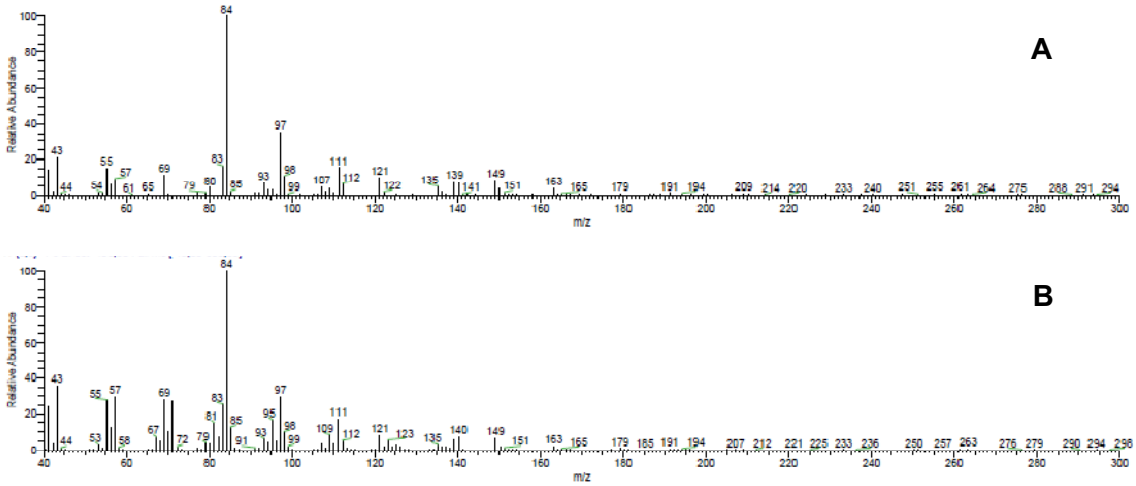


Fig. 3.10. Mass spectra of the two unidentified male-specific compounds (01BP84 (A) and 02BP84 (B)) derived from volatiles of mature male adult Moroccan locusts.

b) Analysis of volatiles of male and female adults collected by SPME

A total of 22 compounds were detected by SPME in the volatiles of mature male and female Moroccan locusts. Qualitative and quantitative differences have been observed between sexes (Fig. 3.11). Peak numbers are in agreement with the nomenclature of male-specific compounds found in headspace volatiles (Fig. 3.9).

The male-specific compounds 2-hexadecanone, hexadecanal, 1-hexadecanol, 2-octadecanone, 01BP84 and 02BP84 were also present in the volatiles, as well as 2-nonadecanone (peaks 4-10; Fig. 3.11 A). On the other hand, ethyl hexadecanoate was only present in female volatiles (compound *) (Fig. 3.11 B). Most of the identified compounds in both sexes, however, were saturated linear or branched hydrocarbons. The chain-length ranged from C_{24} *to* C_{34} with a maximum of one or two methyl groups and four hydrocarbons have not been identified so far (peaks 22, 23, 24 and 27; Fig. 3.11).

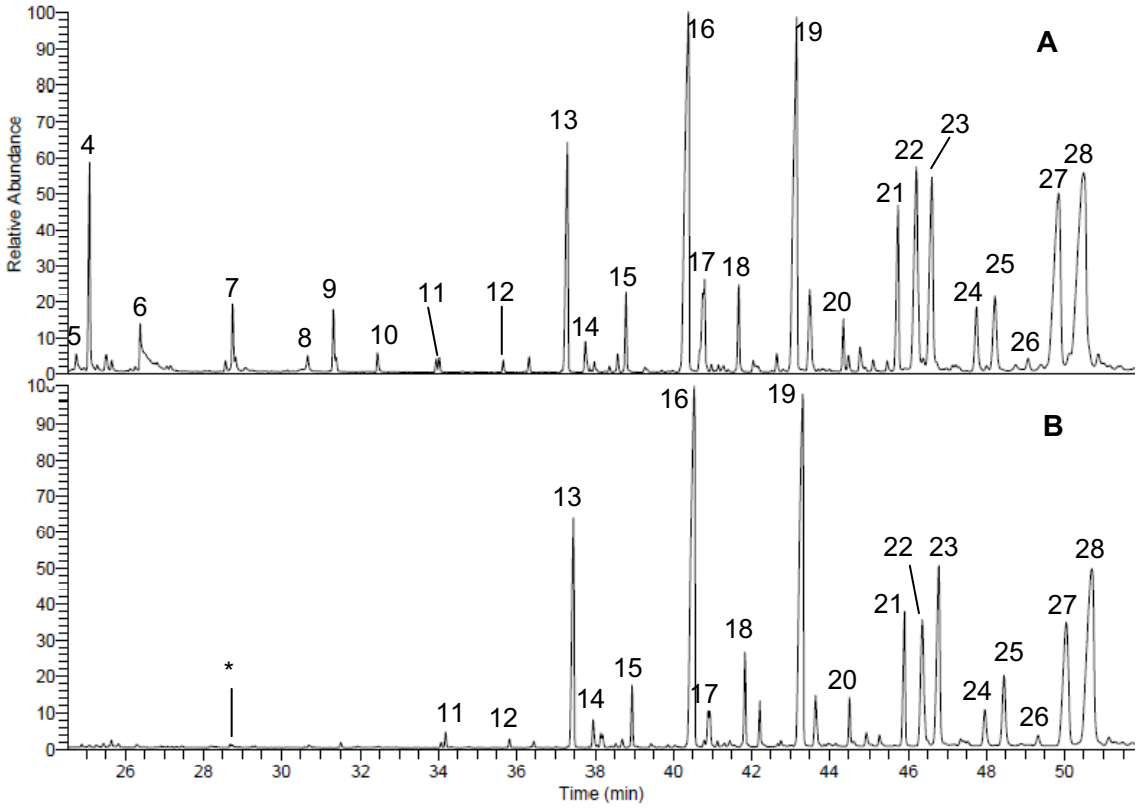


Fig. 3.11. Representative GC-MS profiles of SPME volatiles of seven mature adult males (A) and females (B) of *D.maroccanus*. Numbers on peaks (4-28) refer to identified compounds present in males and/or females and are in accordance with headspace volatiles (Fig. 3.10): 5) 2-hexadecanone, 4) hexadecanal, 6) 1-hexadecanol, 7) 2-octadecanone,*) ethyl hexadecanoate, 8) 01BP84, 9) 02B84, 10) 2-nonadecanone, 11) tricosane, 12) tetracosane, 13) pentacosane, 14) 13-methyl pentacosane, 15) hexacosane, 16) heptacosane, 17) 11,13-dimethylhexacosane, 18) octacosane, 19) nonacosane, 20) triacontane, 21) hentriacontane, 22) non-identified hydrocarbon, 23) non-identified hydrocarbon, 24) non-identified hydrocarbon, 25) dotriacontane, 26) tritriacontane, 27) non-identified hydrocarbon, 28) tetratriacontane.

c) Analysis of natural extracts of mature females

A great number of chemically different compounds were identified from female froth, eggs and fecal extracts (Table 3.2). The extracts of feces and froth, egg froth and eggs contained mostly long-chain (>C_{26}) saturated and unsaturated alcohols and aldehydes and acids (Table 3.2).

In egg froth extracts short-chain acids (≤C_{11}) and unsaturated aldehydes (≤C_{10}) were detected, but also one unsaturated ketone, 3-eicosanone (C_{20}), ethyl hexadecanoate, and furan derivates. These latter chemicals (ketones and furans) were absent in fecal extracts. A representative GC-MS profile of the extracts is shown in Figure 3.12.

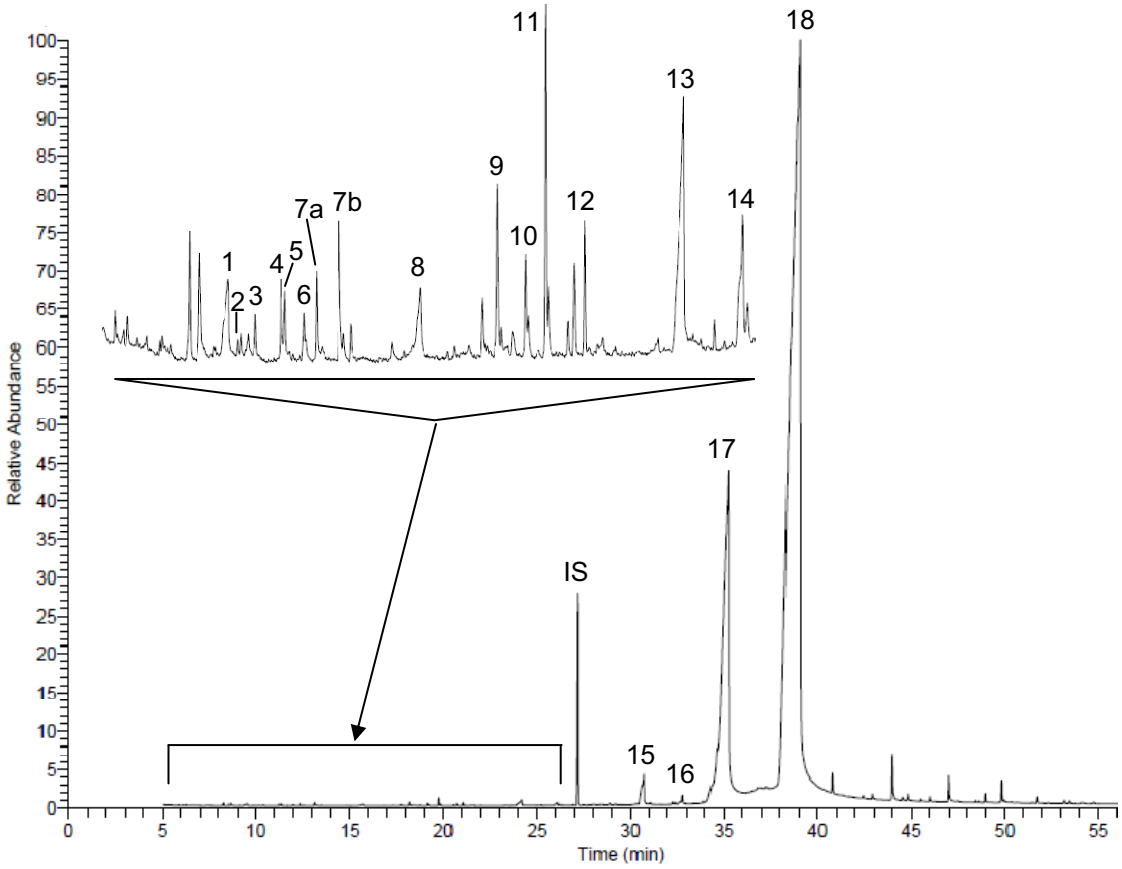


Fig. 3.12. Representative GC-MS profile of froth and fecal extract derived from mature adult females of *D. maroccanus*. Numbers on peaks (1-19) refer to identified compounds: IS = internal standard [100 ng]; 1) hexanoic acid, 2) 2-pentylfuran, 3) (*E*,*E*)-2,4-heptadienal, 4) 5-ethyl-2-(5H)-furanone, 5) (*E*)-3-octen-2-one , 6) (*E*)-2-octenal, 7a) (*E,Z*)-3,5-octadien-2-one, 7b) (*E,E*)-3,5-octadien-2-one, 8) octanoic acid, 9) (*E*)-2-decenal, 10) (*E,Z*)-2,4-decadienal, 11) (*E,E*)-2,4-decadienal, 12) (*E*)-2-undecenal, 13) unidentified acid, 14) dodecanoic acid, 15) tetradecanoic acid, 16) pentadecanoic acid, 17) methyl hexadecanoate, 18) hexadecanoic acid and oleic acid.

3.3.3 Analysis of male-specific compounds after fledging over age

Headspace volatiles of male nymphs and adults were collected to quantify the emitted amount of male-specific compounds (tetradecanal, 1-tetradecanol, pentadecanal, hexadecanal, 1-hexadecanol, 01BP84 and 02BP84) at different ages. Male nymphs don't emit any of these compounds while in 1-4 day-old adults small amounts of the aldehydes and the two unknown compounds have been found (Figs. 3.13 A-D). Total mean amounts of aldehydes released per individual and hour are significantly higher than the corresponding alcohols, such as the amount of 02BP84 compared to 01BP84. The maximum amount of the emitted aldehydes (tetradecanal, pentadecanal and hexadecanal) as well as 01BP84 and 02BP84 was recorded in 6-12 day-old individuals

to decrease from day 21-26. In contrast, at this age male adults produce the highest amount of the alcohols tetradecanol and hexadecanol (Figs. 3.13 A, C). The emission curve of these two compounds is shifted, therefore, ca. 14 days in comparison to the corresponding aldehydes. In 35-36 day-old adults pentadecanal (Fig. 3.13 B), hexadecanol (Fig. 3.13 C) and 01BP84 (Fig. 3.13 D) emissions are practically null.

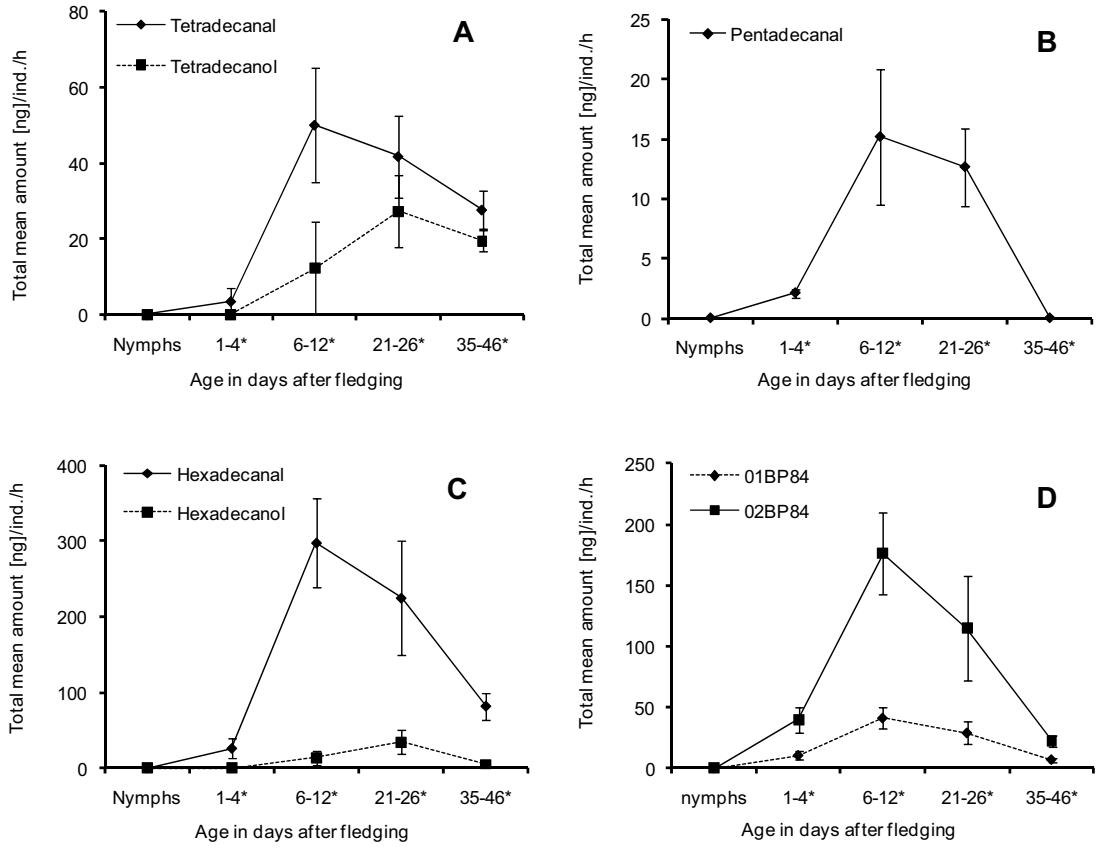


Fig. 3.13 A-D. Total mean amounts (±S.E.M) per individual and hour of male-specific compounds released by nymphs and adult individuals at different ages. Volatiles of 7 individuals were collected by the dynamic headspace method during 4, 8 or 12 h (N=3). Compounds to record were the following: (A) tetradecanal and tetradecanol, (B) pentadecanal, (C) hexadecanal and hexadecanol, (D) 01BP84 and 02BP84. Asterisks indicate period of three repeated volatile collections.

3.3.4 BP84 compund release

Production of the two posible isomers of BP84 from some body parts of 6 day-old mature male Moroccan locusts was determined. The body parts (legs and wings) of 3 locusts were combined and extracted in hexane. By comparison with the headspace volatiles of mature males, the two compounds 01BP84 and 02BP84 were also present

in both legs and wings (Fig. 3.14). Peak numbers are in agreement with the nomenclature of male-specific compounds found in headspace volatiles (peaks 8, 9; Fig. 3.9) and SPME extracts (Fig. 3.11).

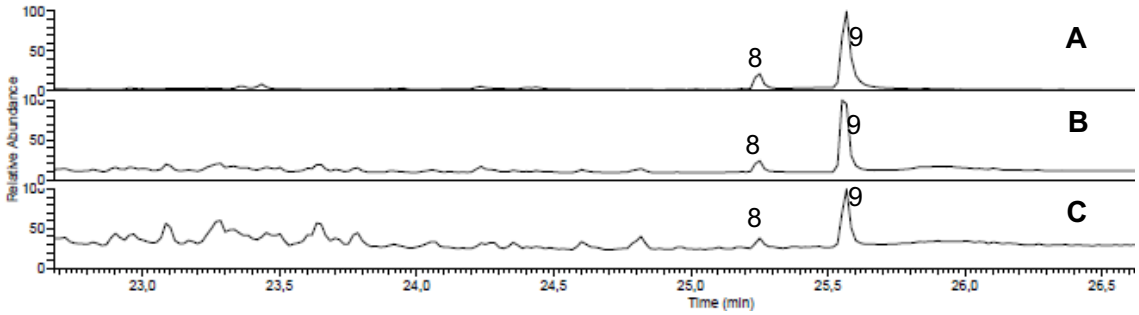


Fig. 3.14. Representative GC-MS profile of headspace volatiles (A), leg (B) and wing extracts (C) from mature adult males of *D. maroccanus*. Numbers on peaks, in accordance with headspace volatiles (Fig. 3.10), represent the unidentified compounds 01BP84 (8) and 02BP84 (9).

The mean amount of the two compounds per individual from leg and wing extracts were matched with the amount, shown in Figure 3.15, present in volatiles of 3-6 day-old male adults collected by dynamic headspace adsorption. The volatile collection for comparism with the body extracts lasted 12 h. The highest amount of 01BP84 and 02BP84 was detected in the 12th day after fledging (208 ± 99 ng/locust for 01BP84; 894 ± 293 ng/locust for 02BP84) (Fig. 3.16). More than half of the total BP84 amount was extracted from the legs and about one fifth from the wings (Fig. 3.16).

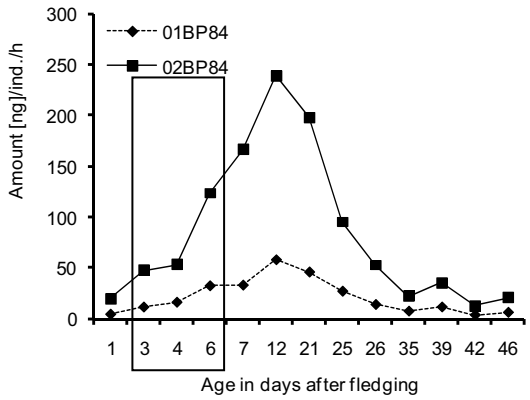


Fig. 3.15. Amount of the two male-specific compounds 01BP84 and 02BP84 released by mature adults at different days after fledging. Volatiles were collected by the dynamic headspace method. Volatile collections of days 3-6 were considered for comparison with the amounts found in wing and leg extract.

In addition to the large variability of the amount of BP84 released from live insects, we also found some variations in the BP84 amount in body extracts indicated by high S.E.M. No significant differences were shown between the released and extracted amounts of 01BP84, but the amount of 02BP84 was significantly higher in headspace volatiles than in wing extracts (p<0.028, n = 3, df = 4; F = 11.347; *Student's t-test*). In contrast, the observed differences of 02BP84 present in wings and legs, and also between leg extract and headspace volatiles are not significant.

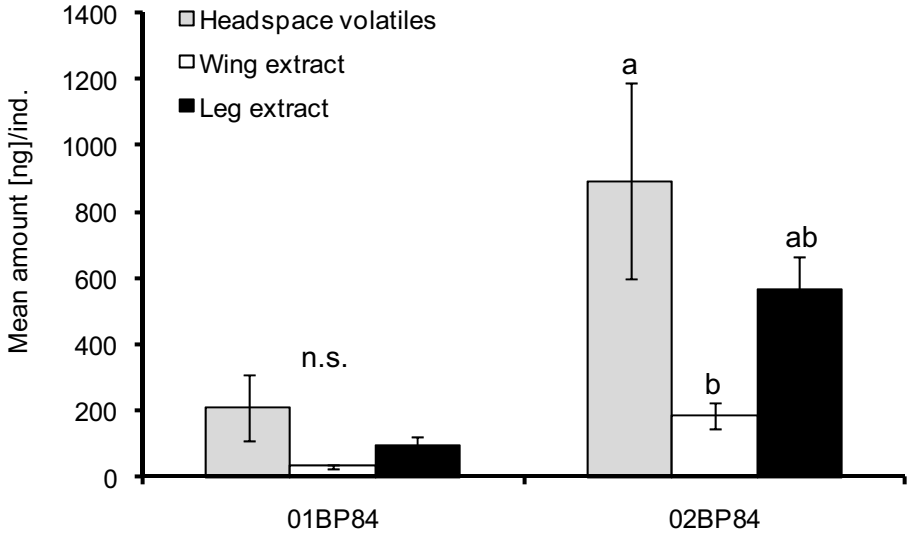


Fig. 3.16. Mean amount (±S.E.M) per individual of the two male-specific compounds 01BP84 and 02BP84 in volatiles released by 3-6 day-old mature adult males (collected during 12 h) and in wing and leg extracts (N=3). Bars with different letters within the same compound indicate significant differences (*Student's t-test*, $P \leq 0.05$; n.s. = not significant).

In a further experiment we examined which part of the legs (1st and 2nd pair and hind legs together) contributed most to the total BP84 amount. The calculated percentages of the total amount of BP84 indicate clear differences between the three leg parts (Fig. 3.17). More than 80% of the total amount of detected 01BP84 and 02BP84 was found in the femur extract. In the tibia 13% and 8% of the two compounds, respectively, were extracted while in the tarsus only small amounts were detectable (5% and 2%, respectively).

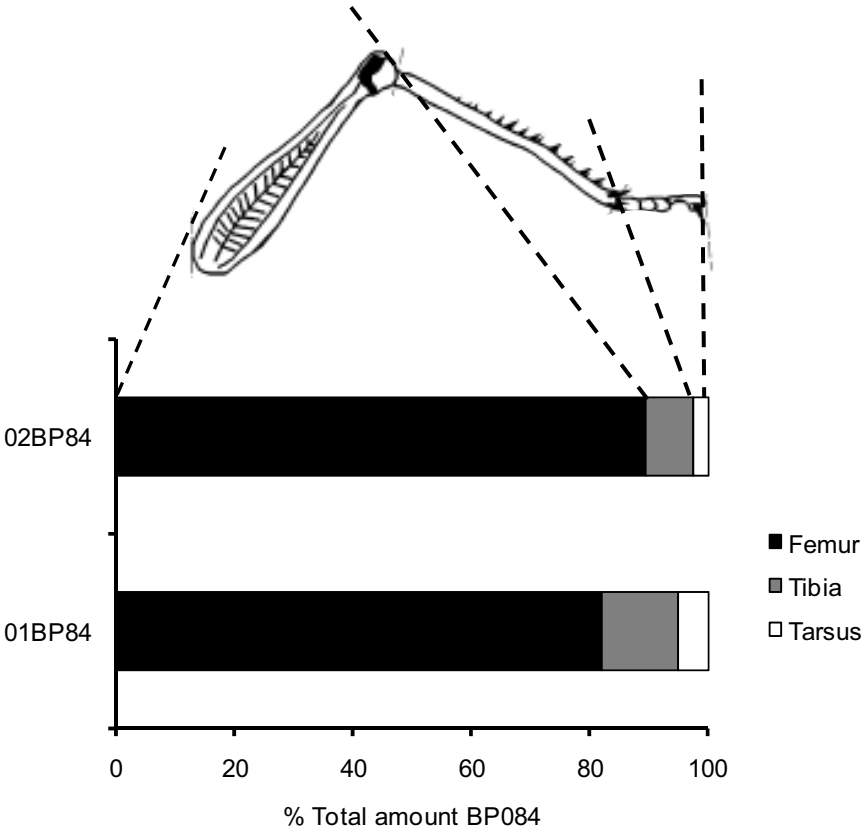


Fig. 3.17. Relative contribution [%] of the major parts of walking and jumping legs (femur, tibia and tarsus) to total the BP84 (both isomers) amount of leg extracts.

3.3.5 GC-EAD recordings

Part of a representative GC-EAD profile of headspace volatiles collected from mature adult *D. maroccanus* and responses of adult male and female antennae is shown in Figure 3.18. Among others, five EAD active compounds were revealed to elicit responses in females (Fig. 3.18 A). These compounds were identified (by comparison of MS and RI with synthetic standards on a nonpolar capillary column) as (2) 1-tetradecanol, (4) hexadecanal, (6) 1-hexadecanol, (7) 2-octadecanone and (9) 02BP84 (numbers on peaks see Figure 3.9 and 3.11). The corresponding amounts of the active compounds were the following: (2) 9 ng, (4) 50 ng, (6) 9 ng, (7) 1.5 ng, (9) 20 ng). In males only the male produced compound (9) 02BP84 provoked antennal responses (Fig. 3.18 B).

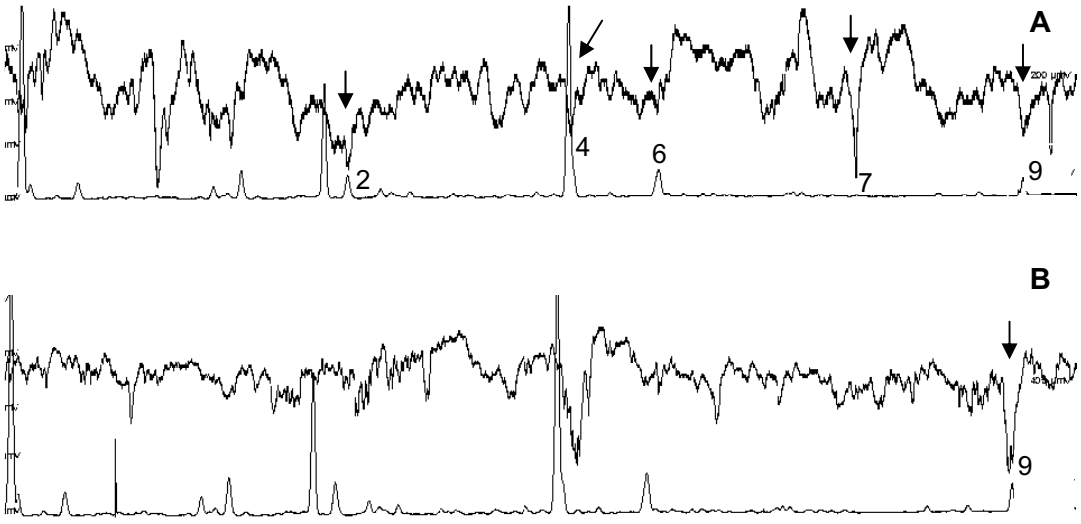


Fig. 3.18. Representative GC-EAD profile using female (A) and male (B) antennae of *D. maroccanus* responding to a 100 µl solution of mature male headspace volatiles collected during 12 h. Active compounds are represented by black arrows. Compound numbers on the peaks (see Figure 3.10): (2) 1-tetradecanol, (4) hexadecanal, (6) 1-hexadecanol, (7) 2-octadecanone, (9) 02BP84.

3.3.6 EAG responses

The electrophysiological responses of male and female antennae of adult Moroccan locusts towards different stimuli were measured in the electroantenogram (EAG). As stimuli were used synthetic compounds and natural extracts derived from conspecific insects and different concentrations of the synthetic compounds detected in desert locust extracts.

a) Natural extracts of D. maroccanus and synthetic compounds

Antennal responses of both, males and females to headspace volatiles of adult males, containing ca. 0.2 µg of 01BP84 and 0.6 µg of 02BP84, and females, as well as egg froth and fecal extracts of both sexes were recorded. Egg froth, female feces and male volatiles elicit the strongest responses (>0.1 mV) in female antennae (Fig. 3.19). In male antennae the highest activity was shown by egg froth extract and male volatiles, provoking significantly higher responses than the other natural extracts (Fig. 3.19).

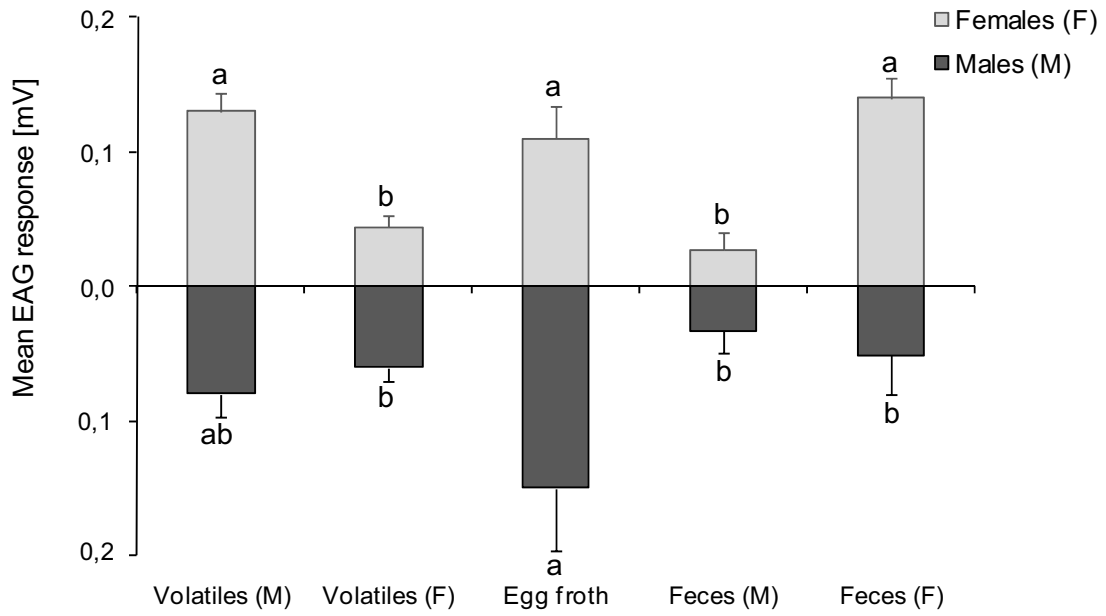


Fig. 3.19. Mean EAG responses (±S.E.M.) of female and male *D. maroccanus* antennae (N=8) to headspace volatiles of adult males (M) and females (F), egg froth and fecal extracts of males (M) and females (F). Bars with different letters within one sex indicate significant differences (*HSD Tukey's post-hoc test*, $P \leq 0.05$).

Furthermore, in adult male and female antenna the activity of three synthetic compounds (10 µg each) that have been detected in headspace volatiles of males (Fig. 3.10) were tested, as well as five compounds (10 µg each) identified from froth plugs secreted by gravid females (Fig. 3.12). For comparison, responses of both sexes were recorded to male volatile samples, containing ca. 1.1 µg of 01BP84 and 3.6 µg of 02BP84, and femur extracts of adult males, containing ca. 0.2 µg of 01BP84 and 1.1 µg of 02BP84 (Fig. 3.20).

Female and male antennae responded significantly different to the tested stimuli (Figs. 3.20 A, B). In general, mean responses were stronger in females. Responses to 2-hexadecanone, 2-octadecanone and 1-hexadecanol were very small, nearly null. The strongest response in both sexes was elicited by the volatile extract of mature male adults: 1.5 times higher in females (Fig.3.20 A: 0,427 ± 0.04 mV) than in males (Fig. 3.20 B: 0.291 ± 0.03). In female antennae, the femur extract and (*E,E*)-2,4-decadienal provoked significantly stronger EAG depolarizations than the other compounds (except male volatile extracts), followed by (*E,E*)-2,4-heptadienal (both found in froth plugs). In contrast, in male antennae no clearly significant responses, except that to male

volatiles and (*E,E*)-2,4-decadienal, were obtained among the different stimuli due to the low intrinsic depolarizations displayed (Fig. 3.20 B).

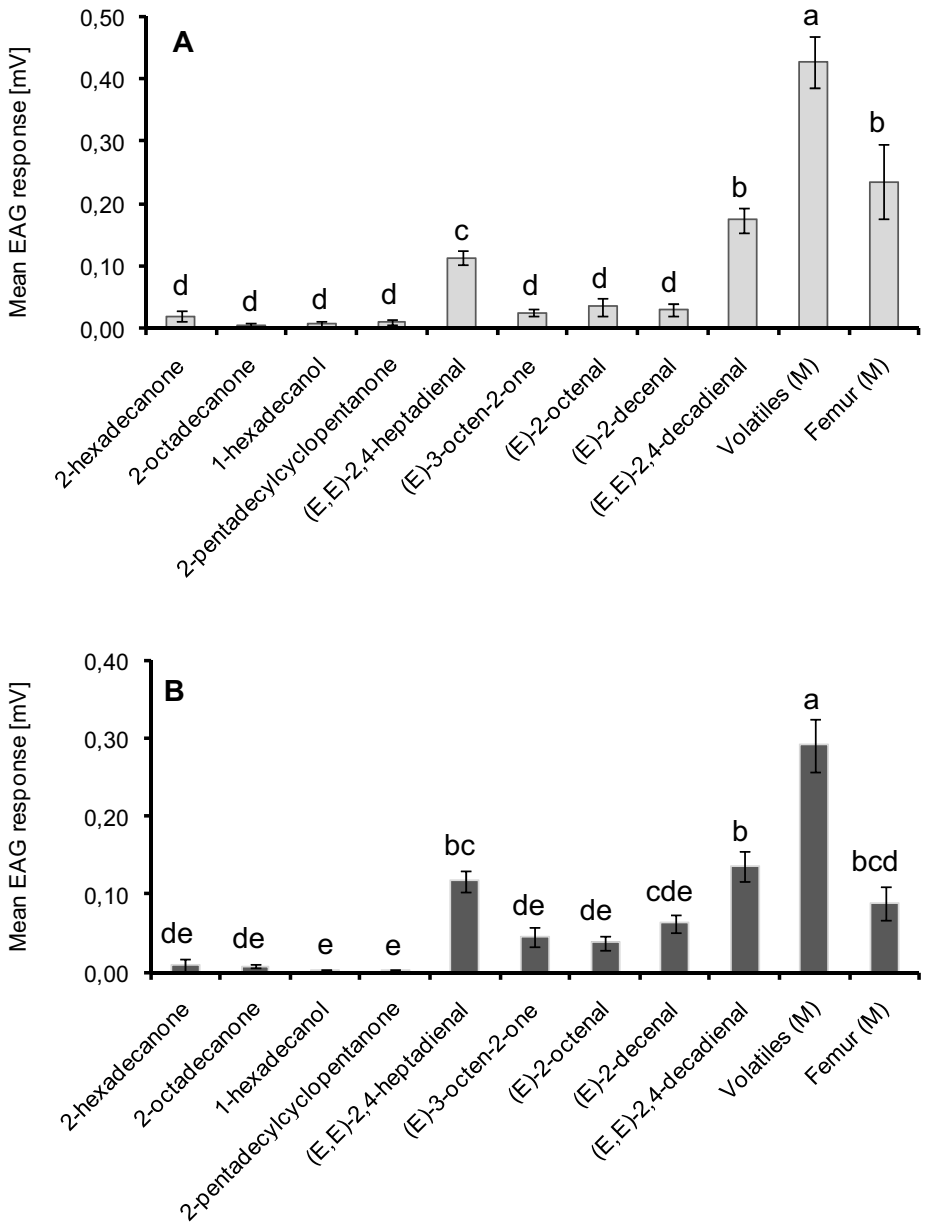


Fig. 3.20. Mean EAG responses (±S.E.M.) of female (A) and male (B) *D. maroccanus* antennae (N=8-13) to nine synthetic compounds [10 µg each] derived from male headspace volatiles (2-hexadecanone, 2-octadecanone and 1-hexadecanol) and from egg froth extracts ((*E*,*E*)-2,4-heptadienal, (*E*)-octen-2-one, (*E*)-2-octenal, (*E*)-2-decenal and (*E,E*)-2,4-decadienal), male (M) headspace volatiles and male (M) femur extract were tested. Bars with different letters indicate significant differences (*DMS post-hoc test*, $P \leq 0.05$).

b) Antennal responses to synthetic compounds from S. gregaria

In EAG assays were analyzed the responses elicited by male and female *D. maroccanus* antennae to three synthetic compounds, PAN, acetophenone and veratrole, identified from mature adult desert locusts, *S. gregaria*. All tested stimuli provoked EAG responses at doses of 1 µg, 10 µg and 50 µg (Figs. 3.21 A, B). All stimuli display a mean response threshold practically identical in both sexes (maximum response of ca. 0.3 mV to the highest concentration of the stimuli). Dose-dependent responses were observed in female antennae to PAN and acetophenone (Fig. 3.21 A). Veratrole only displayed significant responses at 10 and 50 µg doses relative to 1 µg. In males the two highest doses (10, 50 µg) elicited significantly stronger EAG responses than the lowest dose (1 µg) in all treatments (Fig. 3.21 B).

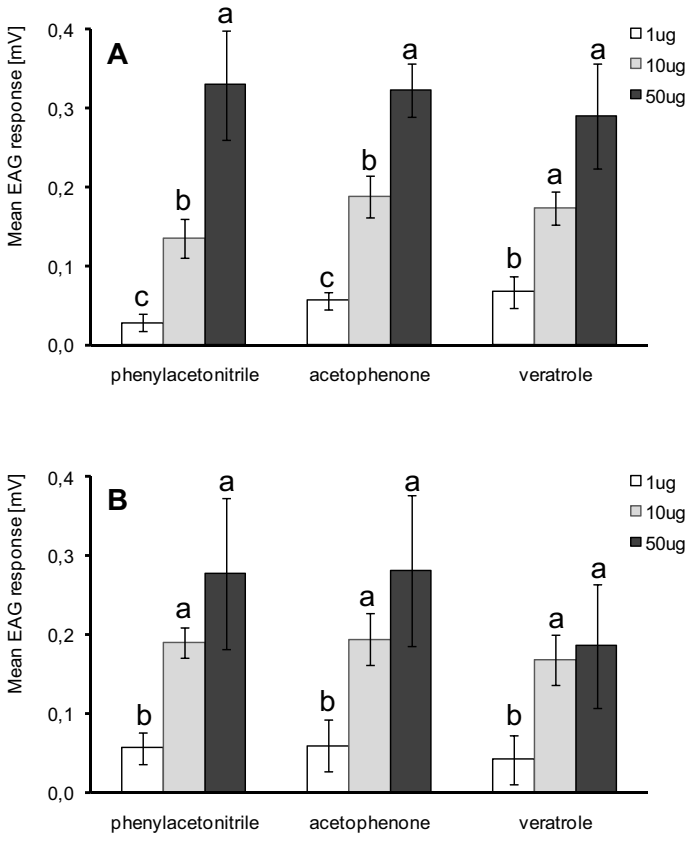


Fig. 3.21. Mean EAG responses (±S.E.M.) of adult *D. maroccanus* female (A) and male (B) antennae (N=8-10) to different doses of phenylacetonitrile, acetophenone and veratrole, active compounds from the desert locust. Bars within the same treatment with different letters are significantly different (*Tukey's HSD test*, $P \leq 0.05$).

3.3.7 Oviposition bioassays

The behavioral response of gravid females to synthetic mixtures and a natural extract was studied in oviposition bioassays. We tested if sand mixed with egg froth extract, a five component blend (compounds identified from egg froth of *D. maroccanus*) and a four component blend (compounds described as part of the pheromone system of *S. gregaria*) have any effect (attraction or repulsion) on the oviposition preference of gravid females. As control untreated sand was offered.

In general, very few egg pods laid by gravid females were found in both years of the experiment (Table 3.3). A total of 18 egg pods were observed in 2009, while the number decreased to 6 in 2010. No significant differences were observed in none of the treatments. Only the egg froth extract from gravid females (treatment 1) showed a clear preference in oviposition compared to control sand, whereas females negatively respond to the 4-component blend (treatment 3).

Table 3.3 Number of egg pods laid by gravid females as response to different treatments

Year	Day	Treatment 1 Test[a]/Control		Treatment 2 Test[b]/Control		Treatment 3 Test[c]/Control		Treatment 4 Con.[d]/Con.	
2009									
	03/07								
	07/07								
	13/07			1	1			2	
	21/09	2							
	29/07				1	1	2		3
	07/08						3	2	
2010									
	16/07					1			
	22/07	1		1					
	30/07								
	05/08						1	1	
	12/08								1
	20/08								
	Σ	3	0	2	2	2	6	5	4

[a] Egg froth 350 mg secreted by gravid females.
[b] Blend five compounds (50 µg each) derived from egg froth of *D. maroccanus*.
[c] Blend four compounds (50 µg each) identified from adult *S. gregaria*.
[d] Control = moistened sterilized sand.

3.4 DISCUSSION

The Moroccan locust, *D. maroccanus*, is found in northern Africa, western Asia and predominantly in the climatic Mediterranean area (Uvarov, 1977). This grasshopper of the family Acrididae is characterized by a solitary appearance that under favorable environmental and climatic conditions can increase drastically in number. In the gregarious phase, the locust congregate and form swarms that represent one of the most serious pests of many cultivated plants threatening agriculture crops of steppes, foothills and arid areas (Latchinisky, 1998; Quesada-Moraga and Santiago-Alvarez, 2001b). To this date, control of the pest is limited to the use of polluting and harmful chemical treatments, so design and development of environmentally-friendly control measures is essential. A better knowledge of the locust's biology, physiology and chemical communication is the first step to establish an integrated pest management to control the pest

In the last decades numerous studies were carried out on the life history of the desert locust, *S. gregaria*, related to oviposition, transformation to gregarious phase and maturation mediated by pheromones (Byers, 1991; Ferenz and Seidelmann, 2003; Hassanali, et al., 2005). Additionally, first attempts have been started to transfrom the multitudinous findings about this threatening locust pest into alternative control strategies (Cressman, 1998; Enserink, 2004). In contrast, almost nothing is known about the chemical ecology of the Moroccan locust. Hence, in the present work we initiated analysis of the volatile emission of nymphs and adults for the first time. Activity of the identified compounds was tested in electrophysiological and behavioral experiments with the aim to establish the presence of possible pheromonal components.

Primarily, the morphology and structure of antennal sensilla was investigated by SEM to obtain information about the olfactory system of *D. maroccanus*. It is assumed that the olfactory system plays an important role both in development and behavior as in other Acrididae (Norris, 1954; 1964; Loher, 1990). Fine structure and distribution of antennal sensilla on the antennae of the Moroccan locust conform in most respects to sensilla types already described in other acridid species (Greenwood and Chapman, 1984; Ochieng, et al., 1998). Thus, four different types of sensilla were identified, similar to those found in *Locusta migratoria* L. (Altner, et al., 1981), and *S. gregaria* (Ochieng, et al., 1998), with no differences between sexes. The nomenclature of sensilla types in the present work is identical to that used by Ochieng and coworkers (1998). Two types, *s. basiconica* and *s. trichodea* were most likely olfactory sensilla. As

observed in the present work, *s. basiconica* possess a multitude of wall pores, while *s. trichodea* dispose of fewer pores. *S. basiconica* of *S. gregaria* have several branched outer segments and *s. trichodea* command only a few sensory neurons. These structural features are typical of olfactory receptors in general (Altner and Prillinger, 1980; Zacharuk, 1980). Receptor neurons present in *s. basiconica* have been shown to respond to aggregation pheromone compounds of *S. gregaria* (Hansson, et al., 1996). In addition, olfactory receptor neurons, sensitive to behaviorally active volatiles (chemosensitive), have been found in *s. trichodea* and *s. coeloconica* of *S. gregaria* (Ochieng and Hansson, 1999; Anton, et al., 2002). In our work the latter type was detected in great number on the entire antenna of the Moroccan locust and its characteristics correspond to the *s. coeloconica* found in other insects. The non-porous *s. chaetica*, mainly found on the terminal segment of *D. maroccanus*, probably function as taste or mechanoreceptor. This assumption arose from comparison with *S. gregaria* antennae (Ochieng, et al., 1998) and the before mentioned strategic location on the terminal segment. Besides, their structural features, such as a flexible socket, a non-porous wall and an apical pore indicate the supposed function of taste reception. But it is still unclear if the apical pores of these sensilla in both locust species are capable of opening and closing as those of gustatory sensilla on the maxillary palps (Blaney and Chapman, 1969).

In oviposition bioassays it was observed that gravid females probe the sand with their antennae before ovipositing (personal observation). Furthermore, EAG recordings have confirmed the presence of antennal receptors responsive to odors from egg froth, as in the desert locust (Saini, et al., 1995). Thus, it is expected that apart from olfactory, contact chemical cues may play a significant role in the chemical communication of locusts and among them *D. maroccanus*. All the recorded analogies in sensilla structure and distribution of the Moroccan locust antennae with the desert locust provide strong evidence that the olfactory perception in both species should be quite similar and therefore, a close phylogenetic relationship is presumed. However, detailed physiological investigations remain to be performed to show whether the four types of sensilla found in *D. maroccanus* respond to chemical (chemoreceptors) and/or mechanical stimulations (mechanoreceptors).

For detection of possible active compounds that could be part of the pheromonal system of *D. maroccanus,* several carboxylic fatty acids (C_5-C_{16}) and unsaturated aldehydes (with one or two double bounds) were identified from egg froth and fecal extracts of females. Two of the carboxylic acids, hexanoic and octanoic acid, have been described to evoke strong aggregation behavior in nymphs of the desert locust

(Torto, et al., 1996). But more interestingly seems the presence of two unsaturated ketones ((*E*)-3-octen-2-one and (*E,E*)-3,5-octadien-2-one) in froth extracts of *D. maroccanus*, due to the fact that the partly identical unsaturated ketones (*Z*)-6-octen-2-one and (*E,E*)-3,5-octadien-2-one enhanced oviposition on *S. gregaria*, and elicited EAG activity in female antennae (Torto, et al., 1999; Malual, et al., 2001). In *D. maroccanus* (*E*)-3-octen-2-one displayed only slight antennal responses and so specific oviposition experiments should be conducted to establish the possible behavioral activity of the two ketones.

Two of the unsaturated aldehydes derived from egg froth of the Moroccan locust, (*E*,*E*)-2,4-decadienal and (*E*,*E*)-2,4-heptadienal, were identified as semiochemicals of the predatory stink bug, *Eocanthecona furcellata*, and aggregation pheromone of the leaf beetle *Diorhabda elongate,* respectively (Ho, et al., 2003; Cossé, et al., 2005). Both compounds elicited significantly stronger EAG responses in male and female antennae of *D. maroccanus* than the other detected unsaturated aldehydes and ketones. Additionally, natural egg froth extracts elicited significantly the strongest antennal responses in adults of both sexes compared to male fecal extracts and female headspace volatiles. In previous observations we noticed that females of the Moroccan locust deposit their egg pods in suitable oviposition sites wherein other females had oviposited in prior years. Therefore, the egg pods should comprise compounds that attract gravid females for oviposition. In this context, Saini and coworkers (1995) demonstrated that chemical signals (pheromonal compounds), originated from the froth of egg pods of the desert locust attracted gravid female *S. gregaria* to common egg laying sites. In addition, two of these compounds were shown to elicit aggregation of females in oviposition assays (Rai, et al., 1997). Therefore, we tested if natural and synthetic extracts derived from female Moroccan and desert locust intermixed with moistened sand have any effect on the oviposition behavior of gravid conspecific females. Froth extract and two blends of synthetic compounds, (*E,E*)-2,4-heptadienal, (*E*)-3-octen-2-one, (*E*)-2-octenal, (*E*)-2-decenal and (*E,E*)-2,4-decadienal identified from *D. maroccanus* egg froth, and PAN, acetophenone, veratrole and benzaldehyde, described as pheromonal components in the desert locust, were applied to the sand. In general, only a few egg pods were laid by females during the two test periods and no significant differences were observed between the different treatments. These results are surprisingly since in the field gravid females can oviposit 2-4 egg pods each. By pooling the results of both years a slight preference towards sand treated with egg froth extract was shown (test = 2 egg pods; control = 0 egg pods), as well as an implied avoidance behavior to the 4-component blend of *S. gregaria* compounds (test = 2 egg

pods; control = 6 egg pods). Nevertheless, in further oviposition experiments the dimensions of cages and oviposition cups and the climatic conditions (temperature, humidity, light…) in the laboratory may have to be redesigned. Besides, the insects were collected directly from the field and determination of age was not precise. May be the adult females were too old, had already oviposited in the field or the texture of sand for egg-laying was not adequate. Thus, new bioassays are essential to establish the possible activity of the natural extracts and the identified compounds, and also to understand their role in the chemical communication and behavior of the Moroccan locust.

By solid phase microextraction (SPME) several saturated linear or branched hydrocarbons were identified in both males and females. In general, this class of compounds is considered to act as close range and contact pheromones, sex attractants and aphrodisiacs, aggregation pheromones and kairomones in many insects (Lockey, 1988; 1991; Lockey and Oraha, 1990; Howard and Blomquist, 2005). Oraha and Lockey (1990) have shown that cuticular lipids of *Locusta m. migratoriodes* and *S. gregaria* comprise five lipid classes including esters, free fatty acids, free primary alcohols, triglycerides and hydrocarbons. Furthermore, it was demonstrated that some hydrocarbons can induce gregarious behavior in solitarious nymphs of the desert locust (Heifetz, et al., 1997). Thus, they expected that cuticular hydrocarbons could function as contact pheromones in the desert locust. It is possible that cuticular compounds of the Moroccan locust are also involved in the kin recognition during the gregarious phase, when several thousand individuals are clumped together. This assumption has to be confirmed in special designed bioassays.

In headspace volatile samples of the Moroccan locust significant differences between ages and sexes were revealed. Mature adult males emitted nine compounds that were absent in nymphs of both sexes and in adult females. Seven of these compounds could be identified but two chemicals (01BP84 and 02BP84) have remained unidentified. Identification of the latter is in process. The unique presence of these compounds in mature was males was confirmed by SPME analysis. Volatile analyses and quantification over time indicated that adult males started the production of some of these compounds (aldehydes and the two unknown compounds) 1-4 days after fledging and reached the maximum emission 12-20 days after fledging. These results correspond to the maturation process in adult males. The highest amounts of the two male-produced primary alcohols with a chain length of 14 carbons (1-tetradecanol) and 16 (1-hexadecanol) were recorded one week later. It is possible that these compounds are metabolic/transformation products of the corresponding

aldehydes. In contrast, in the locust subfamily of *Melanoplus* (Acrididae), the identified free primary alcohols are of longer chain, ranging from 22 to 32 carbons with tetracosanol (C_{24}) and hexacosanol (C_{26}) in the highest proportions (Oraha and Lockey, 1990). We hypothesized that the male-produced compounds detected in volatiles of the Moroccan locust could be involved in the pheromone system of this species. From desert locust it is known that older adult males, in contrast to females, nymphs and immature males, start emission of several male-specific compounds (particularly benzaldehyde, veratrole, and PAN) 10 to 12 days after fledging (Deng, et al., 1996). The maximum amount of the dominant component PAN was recorded in males 15-25 days after the final molt. These compounds represent part of the pheromonal system in *S. gregaria.* Besides, it was demonstrated that this male-specific volatile blend was behaviorally attractive to mature and immature male and female adults but not to nymphs (Torto, et al., 1994; Obeng-Ofori, et al., 1994b). Role of the male-specific compounds in the communication, maturation, aggregation and sexual attraction of *S. gregaria*, especially that of PAN, is being deeply investigated in recent years (Pener and Yerushalmi, 1998; Hassanali and Torto, 1999; Mahamat, et al., 2000; Ferenz and Seidelmann, 2003; Seidelmann, et al., 2005; Seidelmann, 2009; Bashir and Hassanali, 2010). This will be discussed in detail in Chapter 4. In antennae of adult male and female *D. maroccanus* we tested the electrophysiological activity of the major pheromonal compounds of the desert locust, PAN (mature male volatiles), veratrole (volatiles and egg froth) and acetophenone (egg froth). All compounds elicited antennal responses in both sexes in a dose-dependent manner. These results are somewhat surprising, since none of the tested compounds were detected in the Moroccan locust. An explanation could be that both locust species are of the same family (Acrididae) and they may still dispose of the same or similar chemoreceptors in the antennae, although genetically and geographically they may have been separated during evolution.

Nonetheless, in the present work we have put the emphasis on the compounds identified from volatiles of adult male Moroccan locusts. After discovering that male *D. maroccanus* release a bouquet of volatiles when becoming sexually mature, among them large amounts of BP84 and other long-chain compounds such as aldehydes, alcohols and ketones, their origin was investigated. We supposed that they could be produced *de novo* and released directly by mature male Moroccan locust. So far, no pheromone producing sites have been described in this locust species. Headspace volatile collections and analysis of extracts allowed us to identify the isomers of BP84 in other body parts (wings and legs) of adult males. Legs produced up to three times more of both compounds than wings. These results are similar to the findings in desert

locust made by Seidelmann and coworkers (2003). They showed that wings and legs of mature gregarious males of *S. gregaria* are the main releasing sites of the major pheromone PAN, whereas the abdomen and head only contained trace amounts of this compound. They supposed that epidermal gland cells are the putative sites of PAN biosynthesis and perhaps *D. maroccanus* could also dispose of such epidermal cells to synthesize and release the BP84 compounds.

By comparison of the detected amounts of BP84 from different leg parts of the Moroccan locust significant differences were apparent. The largest amounts of the two isomers were produced by the femur, whereas in the outermost part of the legs, the tarsus, only small amounts of the compounds were detected. This is opposed to the results from the desert locust, where the tarsus of the hind legs emitted similar amount of PAN as the tibia and considerable more than the much larger femur (Seidelmann, et al., 2003). High production rate of PAN in the tarsus was considered to proceed from the glandular epidermal cells in tarsi of male desert locusts (Kendall, 1972). Morphological analyses of the different body parts in the Moroccan locust to identify specific epidermal cells and/or glands are required to explain our results.

The electrophysiological activity of volatiles emitted by mature adult males *of D. maroccanus*, male femur extracts and different compounds identified from egg froth and male headspace volatiles was evaluated in GC-EAD and EAG. In addition, 2-pentadecylcyclopentanone was also included since we supposed that this compound could be similar in structure to the two unknown isomers of BP84. In GC-EAD five of the male-specific compounds (1-tetradecanol, hexadecanal, 1-hexadecanol, 2-octadecanone and 02BP84) elicited antennal responses in females but in male antenna only 02BP84 was active. Results from the electroantennogram analysis showed that in male and female antennae headspace volatiles of males provoked the strongest responses. Femur extract of adult males, containing BP84 as the major component, elicited also strong antennal responses in both sexes. 2-Pentadecylcyclopentanone did not provoke any antennal response. Therefore, we presume that the adult male-specific BP84 compounds could be part of the pheromonal components in the Moroccan locust. To confirm this assumption, the structural characterization of the compounds should be determined as well as the type of activity they may be involved in.

Altogether, this work is the first step in the investigation of the chemistry, morphology and biology of *D. maroccanus*. By deploying the knowledge already acquired of *S. gregaria* on the Moroccan locust many parallels have come to light. The

findings, especially those of volatile compounds exclusively produced by mature adult males are very useful to advance in the investigation of this pest species. Further studies, especially focused on the locust's behavior are necessary to confirm the activity and/or the pheromonal character of the identified compounds. Knowledge of how these compounds influence on locusts behavior will be of utmost importance to develop new biorational control methods for the pest.

CHAPTER 4

Maturation studies in gregarious nymphs grouped with conspecific adults of the desert locust, *Schistocerca gregaria* (Orthoptera: Acrididae)

4.1 PREFACE

The desert locust, *Schistocerca gregaria* (Forskål), is an acridid pest which is subject of great research efforts (Hassanali, et al., 2005). The great interest derives from the fact that this swarm-forming species of locusts causes severe damage in agriculture in large parts of Africa and Asia (Anton et al., 2002), as described in the Old Testament. The severity of *S. gregaria* is due to their enormous numbers, their immense dispersal range and the unpredictable invasion strategies used by this pest, which seem to occur in irregular occasions (Enserink 2004; Buhl et al., 2006).

Between 1986 and 1989 (worst invasion in modern time) the locust affected some thirty African countries (Showler and Potter, 1991; Buj, 1995). International aid almost reached 250 million dollars and more than 3 million liters of insecticide were used between January and July, 1988 (Magor, 1989; Lorelle, 1989). In June 1993 the desert locust was devouring the harvests of Ethiopia, Sudan, Eritrea, Somalia and Djibouti (Showler, 1995). During invasions, desert locusts may spread over an area of some 29 million km^2 and extend over or into parts of 60 countries. This is more than 20% of the total land surface of the world. A single swarm can contain billions of insects and travel hundreds of kilometers each day and may consume up to 3000 tons of vegetation per day (Simpson and Sword, 2008). Thus, *S. gregaria* has the potential to damage the livelihood of a tenth of the world's population (FAO, desert locust information service). Therefore, a large number of research projects addressing questions about the biology, physiology and behavior of the pest have been launched, with the aim to find biological control measures against it (Showler, 2002).

The desert locust lives a total of about 3-5 months depending mostly on weather and ecological conditions. The life cycle (development by incomplete metamorphosis) comprises three stages: egg, nymph and adult (Fig. 4.1). After mating, gravid females stretch their abdomen to about twice its normal length, laying eggs (80-150) bound together by a frothy secretion, which forms an egg pod in sandy soils at a depth of 5-10 cm below the surface. Females can oviposit at least three times in their lifetime, usually at intervals of about 6-11 days. After 10-20 days, depending on temperature, the eggs hatch into wingless nymphs, also called hoppers, which resemble miniature, incomplete adults (Ashall and Ellis, 1962; Roffey and Popov, 1968). In order to grow, the nymph needs to shed its cuticle and expand its body before the new cuticle hardens. Each stage between molts is called an *instar*. At each molt, the nymph develops more adult features until, after the 5^{th} instar it is a fully formed adult with wings (The Anti-Locust Research Centre, Ministry of Overseas Development, 1966).

Hoppers develop over a period of about 30-40 days until becoming adult. Following immature adults, called fledglings (pink color), need about 2 weeks to 9 months, according to environmental circumstances, to become mature (yellow color). If food is in adequate supply and the hoppers are not forced to crowd together when they emerge from the eggs, the locusts live their lives separately as do other grasshoppers and are widely dispersed. If, however, the hoppers are crowded together for any reason, they enter into a gregarious phase which represents a 400-fold increase in their numbers. The crowd turn into the before mentioned swarms and the flying gregarious adults are considered the plague (Cressman, 1998). So, the expression of phenotypically plastic traits that transform solitary-living individuals into gregarious, swarm-forming locusts is the result of a drastically increase in local population density (Pener, 1991; Pener and Yerushalmi, 1998).

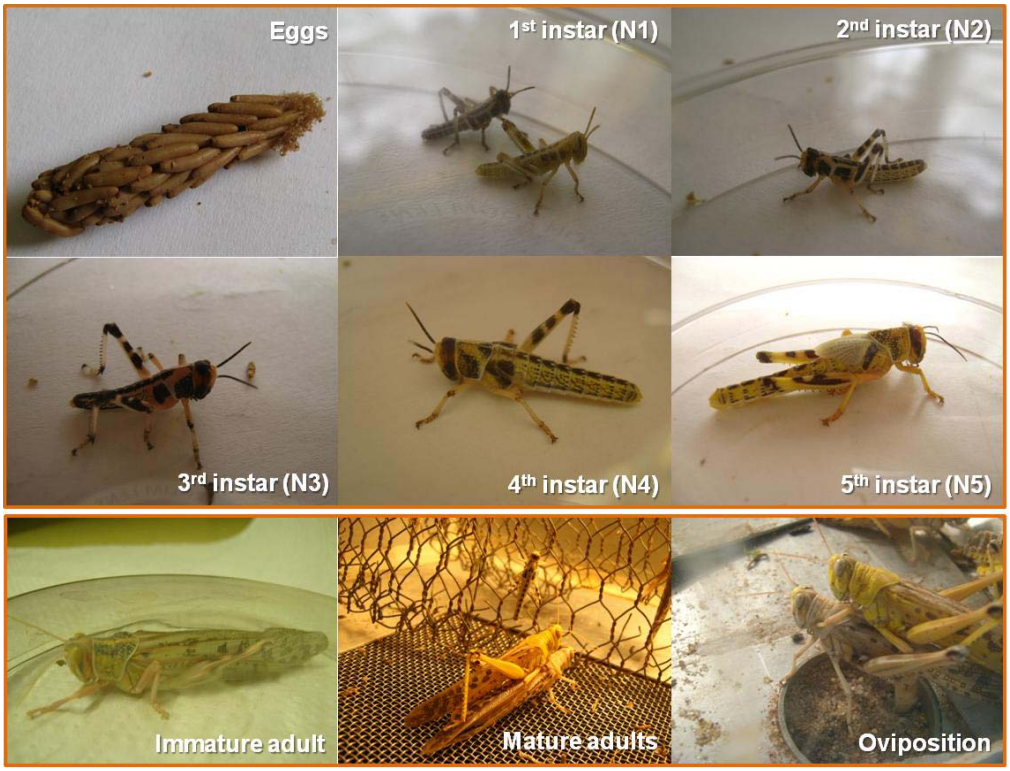


Fig. 4.1. Life cycle of the desert locust *S. gregaria*. Non-flying wingless 1st instar nymphs hatch from eggs laid by gravid females. Hopper phase consists of 5 instars, before immature pink fledglings develop. Copulating yellow mature adults close the cycle by laying eggs.

Depending on the population density, the desert locust switches between gregarious (high density) and solitarious phase (low density), which is accompanied by changes in morphological, physiological and behavioral parameters (Uvarov, 1966; Deng, et al., 1996; Simpson, et al., 2001; Bouaïchi and Simpson, 2003; Simpson and Sword, 2007; Anstey, et al., 2009). The phase change is elicited by a combination of olfactory, visual, and tactile cues (Byers, 1991; Roessingh, et al., 1998). In particular, the aggregation behavior (swarm formation), an essential step in the phase transformation, is supposed to be primarily induced by olfactory cues, especially pheromones (Obeng-Ofori, et al., 1994b; Torto, et al., 1994; 1996; Heifetz, et al., 1996; Ferenz and Seidelmann, 2003), although it has recently been demonstrated that the neurotransmitter serotonin might also play an important role in this process (Anstey, et al., 2009).

It is supposed that the olfactory cues involved in phase shifting include several categories of pheromones, so called primer and releaser pheromones (Loher, 1990; Byers, 1991). Releaser pheromones induce immediate behavioral responses (Obeng-Ofori, et al., 1993), whereas primer pheromones have long-term effects on physiology, development or behavior (Ferenz and Seidelmann, 2003). Resulting hormonal and other physiological effects on locust phase polymorphism are described by Pener and Yerushalmi (1998).

Previous studies revealed that pheromones play an important role in maturation, gregarization, reproduction and oviposition of *S. gregaria*. Interestingly, the pheromone blends can differ between the two phases, sexes and also the developmental stages (Obeng-Ofori, et al., 1994b). Gregarious 2^{nd} to 5^{th} instar nymphs for example employ two different aggregation pheromone systems that emanate from the feces and the body surface (Obeng-Ofori, et al., 1994a; Torto, et al., 1996). Mature adult male locusts, on the other hand, produce a different blend of compounds including phenylacetonitrile (PAN) as its major component (≈80%). Parts of this blend have been shown to be behaviorally attractive to mature and immature male and female adults, but not to nymphs, where they interfere with the aggregation system (Obeng-Ofori, et al., 1993; Torto, et al., 1994). In the odor bouquet of female solitary desert locusts, a potential sex-specific pheromone was identified (Inayatullah, et al., 1994; Njagi and Torto, 1998), whereas in gregarious females oviposition-aggregation pheromones were shown to be deposited with the egg pods (Saini, et al., 1995; Rai, et al., 1997; Torto, et al., 1999).

The effects that can be deduced to the action of pheromones in the gregarious phase are social cohesion (aggregation), synchronous maturation, collective oviposition, and transmission of information about phase traits to the progeny (McCaffery, et al., 1998; Hassanali and Torto, 1999; Hagele and Simpson, 2000; Malual, et al., 2001; Miller, et al., 2008; Verlinden, et al., 2009). Especially the maturation synchrony in gregarious individuals is of major importance for synchronized mating and communal oviposition. Here, different pheromonal signals are involved (Norris, 1954; Loher, 1960; Torto, et al., 1999; Mahamat, et al., 2000; Schmidt and Albütz, 2002). During the maturation process, males change their body color from pink to yellow. This color shift is associated with the emission of sex-specific volatiles (Pener, 1991; Seidelmann and Ferenz, 2002; Seidelmann, et al., 2003). For example, the male-specific PAN displays pheromonal activity by interfering with the maturation process. Previous studies had demonstrated delayed maturation in immature adults grouped with conspecific nymphs in the presence of visual, tactile, and chemical stimuli (Assad, et al., 1997), while maturation was accelerated in young adults grouped with mature adult males (Mahamat, et al., 1993; 2000). Therefore, it has been suggested that the accelerating (provoked by mature adults) and retarding (provoked by nymphs) effects could promote maturation synchrony in the desert locust (Ferenz and Seidelmann, 2003; Hassanali, et al., 2005).

However, the reverse effect of grouping nymphs with conspecific adults on the maturation of nymphs was unknown. The present work was carried out to fill this gap. We investigated the grouping effect and assessed the development and maturation of nymphs regarding: (a) body weight, (b) body length, (c) developmental time (time to fledge), (d) PAN-titer in adult male volatiles, (e) number of egg pods laid by adult females and (f) levels of methyl farnesoate (MF) and juvenile hormone (JH) III in the hemolymph of nymphs of different instars. We supposed that there is an effect on the maturation process of nymphs when grouping them with adults, and hypothesized that the presence of *S. gregaria* adults and their produced volatiles lead to accelerate development and maturation in conspecific gregarious nymphs. This could have important implications for understanding gregarization and swarm formation in the desert locust.

4.2 METHODOLOGY

4.2.1 Insects

Specimens of the desert locust were obtained from the Insect and Animal Breeding Unit (IABU) of ICIPE (International Centre of Insect Physiology and Ecology, Nairobi, Kenya). Insects (50-100) of both sexes were reared under crowded conditions in glass-fronted aluminum cages (50×50×50 cm^3) in a well-ventilated room (4.5×4.5 m^2). The insects were kept separately by developmental stage and age. The temperature was 33 ± 2°C, relative humidity was 60%, and the photoperiod was 12:12 L:D. Fresh grass and wheat bran was provided every day.

4.2.2 Experimental cages

The effect of grouping adults with nymphs on the developmental time and maturation of the nymphs was studied in standard aluminum cages (50×50×50 cm^3), provided with a sliding glass in the front, wire gauze on top, bottom and lateral sides, and the back made of aluminum sheath. For oviposition experiments with gravid females, four aluminum oviposition cups (10 cm high × 4.0 cm ID) were placed into a false floor close to the sliding door. The oviposition cups were filled with sterilized and 15% moistened sand, according to Saini and coworkers (1995) and Bashir and coworkers (2000).

4.2.3 Presence effect of adults on maturation and development of 1st instar nymphs

Ten 2-day-old 1st instar nymphs (referred to as recipients) from the gregarious colony were placed in aluminum cages with twenty 7-day-old immature adult males and females (referred to as signal sources), as described before (treatment I). Recipient insects had visual, tactile, and olfactory contacts with the signal sources. These test assays were replicated 3x. In control experiments ten 2-day-old (1st instar) nymphs as recipients in two different cages (treatment III, two replicates) and 70 nymphs of the same age in three additional cages (treatment II, three replicates) were not grouped with adults (Table 4.1). Insects were supplied with fresh wheat seedlings and wheat bran daily. Recipient individuals were controlled for signs of development and maturation on day 1, 13, 16, 19, 22/23, 26, 29, 32 and 35. We used the following parameters to assess nymph development until becoming pink immature adults: (a) mean body weight of ten individuals, (b) body length of every individual and (c) time until fledging. Thereafter, the closing maturation process of recipient individuals was

monitored by (d) GC titers of trapped PAN from adult male volatiles of different ages (see collection of volatiles), and (e) number of egg-pods laid by adult gravid females 29, 32, 35, 38, 41 and 44 days after fledging. In this last experiment only insect from treatments I and III were compared to guarantee an equal number of recipient individuals (Table 4.1). Each recipient becoming mature was marked to differentiate from signal source insects. Dead signal sources were replaced by new ones of the same age and gender. Furthermore, we analyzed the levels of methyl farnesoate (MF) and juvenile hormone (JH) III in the hemolymph of 2-day-old 4th-instar (L4) and 2-day- and 4-day-old 5th-instar (L5) nymphs from the control (treatment III) and grouping experiment (treatment I). Three insects of the same age and for each treatment were considered.

Table 4.1. Treatments used in studying maturation response of nymphs grouped with conspecific adults and ungrouped nymphs (crowded and uncrowded) of *S. gregaria*.

Treatment	Recipients (R)	Signal sources (S)	No. of insects (R:S)
I[a]	Nymphs	Adults	10:20
II[b]	Nymphs	---	70:0
III[c]	Nymphs	---	10:0

[a] Test treatment: Nymphs grouped with adults (10 males and females) (N=3).
[b] Control treatment: Crowded nymphs (N=3).
[c] Control treatment: Uncrowded nymphs (N=2).

4.2.4 Collection of volatiles

Volatiles were collected by aeration of the insects as described by Torto and coworkers (1996). Briefly, air from a compressed air cylinder was passed through a charcoal filter over locusts in a trapping chamber (25 cm long × 3.5 cm ID), and the volatiles were trapped with Super Q glass tubes (6 cm long × 8 mm ID) (Ars Inc., Gainesville, FL, USA) containing 30 mg of the adsorbent (Fig. 4.2). A flow rate of 350 ml min^{-1} was maintained for 6 h (10.30 am–16.30 pm) at 30 ± 1°C. Volatiles were collected from one adult male (former recipient nymph) exposed to: (I) mature adult males and females (nymphs grouped with adults; test); (II) no adult volatiles (crowded nymphs, control 1); (III) no adult volatiles (uncrowded nymphs, control 2) (Table 4.1).

Crowded and uncrowded nymphs refer to the number of recipient individuals (70 and 10, respectively) in the specified treatment. For each test, collections were done on insects 13-14, 19-20, 25-26, 32-33, and 39-40 days from fledging. Each set was replicated 2-4 times. Trapped volatiles were eluted with 200 µl HPLC grade dichloromethane (DCM) (Sigma-Aldrich Ltd., Milwaukee, WI, USA) and stored at -15°C until used.

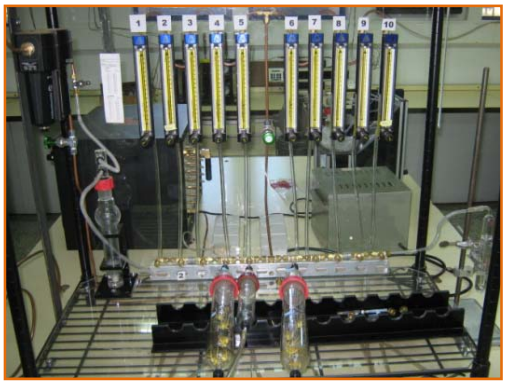


Fig. 4.2. Volatile collection of *S. gregaria* males by dynamic headspace extraction.

4.2.5 Analysis of volatiles

GC-MS analyses of volatiles were carried out as described by Torto and coworkers (1999). Methyl salicylate (600 ng) was added as internal standard to 40 µl of each volatile extract. An aliquot of 1 µl was analyzed by GC coupled to a mass spectrometer (MS) under electron impact (EI, 70 eV) conditions. Analyses were performed on an Agilent 7890 A gas chromatograph equipped with a flame ionization detector (FID) and a HP-5MS glass silica capillary column (5% phenylmethylsiloxane, 30 m×0.25 mm ID×0.25 µm) with nitrogen as carrier gas at a flow rate of 1.2 ml min^{-1}. The oven temperature was initially set at 35°C for 5 min, then programmed at 10°C min^{-1} to 280°C, and held at this temperature for 10 min. Chromatographic peaks of interest were integrated using a 5975 C Inert XL EI/CI MSD with Triple-Axis Detector (Agilent Technologies, Inc., Santa Clara, CA, USA) and compounds were identified by comparison of their mass spectra with those of authentic standards and/or with those from a commercial library (NIST Registry of Mass Spectral Data, 1995).

4.2.6 Collection and analysis of JH III and MF in hemolymph

To collect hemolymph, the junction between the coxa, trochantin and the metathorax of desert locust nymphs exposed and unexposed to adults were punctured with a sterilized scalpel. The hemolymph was allowed to flow into a 200 µl glass micropipette and later transferred into a vial containing 150 µl of distilled water: 300 µl methanol and stored in the freezer at -20^0C until used. To extract hormones, 300 µl of pentane and 10 ng of internal standard were added to the hemolymph samples and then vortexed

for 3 min before centrifuging the samples at 12,000 rpm for 5 min. After centrifugation, the upper pentane layer was removed and then analyzed for JH III and MF by MS. The amounts of JH III and MF in the hemolymph were calculated relative to the amount of internal standard.

4.2.7 Statistical analysis

The collected data were analyzed with PASW 18 (SPSS Inc., Chicago, IL, USA). *One-way ANOVA* on normalized ranks and *DMS post-hoc* analyses were used with the results of growth (length and weight) and PAN production of recipient males. For percentages of fledged individuals *Tukey's HSD post-hoc* test was employed. Means of laid egg-pods by gravid females and hormone titers in the hemolymph of 4^{th}- and 5^{th}-instars nymphs were compared by *Student's t-test* after $Log_{(x+1)}$ transformation of the data.

4.3 RESULTS

4.3.1 Effect of grouping nymphs with adults on development time of nymphs

The maturation effects of grouping nymphs with conspecific adults on growth regarding body weight and length were checked until day 22 after eclosion, when the first individuals of nymphs grouped with adults (test; treatment I) started fledging (Figs. 4.3; 4.4). Significant accelerated development (faster growing) was observed in nymphs that have been grouped with adults on every revision day compared with nymphs that were not grouped and stayed uncrowded (control 2; treatment III) (Figs. 4.3; 4.4). Differences between ungrouped crowded nymphs (control 1; treatment II) and nymphs grouped with adults (test, treatment I), demonstrating faster growing of the latter, were visible but not significant at all. Significant differences on body length were determined between nymphs grouped with adults and ungrouped crowded nymphs of the same age from day 16 after eclosion (Fig. 4.4).

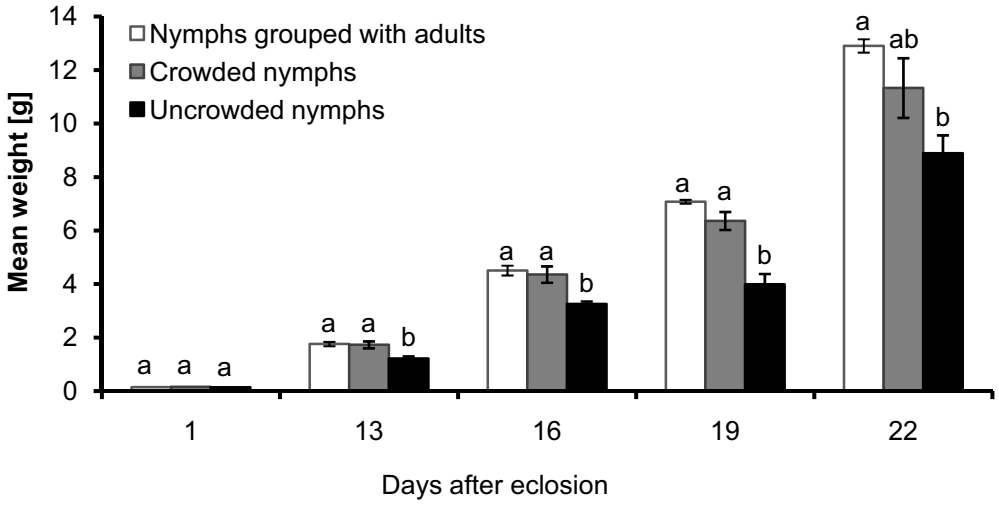


Fig. 4.3. Effects of grouping nymphs with adults on the development time of nymphs concerning body weight. Mean weight (±S.E.M.) of ten individuals of *S. gregaria* nymphs that were grouped with adults (treatment I), ungrouped-crowded (treatment II) and ungrouped-uncrowded (treatment III) at different days after eclosion is displayed. Bars within the same day with different letters are significantly different (*DMS post-hoc test*, $P \leq 0.05$).

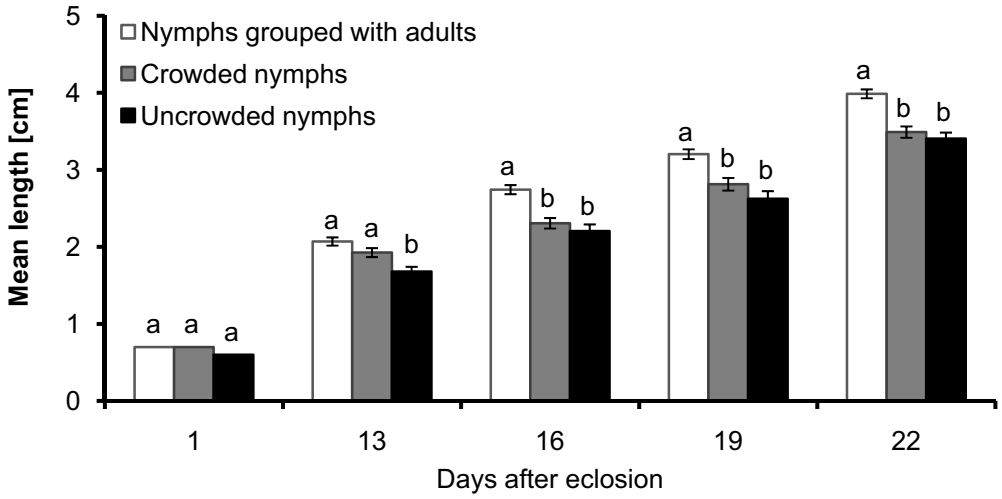


Fig. 4.4. Effects of grouping nymphs with adults on the development time of nymphs concerning body length. Mean length (±S.E.M.) per individual of 10 *S. gregaria* nymphs that were grouped with adults (treatment I), ungrouped-crowded (treatment II) and ungrouped-uncrowded (treatment III) at different days after eclosion is displayed. Bars within the same day with different letters are significantly different (*DMS post-hoc test*, $P \leq 0.05$).

In addition, 27% of nymphs grouped with adults started significantly earlier (day 23) to become immature adults than crowded and uncrowded nymphs (Table 4.2). After 29 days 100% of the grouped nymphs have fledged, whereas the ungrouped crowded nymphs (CII) took 32 days and the uncrowded (CIII) 35 days (Fig. 4.5).

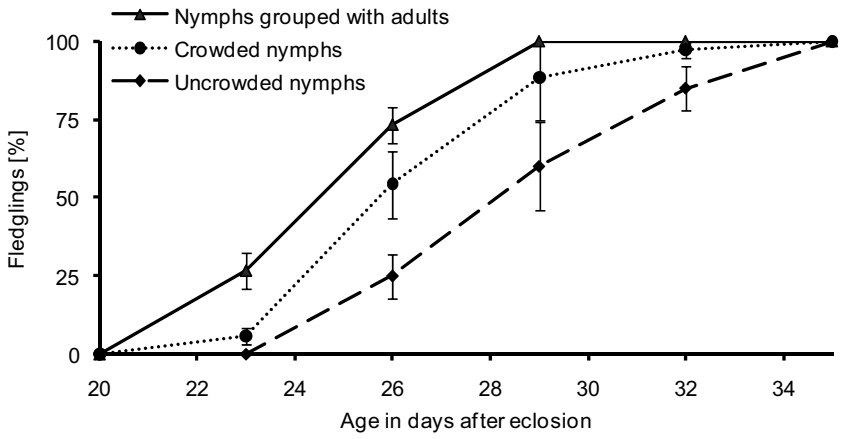


Fig. 4.5. Effects of grouping nymphs with adults on the development time of nymphs concerning time to become immature adults. Percentage of fledged *S. gregaria* nymphs that were grouped with adults (treatment I), ungrouped-crowded (treatment II) and ungrouped-uncrowded (treatment III) at different days after eclosion is displayed.

Table 4.2. Statistical analysis on percentage of fledged *S. gregaria* nymphs grouped with adults, ungrouped-crowded and ungrouped-uncrowded at different ages. Percentages with different uppercase letters are significantly different (*DMS post-hoc test*, *P≤0.05*).

Age in days	*F*-values[a]	*P*[b]	Grouped nymphs (% ± S.E.M)	Crowded nymphs (% ± S.E.M)	Uncrowded nymphs (% ± S.E.M.)
23	31.606	***	27±5.8 a	6±2.5 b	--- b
26	12.313	**	73±5.8 a	54±15.1 ab	26±7.1 b
29	8.442	*	100±0.0 a	89±13.8 ab	60±14.1 b
32	10.906	*	100±0.0 a	97±2.5 a	85±7.1 b
35	---		100±0.0 a	100±0.0 a	100±0.0 a

[a] Variance of group means.
[b] On ranked normalized data (*one-way Anova*, **P*<0.05, ***P*<0.01, ****P*<0.001).

4.3.2 Effect of grouping nymphs with adults on maturation time of nymphs

For evaluation of the maturation time after fledging of individuals that were grouped with adults, the PAN-titer in volatiles of matured recipient males was determined, as well as the number of egg pods laid by matured recipient females. In both cases, altered PAN emission patterns and elevated oviposition rates compared to control individuals demonstrate accelerated maturation. The body coloration confirmed these findings and was used as control; pink colored adults are immature and yellow colored males mature (Schmidt and Albütz, 2002). The dispersion of sexes of recipient individuals was considered equal for these analyses. Male matured individuals grouped with adults started with the emission of PAN 19/20 days after fledging. The maximum PAN levels were reached earlier and test individuals emitted significantly more PAN 25/26 days after fledging than uncrowded nymphs that were not grouped with adults (Fig. 4.6). PAN levels decreased significantly from day 32 after fledging. Crowded nymphs followed the same trend as nymphs grouped with adults with a maximum PAN production at the same nymphs' age (25/26 days after fledging). Ungrouped and uncrowded insects emitted the highest amount of pheromone one week later. During the last examination (39/40 days after fledging) significantly more PAN was emitted by uncrowded nymphs than by insects of treatments I and II.

A similar maturation pattern was observed in gravid females. Female recipient individuals (former nymphs) grouped with adults that had become mature started laying egg pods earlier (29 days after fledging) in significantly higher number than uncrowded nymphs (III) until 32 days after fledging (Fig. 4.7). Uncrowded nymphs (control) that

were not grouped with adults laid egg pods over time followed also a parabolic curve, but with a displaced maximum at 38 days after fledging (Fig. 4.7).

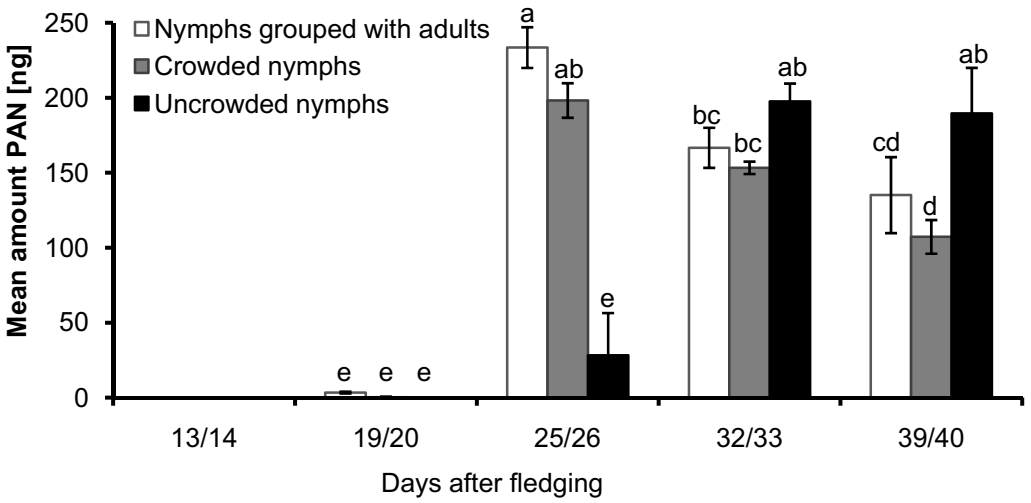


Fig. 4.6. Effects of grouping nymphs with adults on the maturation time of nymphs concerning PAN production in males. Mean amount (±S.E.M) per individual and hour of PAN produced by *S. gregaria* adult males that were grouped with adults (treatment I), ungrouped-crowded (treatment II) and ungrouped-uncrowded (treatment III) at different ages after fledging is displayed. Bars with different letters indicate significant differences between treatments and ages (*DMS post-hoc test*, $P \leq 0.05$).

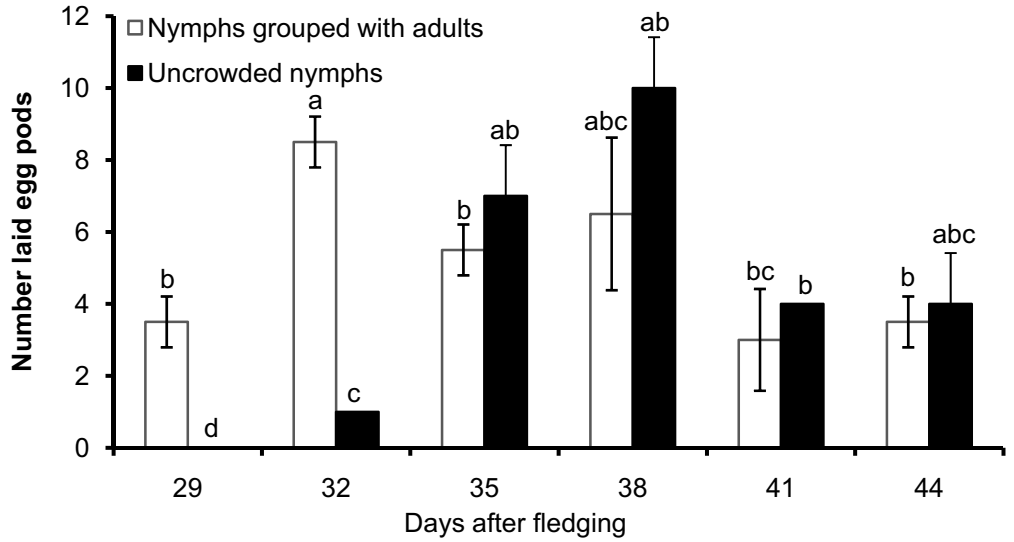


Fig. 4.7. Effects of grouping nymphs with adults on the maturation time of nymphs concerning oviposition in gravid females. Mean number of egg pods (±S.E.M.) laid by *S. gregaria* adult females that were grouped with adults (treatment I) and ungrouped-uncrowded (treatment III) at different days after fledging is displayed. Bars with different letters indicate significant differences between treatments and ages (*Student's t-test*, $P \leq 0.05$).

4.3.3 Effect of grouping nymphs with adults on hormone levels in the hemolymph of nymphs

The levels of MF and JH III in the hemolymph of nymphs grouped with adults were evaluated at different nymphal stages and compared to control treatments. In 2-day-old 4th- and 5th–instar nymphs the levels of both hormones were reduced in grouped individuals compared to uncrowded nymphs. The opposite effect was observed with MF levels in 4-day-old 5th–instar nymphs. At this stage no JH III was detected. The amount of MF in uncrowded insects was almost close to zero (Fig. 4.8). Furthermore, the levels of JH III increased dramatically from 2-day-old 4th instar to 5th instar in uncrowded nymphs, whereas in grouped insects the amount of the hormone was nearly identical (Fig. 4.9). In both experiments the MF levels were notably higher (up to 600x) than those of JH, but in all cases no significant differences were observed.

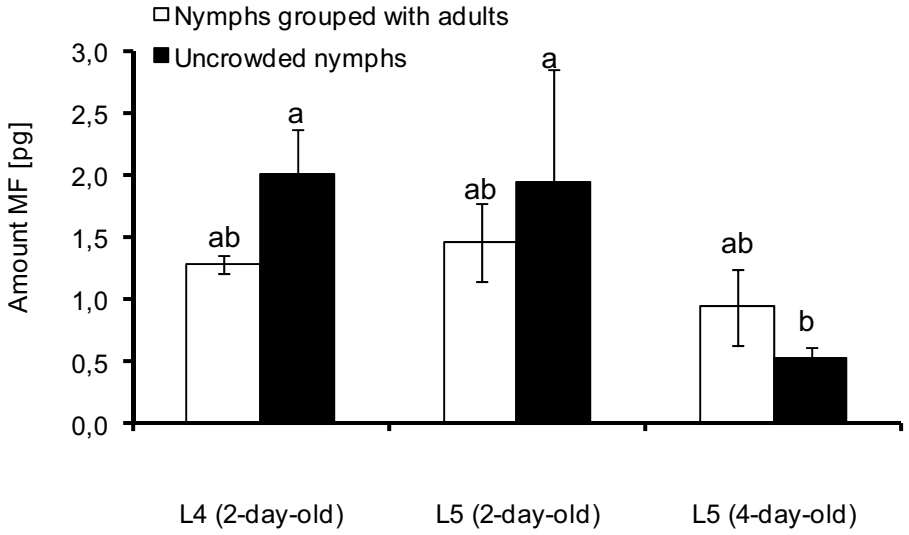


Fig. 4.8. Effects of grouping nymphs with adults on the maturation time of nymphs concerning hormone levels in the hemolymph of different instars nymphs. Mean amount of methyl farnesoate (MF) (±S.E.M.) per mg of hemolymph of *S. gregaria* nymphs (N=3) that were grouped with adults (treatment I) and ungrouped-uncrowded (treatment III) at different ages is displayed. Different letters above bars indicate significant differences between the two treatments and the nymphal stages (*DMS post-hoc test*, $P \leq 0.05$).

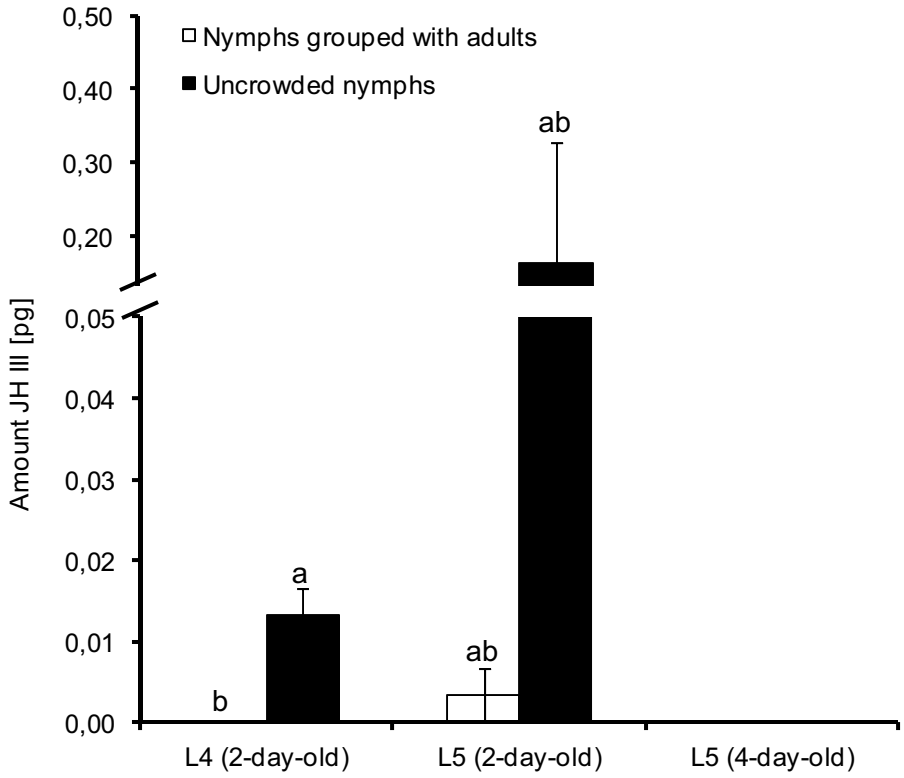


Fig. 4.9. Effects of grouping nymphs with adults on the maturation time of nymphs concerning hormone levels in the hemolymph of different instars nymphs. Mean amount of juvenile hormone (JH) III (±S.E.M.) per mg of hemolymph of *S. gregaria* nymphs (N=3) that were grouped with adults (treatment I) and ungrouped-uncrowded (treatment III) at different ages is displayed. Different letters above bars indicate significant differences between the two treatments and the nymphal stages (*Student's t-test*, $P \leq 0.05$).

4.4 DISCUSSION

The desert locust, *S. gregaria*, is considered the most dangerous of all locust species. Under specific environmental conditions such as the spatial distributions of food plants and/or convergent wind that round up locusts in relatively small areas, solitary living individuals scattered in low population density in a wide area can dramatically multiply into dense gregarious groups. As a consequence, juvenile stages transform into large adult swarms that are able to migrate great distances, threatening agricultural crops over a large part of Africa, the Middle East and Southwest Asia (Cressman, 1998). This density-dependent population shifting from solitarious to the hazardous gregarious phase, known as 'phase change' or 'phase transition' (Verlinden, et al., 2009), is characterized by morphological, physiological and behavioral changes (polyphenism) (Bouaïchi and Simpson, 2003; Ferenz and Seidelmann, 2003; Anstey, et al., 2009). This transformation can rapidly occur within a few hours of crowding (Roessingh and Simpson, 1994), and the capacity to behave gregariously can be passed from parents to offspring, a trait that can increase over several generations of crowding (Islam, et al., 1994a, b).

In the last two decades several research groups have been investigating the biology, physiology, behavior and chemical ecology of *S. gregaria* with the aim to discover novel environmental friendly IPM tools for locust control. In studies concerning the chemical ecology of the locust a variety of pheromones have been identified, including those that mediate social cohesion, maturation synchrony and communal oviposition behavior (Torto, et al., 1994; 1996; 1999; Hassanali and Torto, 1999; Mahamat, et al., 2000). Maturation synchrony in gregarious desert locust is of great importance to ensure synchronized mating of the sexes and communal oviposition to guarantee sustainment of the gregarious status and critical locust population densities (McCaffery, et al., 1998; Mahamat, et al., 2000). Previous work has concentrated on maturation of the desert locust, demonstrating that the presence of 5^{th} instar nymphs have a strong retarding effect on the maturation of conspecific young adults (Assad, et al., 1997). However, the reverse effect of adults on the maturation of nymphs has not been investigated so far.

In the current study we tested if adults grouped with nymphs in experimental cages have an influence on the development and maturation of the young hoppers and thereby promote synchronized maturation in the nymphs. For comparison, maturation time of uncrowded and crowded nymphs that were not grouped with adults was observed. The grouping experiments demonstrate a faster growing (increase in weight

and length) of nymphs that were grouped with mature adults in the presence of physical, visual, and olfactory cues than nymphs that had no contact to adults. Furthermore, the nymphs exposed to adults needed less time to form wings and become immature pink-colored adults compared to control individuals. Altogether, these results indicated that the presence of adults accelerated the developmental time of conspecific nymphs. The first study to describe development and maturation in young adults regulated by adult pheromones was conducted by Norris (1954). Further studies led to the suggestion of a two-set primer pheromone system regulating maturation in the gregarious desert locust, comprising a maturation-accelerating pheromone associated with mature male adults (Loher, 1960; Amerasinghe, 1978), and a maturation-retarding pheromone associated with the immature stages of the insect (Norris, 1954; 1964).

To verify our assumption that the presence of adults has an effect on the development of nymphs, the closing maturation process of nymphs that were grouped with conspecific adults was assessed by both body color and quantity of male produced pheromone PAN. It is known that PAN is the major pheromone compound, exclusively released by male desert locusts in the gregarious phase when they become sexually mature (Pener, 1991; Torto, et al., 1994; Pener and Yerushalmi, 1998; Seidelmann and Ferenz, 2002; Seidelmann, et al., 2003). Thus, PAN production represents a perfect indicator for studying development stages of the desert locust. Besides, mature males change their color from pink to yellow (Schmidt and Albütz, 2002). On the other hand, females (former recipient nymphs) were checked for maturation by recording the start of the oviposition (age of insects) after fledging and by counting the number of laid egg pods.

Results of PAN titers in male adult locusts revealed that male individuals (former recipient nymphs) that had been grouped with adults initiated integument yellowing and PAN production significantly earlier than ungrouped desert locust individuals. First notable amounts of PAN were observed 19 days after fledging, which is in contrast to previous results where it was reported >24 days after fledging (Torto, et al., 1994; Deng, et al., 1996). Grouped nymphs reached their maximum emission of PAN 25 days after fledging, one week before uncrowded individuals that were not grouped, and interestingly the amount decreased significantly earlier. An accelerated maturation in females grouped with adults was also observed. They got gravid and laid significantly more egg-pods earlier than ungrouped individuals. The results obtained from females match perfectly well with the monitored PAN-emission in males, with one week shifting between individuals that had been grouped and ungrouped nymphs. The time slot

between maximum emission of PAN in males and highest number of laid egg pods in females was ca. 7 days. This period could represent the moment of highest sexual activity in mature adults and the time from fecundation to oviposition.

Our data demonstrates that male and female nymphs grouped with adults display accelerated maturation compared to those grouped without adults in all the parameters monitored. So far, the grouping effect of nymphs was exclusively tested by mixing them with young adults. Previous laboratory experiments had revealed that the presence of nymphs, producing age-specific volatiles that are different from adult volatiles (e.g. PAN is lacking), delay maturation of young immature adults (Assad, et al., 1997). In contrast, our results confirm a previous finding of synchronous maturation in the gregarious locust, that is, a delayed maturation in immature adults, with accelerated maturation in nymphs in a mixed population of nymphs and immature adults. It was shown that PAN, among other male-specific volatiles of mature individuals, is responsible for the accelerated sexual maturation in young (immature) males (Mahamat, et al., 1993). It is possible that PAN may also be involved in the accelerated maturation observed in nymphs in the present study, but this would require further investigation.

However, the precise role of the male-produced pheromones, including PAN (apart from the accelerating effect) remains unknown, since it seems, on the one hand, to have a male-male avoidance function at relatively high concentrations near the source (Seidelmann and Ferenz, 2002), and, on the other hand, a cohesive effect promoting aggregation at lower concentrations away from the source (Schmidt and Albütz, 2002; Hassanali, et al., 2005; Rono, et al., 2008). With respect to the ultimate goal of controlling desert locust in an environmental friendly manner, PAN has been utilized in field assays to control nymphs (Bashir and Hassanali, 2010). These researchers showed that field gregarious hopper bands and laboratory crowd-reared nymphs treated with different doses of synthetic PAN caused hyperactivity, disorientation and dispersion in the treated nymphs compared to untreated ones. The nymphs of a PAN-treated population reduced feeding on plants and, moreover, increased predation by birds and cannibalism among them was observed. It was concluded that PAN had a solitarising effect on hoppers.

In our cage experiments nymphs were exposed to all cues offered by the present adults and thus, PAN was internally produced and not externally applied, as described before. The results revealed that in our laboratory experiments grouping nymphs with mature adults neither led to reduce feeding nor to solitarization in gregarious nymphs.

Nevertheless, it is not possible to compare the two different findings, since the experimental set up (laboratory >< field) and hypothesis were totally different.

In the present study, hormonal titers (juvenile hormone JH III and methyl farnesoate, MF) were compared in gregarious nymphs grouped and ungrouped with adults. The effect of ecdysone and JH hormone on insect development and metamorphosis has been well documented (Verlinden, et al., 2009). In *S. gregaria* hormonal control of phase-related variations in body color, morphological traits (Tanaka, 2005) and on ovarian development has been investigated (Applebaum, et al., 1997). It has been shown that yellowing in gregarious adults, indicating maturation, was dependent on JH production. Removal of the corpora allata, the endocrine gland for biosynthesis of the JH, resulted in fading of the yellow color in sexually mature gregarious adults (Pener, 1991). In this work it was observed that JH III titers of 2-day old 4^{th} and 5^{th} nymphs that were grouped with adults decreased in comparison to the titers in ungrouped nymphs. These results are in accordance with the assumption that high levels of JH might inhibit the transition to the adult stage. In past studies it was reported that gregarious desert locusts showed accelerated sexual maturation (five instars) compared to their solitarious congeners (six instars) (Uvarov, 1966). The JH titer in the hemolymph was lower in the gregarious phase compared to the solitarious phase (Injeyan and Tobe, 1981). Such difference in JH titers could be involved in the occurrence of the extra instar in the solitarious phase (delaying decrease of the JH titer could produce an extra instar). Our results suggest that the lower JH level observed in nymphs grouped with adults could account for their accelerated maturation, rather than produce an extra instar, as was observed for solitarious locusts.

Methyl farnesoate (MF) is the unepoxidated analogue of JH III and its immediate biosynthetic precursor. Its role in desert locust has been rarely described (Marchal et al., in press), but is assumed to accelerate molting and development in invertebrates (Ahl and Brown, 1990; Laufer and Biggers, 2001). Thus, it is expected that the higher amounts of MF, detected in the hemolymph of 4-day old 5^{th} instar nymphs of *S. gregaria* that were grouped with adults, could be a sign for the accelerated maturation observed in this work compared to ungrouped nymphs.

The results of the present study provide further insight into the understanding of development (molting), gregarization and swarm formation in the desert locust. Pheromones mediating maturation synchrony in gregarious populations of the desert locust ensure cohesiveness and sustain the gregarious phase (Norris, 1964; Hassanali, et al., 2005). Therefore, if the population of gregarious desert locust is dominated by

hoppers, the retarding effect of their pheromonal signals will prevent maturation of young adults, whereas a predominance of mature adults could inhibit JH production inducing an accelerated maturation in nymphs and young adults. Thus, it appears that synchronized maturation guarantees group oviposition behavior, which ensures high egg pod densities at oviposition sites and resultant spatial cohesiveness of the progeny. This is of great importance since oviposition in gregarious-phase acridids constitutes the principal means of transmitting gregarious traits to the progeny and facilitating accumulation of these traits across generations (Norris, 1968; 1970; Roffey and Popov, 1968).

CONCLUSIONS

Chapter 1 Analytical and behavioral studies on the oak branch borer (OBB) *Coroebus florentinus* (Coleoptera: Buprestidae)

- SEM analysis of male and female antennae revealed the presence of four types of sensilla. *S. basiconica* type 1 and 2 (olfactory), *uniporous sensilla* (gustatory) and *s. chaetica* (mechanoreceptor) were identified by comparison with the antennal structure of the emerald ash borer (EAB), *A. planipennis* (Buprestidae). Male antennae are longer, have a bigger surface and dispose of more *uniporous sensilla* than females, indicating that there may be short-range, contact cues for mate recognition in *C. florentinus*.
- In male and female volatiles collected by dynamic headspace and SPME and in abdominal extracts of both sexes a total of 28 compounds were identified. Most of these compounds are saturated linear or branched hydrocarbons, typically used as contact pheromones in other insects.
- Contrary to our hypothesis, no qualitative differences in the headspace composition between adult males and females were noticed. Nevertheless, in Y-tube olfactometer males were significantly attracted to conspecific live females and female body extract, but not to males (acceptance of hypothesis). Apparently, a possible sex pheromone produced by females, not detected so far, is involved.
- In electrophysiological studies three of the identified volatile compounds (nonanal, decanal and geranylacetone) elicit strong antennal responses in males and females. Furthermore, they are behaviorally attractive to both sexes in different ways (males choose the three component blend and decanal; females choose the three component blend, geranylacetone and in part nonanal). It is assumed that these compounds could be components of a possible aggregation pheromone.
- Thirty two compounds have been identified in headspace volatiles of freshly cut branches and leaf extracts of the host plant *Q. suber*; being the green leaf volatiles (GLVs) the most abundant. We consider that feeding of *Coroebus spp.* adults would induce production of these compounds or rather provoke an increased emission of GLVs.
- Females show strong electrophysiological and behavioral responses to host plant odors and five GLVs identified from *Q. suber* ((*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, (*Z*)-3-hexenyl acetate and *n*-hexyl acetate). Significant preferences were observed to the green leaf alcohols (*E*)-2-hexenol and 1-hexanol (acceptance of

hypothesis). The results point out that these compounds could play an important role in the foraging or oviposition behavior of the OBB.

- In field trapping experiments with different trap types and semiochemical lures not a single individual of *C. florentinus* was captured in a *Coroebus spp.* infested cork oak forest. Instead, a relatively high number of *C. undatus* females, its sister species, was trapped with host plant volatiles and the three component blend of the insect volatiles. The reason for these results could be that traps were placed at the height of the trunk and not in the canopy of the tree were OBB adults are supposed to stay.

Chapter 2 Analytical studies and field trapping experiments on the oak flathead borer (OFB) *Coroebus undatus* (Coleoptera: Buprestidae)

- Antennae of both sexes of the OFB are identical in form and size and dispose of the same four sensilla types as *C. florentinus* and *A. planipennis*. In conformity with these two species of Buprestidae, males appear to possess noticeably more olfactory *sensilla basiconica* and gustatory *uniporous sensilla* than females. Apparently, there is growing evidence for the importance of odor perception by males especially for mate recognition, in this beetle family.
- Analysis of the cuticle from the abdomen of both sexes by SPME showed the presence of 18 saturated linear or branched hydrocarbons, and two of them (possibly dimethyl branched) exclusively in females (acceptance of hypothesis). Since in the EAB two female-specific methyl-branched hydrocarbons, structurally similar to the OFB compounds, have been identified as contact pheromones, it can be assumed that *C. undatus* males could also use the hydrocarbons produced by females as contact chemical cues for mate recognition.
- In field tests, significantly more individuals (only females) were captured with purple-colored Prism traps compared to Lindgren funnel traps and single Panel traps. Thus, trap design and visual cues (e.g. purple) play an important role in the attraction of the cork oak pest (acceptance of hypothesis).
- Lures of a three component blend, comprising nonanal, decanal and geranylacetone, and a mixture of 5 GLVs ((*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, (*Z*)-3-hexenyl acetate and *n*-hexyl acetate), caught a significant

number of *C. undatus* adults in Prism traps in comparison to control (acceptance of hypothesis). These results reinforce the idea that host plant GLVs are possibly involved in a foraging or ovipostion kairomone of *Coroebus spp.* and furthermore, that nonanal, decanal and geranylacetone, identified from *C. florentinus* headspace volatiles could be part of a possible aggregation pheromone.

Chapter 3 Analytical and electrophysiological studies on the Moroccan locust *Dociostaurus maroccanus* (Orthoptera: Acrididae), pest of the Iberian Peninsula

- Male and female antennae of Moroccan locust adults are identical in structure and distribution of sensilla. By comparison of SEM micrographs with antennae of the desert locust and other acridid species three types of olfactory sensilla (*s. basiconica, s. trichodea* and *s. coeloconica*) and one taste/mechanoreceptor (*s. chaetica*) were identified. These results reflect the importance of the olfactory perception for behavior and development of Acrididae, in males and females.
- Nine long-chain compounds (≥C_{24}) (three aldehydes, two alcohols, two ketones and two unknown compounds) were detected in headspace volatiles and SPME extracts of adult males, not in adult females or nymphs of both sexes (acceptance of hypothesis). The maximum amounts of these compounds are emitted by adult males 12-20 days after fledging. These age-dependent and sex-specific volatile organic compounds could be involved in the chemical communication of *D. maroccanus.*
- The two compounds, so far unidentified, 01BP84 and 02BP84 were found in wing and leg extracts of adult males. Significantly higher amounts were detected in legs and especially in extracts of the femur, indicating possible production and/or emission sites.
- Headspace volatiles and femur extracts of adult males provoke significant EAG responses in antennae of both sexes. In GC-EAD analysis five of the male-produced compounds, 1-tetradecanol, hexadecanal, 1-hexadecanol, 2-octadecanone and 02BP84, were electrophysiologically active on adult females. In contrast, on adult males only 02BP84 elicit antennal responses (acceptance of hypothesis). The male produced volatiles, and in particular the unidentified compounds 01BP84 and 02BP84, are possibly part of the pheromone complex of the Moroccan locust.

- From freshly secreted egg froth a high number of saturated and unsaturated acids, aldehydes and ketones have been identified. Among them, (*E*)-3-octen-2-one and (*E,E*)-3,5-octadien-2-one have been described to elicit EAG responses in gravid females and to enhance group oviposition in gregarious desert locust, *S. gregaria*. In males and females of the Moroccan locust these compounds do not display electrophysiological activity. In contrast, egg froth extracts and the unsaturated aldehydes (*E,E*)-2,4-heptadienal and (*E,E*)-2,4-decadienal elicit significant antennal responses. Although, no significant effect of these compounds and fresh egg froth was observed in oviposition bioassays (rejection of hypothesis), a possible role of female secreted froth in recognition of suitable oviposition sites is expected.

Chapter 4 Maturation studies in gregarious nymphs of the desert locust *Schistocerca gregaria* (Orthoptera: Acrididae), important crop pest in Africa.

- Nymphs of the desert locust grouped with conspecific adults display accelerated maturation compared to those grouped without adults with respect to growth, levels of JH III and methyl farnesoate in the hemolymph and formation of wings. After fledging accelerated maturation of the recipient individuals is demonstrated by change of body color from pink to yellow, titer of the male produced pheromone PAN and number of laid egg pods by females (acceptance of hypothesis).
- A previous finding of synchronous maturation in the gregarious locust, that is, a delayed maturation in immature adults, with accelerated maturation in nymphs in a mixed population of nymphs and immature adults is confirmed. It is considered that pheromones, primarily PAN, mediate maturation synchrony in gregarious populations of the desert locust, ensure cohesiveness and, thereby, sustain the gregarious phase. These results have important implications for understanding gregarization and swarm formation in the desert locust.

LITERATURE CITED

Acin, P., Rosell, G., Guerrero, A. and Quero, C. (2010). Sex Pheromone of the Spanish Population of the Beet Armyworm *Spodoptera exigua*. *J. Chem. Ecol.* **36,** 778-786.

Adams, P. R. (2007). In *Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry.*, pp. 804. Carol Stream, Illinois, USA: Allured Publishing Corporation.

Agelopoulos, N. G. and Pickett, J. A. (1998). Headspace Analysis in Chemical Ecology: Effects of Different Sampling Methods on Ratios of Volatile Compounds Present in Headspace Samples. *J. Chem. Ecol.* **24,** 1161-1172.

Ahl, J. S. B. and Brown, J. J. (1990). Salt-Dependent Effects of Juvenile Hormone and Related Compounds in Larvae of the Brine Shrimp, *Artemia*. *Comp. Biochem. Physiol.* **95A,** 491-496.

Altner, H. and Prillinger, L. (1980). Ultrastructure of Invertebrate Chemo-, Thermo-, and Hygroreceptors and its Functional Significance. *Int. Rev. Cytol.* **67,** 69-139.

Altner, H., Routil, C. and Loftus, R. (1981). The Structure of Bimodal Chemo-, Thermo-, and Hygroreceptive Sensilla on the Antenna of *Locusta migratoria*. *Cell Tissue Res.* **215,** 289-308.

Amerasinghe, F. P. (1978). Pheromonal Effects on Sexual Maturation, Yellowing, and the Vibration Reaction in Immature Male Desert Locusts (*Schistocerca gregaria*). *J. Insect Physiol.* **24,** 309-314.

Anderbrant, O., Hansson, B. S., Hallberg, E., Geri, C., Varama, M., Hedenstrom, E., Hogberg, H., Fagerhag, J., Edlund, H., Wassgren, A. et al. (1995). Electrophysiological and Morphological Characteristics of Pheromone Receptors in Male Pine Sawflies, *Diprion pini* (Hymenoptera: Diprionidae), and Behavioural Response to some Compounds. *J. Insect Physiol.* **41,** 395-401.

Anderson, P., Hallberg, E. and Subchev, M. (2000). Morphology of Antennal Sensilla Auricillica and their Detection of Plant Volatiles in the Herald Moth, *Scoliopteryx libatrix* L. (Lepidoptera: Noctuidae). *Arthropod Struct. Dev.* **29,** 33-41.

Anstey, M. L., Rogers, S. M., Ott, S. R., Burrows, M. and Simpson, S. J. (2009). Serotonin Mediates Behavioral Gregarization Underlying Swarm Formation in Desert Locusts. *Science* **323,** 627-630.

Anton, S., Ignell, R. and Hansson, B. S. (2002). Developmental Changes in the Structure and Function of the Central Olfactory System in Gregarious and Solitary Desert Locusts. *Microsc. Res. Techniq.* **56,** 281-291.

Applebaum, S. W., Avisar, E. and Heifetz, Y. (1997). Juvenile Hormone and Locust Phase. *Arch. Insect Biochem. Physiol.* **35,** 375-391.

Arias, A., Alvez, C., Garcia, F., Martinez de Velasco, D., Olivera, J., Prieto, A. and Santos, R. (1993). La Lucha contra la Langosta Marroquí (*Dociostaurus maroccanus* Thunb.) en Extremadura durante el Decenio 1983–1992. *Bol. San. Veg. Pl.* **19,** 425-453.

Arn, H., Staedler, E. and Rauscher, S. (1975). The Antennographic Detector, a Selective and Sensitive Tool in the Gas Chromatographic Analysis of Insect Pheromones. *Z. Naturforsch. C* **30,** 722-725.

Ashall, C. and Ellis, P. E. (1962). Studies on the Numbers and Mortality in Field Populations of the Desert Locust (*Schistocerca gregaria*). *Anti-Locust Bull., London* **38,** 1-59.

Assad, Y. O. H., Hassanali, A., Torto, B., Mahamat, H., Bashir, N. H. H. and El Bashir, S. (1997). Effects of Fifth-Instar Volatiles on Sexual Maturation of Adult Desert Locust *Schistocerca gregaria*. *J. Chem. Ecol.* **23,** 1373-1388.

Bachiller, P. (1981). In *Plagas de Insectos en las Masas Forestales Españolas. Colección Técnica ICONA.,* pp. 272.

Barclay, H. and Van den Driessche, P. (1983). Pheromone Trapping Models for Insect Pest Control. *Res. Popul. Ecol.* **25,** 105-115.

Barclay, H. J. and Judd, G. J. R. (1995). Models for Mating Disruption by Means of Pheromone for Insect Pest Control. *Res. Popul. Ecol.* **37,** 239-247.

Barr, D. B. and Needham, L. L. (2002). Analytical Methods for Biological Monitoring of Exposure to Pesticides: A Review. *J. Chromatogr. B* **778,** 5-29.

Bartelt, R. J., Cossé, A. A., Zilkowski, B. W. and Fraser, I. (2007). Antennally Active Macrolide from the Emerald Ash Borer *Agrilus planipennis* Emitted Predominantly by Females. *J. Chem. Ecol.* **33,** 1299-1302.

Bashir, M. O., Hassanali, A., Rai, M. M. and Saini, R. K. (2000). Changing Oviposition Preferences of the Desert Locust, *Schistocerca gregaria*, Suggest a Strong Species Predisposition for Gregarization. *J. Chem. Ecol.* **26,** 1721-1733.

Bashir, M. O. and Hassanali, A. (2010). Novel Cross-Stage Solitarising Effect of Gregarious-Phase Adult Desert Locust (*Schistocerca gregaria* (Forskål)) Pheromone on Hoppers. *J. Insect Physiol.* **56,** 640-645.

Belahbib, N., Pemonge, M. H., Ouassou, A., Sbay, H., Kremer, A. and Petit, R. J. (2001). Frequent Cytoplasmic Exchanges between Oak Species that are not closely related: *Quercus suber* and *Q. ilex* in Morocco. *Mol. Ecol.* **10,** 2003-2012.

Bellamy, C. L. (1985). A Catalogue of the Higher Taxa of the Family Buprestidae (Coleoptera). *Navors. Nas. Mus. Bloemfontain* **4,** 406-472.

Bennett, G. W., Owens, J. M. and Corrigan, R. M. (2005). *Truman's Scientific Guide to Pest Management Operations.* Cleveland: Advanstar Communications, Inc.

Bernal, C. J. and Cardillo, E. (2004). Plagas y Enfermedades del Alcornorcal - La Culebra del Corcho. *Cuadern. For.* **2,** 1-29.

Birch, M. C. and Haynes, K. F. (1990). In *Feromonas de Insectos.*, pp. 96: Oikos-Tau, S.A. Ediciones.

Blaney, W. M. and Chapman, R. F. (1969). The Anatomy and Histology of the Maxillary Palp of *Schistocerca gregaria*. *J. Zool. London* **157,** 509-535.

Blaney, W. M. and Simmons, M. S. J. (1990). The Chemoreceptors. In *Biology of Grasshoppers.* (ed. R. F. Chapman and A. Joern), pp. 1-37. New York: John Wiley & Sons.

Boland W., Ney P., Jaenicke L. and Gassman G. (1984). A “Closed-Loop-Stripping” Technique as Versatile Tool for Metabolic Studies of Volatiles. In *Analysis of Volatiles.* (ed. P. Schreier), pp. 371-380. Berlin: Walter de Gruyter.

Bonnemaison, L. (1976). *Enemigos Naturales de las Plantas Cultivadas y Forestales. Vol. I.*, pp. 605. Barcelona: Oikos-Tau, S.A. Ed.

Borden, J. (1993). Strategies and Tactics for the use of Semiochemicals Against Forest Insect Pests in North America. In *Pest Management: Biologically Based Technologies.* (ed. R. D. Lumsden and J. L. Vaughn), pp. 265-276. Washington: ACS

Borges, J. G., Oliveira, A. C. and Costa, M. A. (1997). A Quantitative Approach to Cork Oak Forest Management. *For. Ecol. Manage.* **97,** 223-229.

Bouaïchi, A. and Simpson, S. J. (2003). Density-Dependent Accumulation of Phase Characteristics in a Natural Population of the Desert Locust *Schistocerca gregaria.*. *Physiol. Entomol.* **28,** 25-31.

Brasier, C. M., Robredo, F. and Ferraz, J. F. (1993). Evidence for *Phytophthora cinnamoni* Involvement in Iberia Oak Decline. *Plant Pathol.* **42,** 140-145.

Breer, H., Boekhoff, I., Strotmann, J., Raming, K. and Tareilus, E. (1990a). Molecular Elements of Olfactory Signal Transduction in Insect Antennae. *NATO ASI Series H Cell Biol.* **39,** 77-86.

Breer, H., Krieger, J. and Raming, K. (1990b). A Novel Class of Binding Proteins in the Antennae of the Silk Moth *Antheraea pernyi*. *Insect Biochem.* **20,** 735-740.

Brockerhoff, E. G., Jones, D. C., Kimberley, M. O., Suckling, D. M. and Donaldson, T. (2006a). Nationwide Survey for Invasive Wood-Boring and Bark Beetles (Coleoptera) Using Traps Baited with Pheromones and Kairomones. *For. Ecol. Manage.* **228,** 234-240.

Brockerhoff, E. G., Bain, J., Kimberley, M. and Knizek, M. (2006b). Interception Frequency of Exotic Bark and Ambrosia Beetles (Coleoptera: Scolytinae) and Relationship with Establishment in New Zealand and Worldwide. *Can. J. For. Res.* **36,** 289-298.

Buhl, J., Sumpter, D. J. T., Couzin, I. D., Hale, J. J., Despland, E., Miller, E. R. and Simpson, S. J. (2006). From Disorder to Order in Marching Locusts. *Science* **312,** 1402-1406.

Buj, A. (1995). International Experimentation and Control of the Locust Plague. Africa in the First Half of the 20th Century. In *Les Sciences Hors D'Occident au XX^{e} Siècle. Nature and Environment.* (ed. Y. Chatelin and C. Bonneuil), pp. 93-105. Paris: Orstom.

Butenandt, A., Beckmann, R., Stammd, D. and Hecker, E. (1959). On the Sex Attractant of the Silk Moth *Bombyx mori*. Purification and Structure Determination. (Über den Sexuallockstoff des Seidenspinners *Bombyx mori*. Reindarstellung und Konstitutionsermittlung). *Z. Naturforsch. B* **14,** 283-284.

Byers, J. A. (1991). Pheromones and Chemical Ecology of Locusts. *Biol. Rev. Camb. Philos. Soc.* **66,** 347-378.

Cañizo, J. (1943). Parásitos de la Langosta en España. II. Dípteros Bombilidos. *Bol. Patol. Veg. Entomol. Agr.* **12,** 77-99.

Cañizo, J. (1956). Parásitos de la Langosta en España. II. Los Trichodes (Col. Cleridae). *Bol. Patol. Veg. Entomol. Agr.* **22,** 297-312.

Carlson, R. W. and Knight, F. B. (1969). Biology, Taxonomy and Evolution of Four Sympatric *Agrilus* Beetles (Coleoptera: Buprestidae). *Contrib. Am. Ent. Inst.* **4,** 1-105.

Carson, R. (1962). In *Silent Spring.*, pp. 400. Boston: Houghton Mifflin.

Carter, P. C. S. (1989). Risk Assessment and Pest Detection Surveys for Exotic Pests and Diseases which Threaten Commercial Forestry in New Zealand. *New Zeal. J. For. Sci.* **19,** 353-374.

Chenier, J. V. R. and Philogene, B. J. R. (1989). Field Responses of Certain Forest Coleoptera to Conifer Monoterpenes and Ethanol. *J. Chem. Ecol.* **15,** 1729-1745.

Cobos, A. (1986). In *Fauna Ibérica de Coleópteros Buprestidae*., pp.364. Madrid: CSIC.

Collar-Urquijo, J. L., Celma-Calamita, J., Blandford, S. and Thomas, M. B. (2002). Control de *Dociostaurus maroccanus* y *Calliptamus italicus* (Orthoptera: Acrididae) Mediante Aplicaciones en Campo de *Metarhizium anisopliae* var *Acridum*. *Bol. San. Veg. Pl.* **28,** 185-192.

Cossé, A. A., Bartelt, R. J., Zilkowski, B. W., Bean, D. W. and Petroski, R. J. (2005). The Aggregation Pheromone of *Diorhabda elongata*, a Biological Control Agent

of Saltcedar (Tamarix Spp.): Identification of Two Behaviorally Active Components. *J. Chem. Ecol.* **31,** 657-670.

Cressman, K. (1998). Monitoring Desert Locusts in the Middle East: An Overview. *Yale F. ES Bull.* **103,** 123-141.

Crook, D. J., Francese, J. A., Zylstra, K. E., Fraser, I., Sawyer, A. J., Bartels, D. W., Lance, D. R. and Mastro, V. (2009). Laboratory and Field Response of the Emerald Ash Borer, *Agrilus planipennis* (Coleoptera: Buprestidae), to Selected Regions of the Electromagnetic Spectrum. *J. Econ. Entomol.* **102,** 2160-2169.

Crook, D. J., Kerr, L. M. and Mastro, V. C. (2008a). Distribution and Fine Structure of Antennal Sensilla in Emerald Ash Borer (Coleoptera: Buprestidae). *Ann. Entomol. Soc. Am.* **101,** 1103-1111.

Crook, D. J., Khrimian, A., Fraser, I., Francese, J. A., Poland, T. M. and Mastro, V. C. (2008b). Electrophysiological and Behavioral Responses of *Agrilus planipennis* (Coleoptera: Buprestidae) to Host Bark Volatiles. *Environ. Entomol.* **37,** 356-365.

Crook, D. J., Khrimian, A., Francese, J. A., Fraser, I., Poland, T. M., Sawyer, A. J. and Mastro, V. C. (2008c). Development of a Host-Based Semiochemical Lure for Trapping Emerald Ash Borer *Agrilus planipennis* (Coleoptera: Buprestidae). *Environ. Entomol.* **37,** 356-365.

Crook, D. J. and Mastro, V. C. (2010). Chemical Ecology of the Emerald Ash Borer *Agrilus planipennis*. *J. Chem. Ecol.* **36,** 101-112.

Dahanukar, A., Hallem, E. A. and Carlson, J. R. (2005). Insect Chemoreception. *Curr. Opin. Neurobiol.* **15,** 423-430.

Dajoz, R. (2000). In *Insects and Forests. The Role and Diversity of Insects in the Forest Environment.*, pp. 668. Paris, France: Lavoisier.

Davies, R. G. (1988). In *Outlines of Entomology.*, pp. 425. London, UK: Chapman and Hall.

De Bruyne, M., Foster, K. and Carlson, J. R. (2001). Odor Coding in the *Drosophila* Antenna. *Neuron* **30,** 537-552.

De Groot, P., Grant, G. G., Poland, T. M., Scharbach, R., Buchan, L., Nott, R. W., Macdonald, L. and Pitt, D. (2008). Electrophysiological Response and Attraction of Emerald Ash Borer to Green Leaf Volatiles (GLVs) Emitted by Host Foliage. *J. Chem. Ecol.* **34,** 1170-1179.

De Groot, P. and Poland, T. M. (2003). Attraction of *Hylastes opacus* (Coleoptera: Scolytidae) to Nonanal. *Can. Entomol.* **135,** 309-311.

Del Moral de la Vega, J. (1986). Discusión sobre la Situación Actual de la Plaga de Langosta (*Dociostaurus maroccanus* Thunb.) en Badajoz y Resultados de un Ensayo de Insecticidas para su Control. *Bol. San. Veg. Pl.* **12,** 221-235.

Deng, A. L., Torto, B., Hassanali, A. and Ali, E. E. (1996). Effects of Shifting to Crowded or Solitary Conditions on Pheromone Release and Morphometrics of the Desert Locust, *Schistocerca gregaria* (Forskål) (Orthoptera: Acrididae). *J. Insect Physiol.* **42,** 771-776.

Dicke, M. and Sabelis, M. W. (1988). Infochemical Terminology: Based on Cost-Benefit Analysis rather than Origin of Compounds? *Funct. Ecol.* **2,** 131-139.

Dickens, J. C. (2006). Plant Volatiles Moderate Response to Aggregation Pheromone in Colorado Potato Beetle. *J. Appl. Entomol.* **130,** 26-31.

Dreistadt, S. H., Flint, M. L. and Clark, J. K. (2004). In *Pests of Landscape Trees and Shrubs: An Integrated Pest Management Guide.*, pp. 502. University of California, Oakland: ANR Publications.

Ehrlich, P. R. and Raven, P. H. (1964). In Butterflies and Plants: A Study in Co-Evolution. *Evolution* **18,** 586-608.

El Ghadraoui, L., Petit, D., Picaud, F. and El Yamani, J. (2002). Relationship between Labrum Sensilla Number in the Moroccan Locust *Dociostaurus maroccanus* and the Nature of its Diet. *J. Orthoptera. Res.* **11,** 11-18.

Enserink, M. (2004). Can the War on Locusts Be Won? *Science* **306,** 1880-1882.

Ernst, K. D. (1969). Die Feinstruktur von Riechsensillen auf der Antenne des Aaskäfers Necrophorus. *Z. Zellforsch.* **94,** 72-102.

Evans, H. F., Moraal, L. G. and Pajares, J. A. (2007). Biology, Ecology and Economic Importance of Buprestidae and Cerambicidae. In *Bark and Wood Boring Insects in Living Trees in Europe, a Synthesis.* (ed. F. Lieutier, K. R. Day, A. Battisti, J. C. Grégoire and H. F. Evans), pp. 447. Netherlands: Springer.

Faria, T., García-Plazaola, J. I., Abadía, A., Cerasoli, S., Pereira, J. S. and Chaves, M. M. (1996). Diurnal Changes in Photoprotective Mechanisms in Leaves of Cork Oak (*Quercus suber*) during Summer. *Tree Physiol.* **16,** 115-123.

Ferenz, H. J. and Seidelmann, K. (2003). Pheromones in Relation to Aggregation and Reproduction in Desert Locusts. *Physiol. Entomol.* **28,** 11-18.

Flodin, O., Frederiksson, M., Persson, B. and Axelson, O. (1988). Chronic Lymphatic Leukaemia and Engine Exhausts, Fresh Wood and DDT: A Case-Referent Study. *Brit. J. Ind. Med.* **45,** 33-38.

Flower, N. E. and Helson, G. A. H. (1974). Variation in Antennal Sensilla of some Noctuid Moths; a Scanning Electron Microscope Study. *New Zeal. J. Zool.* **1,** 59-66.

Fonseca, M. G., Vidal, D. M. and Zarbin, P. H. G. (2010). Male-Produced Sex Pheromone of the Cerambycid Beetle *Hedypathes betulinus*: Chemical Identification and Biological Activity. *J. Chem. Ecol.* **36,** 1132-1139.

Francese, J. A., Mastro, V. C., Oliver, J. B., Lance, D. R., Youssef, N. and Lavallee, S. G. 4. (2005). Evaluation of Colors for Trapping *Agrilus planipennis* (Coleoptera: Buprestidae). *J. Entomol. Sci.* **40,** 93-95.

Francese, J. A., Oliver, J. B., Fraser, I., Lance, D. R., Youssef, N., Sawyer, A. J. and Mastro, V. C. (2008). Influence of Trap Placement and Design on Capture of the Emerald Ash Borer (Coleoptera: Buprestidae). *J. Econ. Entomol.* **101,** 1831-1835.

Francke, W., Levinson, A. R., Jen, T. L. and Levinson, H. Z. (1979). Isopropyl Carboxylates - A New Class of Insect Pheromones. *Angew. Chem.* **18,** 796-797.

Francke, F. and Schulz, S. (1999). Pheromones. In *Comprehensive Natural Products Chemistry.* (ed. K. Mori), pp. 197-261. Amsterdam: Elsevier.

Francke, W. and Dettner, K. (2005). Chemical Signaling in Beetles. *Top. Curr. Chem.* **240,** 85-166.

Gibbs, A. G. (1998a). The Role of Lipid Physical Properties in Lipid Barriers. *Am. Zool.* **38,** 268-279.

Gibbs, A. G. (1998b). Water-Proofing Properties of Cuticular Lipids. *Am. Zool.* **38,** 471-482.

Gilden, R. C., Huffling, K. and Sattler, B. (2010). Pesticides and Health Risks. *J. Obstet. Gynaecol. Neonatal Nurs.* **39,** 103-110.

Ginzel, M. D., Blomquist, G. J., Millar, J. G. and Hanks, L. M. (2003a). Role of Contact Pheromones in Mate Recognition in *Xylotrechus colonus*. *J. Chem. Ecol.* **29,** 533-545.

Ginzel, M. D. and Hanks, L. M. (2003b). Contact Pheromones as Mate Recognition Cues of Four Species of Longhorned Beetles (Coleoptera: Cerambycidae). *J. Insect Behav.* **16,** 181-187.

Ginzel, M. D., Millar, J. G. and Hanks, L. M. (2003c). (*Z*)-9-Pentacosene: Contact Sex Pheromone of the Locust Borer, *Megacyllener robiniae*. *Chemoecology* **13,** 135-141.

Gonzalez-Adrados, J. R., Lopes, F. and Pereira, H. (2000). Quality Grading of Cork Planks with Classification Models Based on Defect Characterisation. *Holz. Roh. Werkstoff* **58,** 39-45.

Grant, A. J., O'Connell, R. J. and Eisner, T. (1989). Pheromone-Mediated Sexual Selection in the Moth *Utetheisa ornatrix*: Olfactory Receptor Neurons Response to Male-Produced Pheromone. *J. Insect Behav.* **2,** 371-385.

Grant, G. G., Ryall, K. L., Lyons, D. B. and Abou-Zaid, M. M. (2010). Differential Response of Male and Female Emerald Ash Borers (Col., Buprestidae) to (*Z*)-3-Hexenol and Manuka Oil. *J. Appl. Entomol.* **134,** 26-33.

Greenwood, M. and Chapman, R. F. (1984). Differences in Numbers of Sensilla on the Antennae of Solitarious and Gregarious *Locusta migratoria* L. (Orthoptera: Acrididae). *Int. J. Insect Morphol. Embryol.* **13,** 295-301.

Grob, K., Grob, K. J. and Grob, G. (1975). Organic Substances in Potable Water and in its Precursor. III. The Closed-Loop Stripping Procedure Compared with Rapid Liquid Extraction. *J. Chromatogr.* **106,** 299-315.

Gullan, P. J. and Cranston, P. S. (2004). In *The Insects. An Outline of Entomology.*, pp. 528: Blackwell-Science.

Gwynne, D. T. and Rentz, D. C. F. (1983). Beetles on the Bottle: Male Buprestids Mistake Stubbies for Females (Coleoptera). *J. Aust. Entomol. Soc.* **22,** 79-80.

Haack, R. A., Jendek, E., Liu, K., Marchant, T. R., Petrice, T. R., Poland, T. M. and Ye, H. (2002). The Emerald Ash Borer: A New Exotic Pest in North America. *Newslett. Mich. Entomol. Soc.* **47,** 1-5.

Haberer, W., Schmitt, T., Peschke, K., Schreier, P. and Mueller, J. K. (2008). Ethyl 4-Methyl Heptanoate: A Male-Produced Pheromone of *Nicrophorus vespilloides*. *J. Chem. Ecol.* **34,** 94-98.

Hagele, B. F. and Simpson, S. J. (2000). The Influence of Mechanical, Visual and Contact Chemical Stimulation on the Behavioural Phase State of Solitarious Desert Locusts (*Schistocerca gregaria*). *J. Insect Physiol.* **46,** 1295-1301.

Hallem, E. A., Ho, M. G. and Carlson, J. R. (2004). The Molecular Basis of Odor Coding in the *Drosophila* Antenna. *Cell* **117,** 965-979.

Hansson, B. S. (1995). Olfaction in Lepidoptera. *Experientia* **51,** 1003-1027.

Hansson, B. S., Ochieng, S. A., Grosmaitre, X., Anton, S. and Njagi, P. G. N. (1996). Physiological Responses and Central Nervous Projections of Antennal Olfactory Neurons in the Adult Desert Locust, *Schistocerca gregaria* (Orthoptera: Acrididae). *J. Comp. Physiol. A* **179,** 157-167.

Harborne, J. B. (1995). In *Ökologische Biochemie.*, pp. 383. Heidelberg, Berlin, Oxford: Spektrum Akademischer Verlag.

Hardie, J. and Minks, A. K. (1999). In *Pheromones of Non-Lepidopteran Insects Associated with Agricultural Plants.*, pp. 466. Wallingford: CABI Publishing.

Hassanali, A. and Torto, B. (1999). In *Grasshoppers and Locusts.*, pp. 328. Wallingford: CABI Publishing.

Hassanali, A., Njagi, P. G. N. and Bashir, M. O. (2005). Chemical Ecology of Locusts and Related Acridids. *Annu. Rev. Entomol.* **50,** 223-245.

Hatanaka, A., Kajiwara, T. and Matsui, K. (1995). The Biogeneration of Green Odour by Green Leaves and its Physiological Functions – Past, Present and Future. *Z. Naturforsch. C* **50,** 467-472.

Hatanaka, A., Kajiwara, T. and Sekiya, J. (1987). Biosynthetic Pathway for C6 Aldehyde Formation from Linolenic Acid in Green Leaves. *Chem. Phys. Lipids* **44,** 341-361.

Headrick, D. H. and Gordh, G. (2001). In *A Dictionary of Entomology.*, pp. 1031. New York: CABI Publishing.

Heifetz, Y., Boekhoff, I., Breer, H. and Applebaum, S. W. (1997). Cuticular Hydrocarbons Control Behavioural Phase Transition in *Schistocerca gregaria* Nymphs and Elicit Biochemical Responses in Antennae. *Insect Biochem. Mol. Biol.* **27,** 563-568.

Heifetz, Y., Voet, H. and Applebaum, S. W. (1996). Factors Affecting Behavioral Phase Transition in the Desert Locust, *Schistocerca gregaria* (Forskål) (Orthoptera: Acrididae). *J. Chem. Ecol.* **22,** 1717-1734.

Hernández-Crespo, P. and Santiago-Álvarez, C. (1997a). Description of *Hexamermis serenensis* (Nematoda; Mermithidae), a Parasite of *Dociostaurus maroccanus* (Thunberg) (Orthoptera: Acrididae) in Spain. *Fund. Appl. Nematol.* **20,** 37-42.

Hernández-Crespo, P. and Santiago-Álvarez, C. (1997b). Entomopathogenic Fungi Associated with Natural Papulations of the Moroccan Locust *Dociostaurus maroccanus* (Thunberg) (Orthoptera: Gomphocerinae) and Other Acridoidea in Spain. *Bio. Sci. Tech.* **7,** 357-363.

Hildebrand, J. G. (1996). Olfactory Control of Behavior in Moths: Central Processing of Odor Information and the Functional Significance of Olfactory Glomeruli. *J. Comp. Physiol. A* **178,** 5-19.

Hildebrand, J. G. (1997). Sensory Processing of Pheromone Signals. In *Insect Pheromone Research. New Directions.* (ed. R. T. Cardé and A. K. Minks), pp. 111-113. New York: Chapman & Hall.

Ho, H., Kou, R. and Tseng, H. K. (2003). Semiochemicals from the Predatory Stink Bug *Eocanthecona furcellata* (Wolff): Components of Metathoracic Gland, Dorsal Abdominal Gland, and Sternal Gland Secretions. *J. Chem. Ecol.* **29,** 2101-2114.

Howard, W. and Blomquist, G. J. (2005). Ecological, Behavioral, and Biochemical Aspects of Insect Hydrocarbons. *Annu. Rev. Entomol.* **50,** 371-393.

Howe H.F. and Westley L.C. (1993). In *Anpassung und Ausbeutung – Wechselbeziehungen Zwischen Pflanzen und Tieren*., pp. 310. Heidelberg: Spektrum Akademischer Verlag.

Huber, D. P. and Borden, J. H. (2001). Angiosperm Bark Volatiles Disrupt Response of Douglas-Fir Beetle, *Dendroctonus pseudotsugae*, to Attractant-Baited Traps. *J. Chem. Ecol.* **27,** 217-233.

Huber, D. P., Borden, J. H. and Stastny, M. (2001). Response of the Pine Engraver, *Ips pini* (Say) (Coleoptera: Scolytidae), to Conophthorin and Other Angiosperm Bark Volatiles in the Avoidance of Non-Hosts. *Agr. Forest Entomol.* **3,** 225-232.

Hunter, D. M. (2004). Advances in the Control of Locusts (Orthoptera : Acrididae) in Eastern Australia: From Crop Protection to Preventive Control. *Aust. J. Entomol.* **43,** 293-303.

Inayatullah, C., El Bashir, S. and Hassanali, A. (1994). Sexual Behavior and Communication in the Desert Locust, *Schistocerca gregaria* (Orthoptera: Acrididae): Sex Pheromone in Solitaria. *Environ. Entomol.* **23,** 1544-1551.

Injeyan, H. S. and Tobe, S. S. (1981). Phase Polymorphism in *Schistocerca gregaria* - Assessment of Juvenile-Hormone Synthesis in Relation to Vitellogenesis. *J. Insect Physiol.* **27,** 203-210.

Ishida, Y. and Leal, W. S. (2005). Rapid Inactivation of a Moth Pheromone. *P. Natl. Acad. Sci. USA.* **102,** 14075-14079.

Islam, M. S., Roessingh, P., Simpson, S. J. and McCaffery, A. R. (1994a). Effects of Population Density Experienced by Parents during Mating and Oviposition on the Phase of Hatchling Desert Locusts, *Schistocerca gregaria*. *P. Roy. Soc. London, B* **257,** 93-98.

Islam, M. S., Roessingh, P., Simpson, S. J. and McCaffery, A. R. (1994b). Parental Effects on the Behaviour and Colouration of Nymphs of the Desert Locust *Schistocerca gregaria*. *J. Insect Physiol.* **40,** 173-181.

Isman, M. B. (2004). In Plant Essential Oils as Green Pesticides for Pest and Disease Management. *ACS Symp. Ser.* **887,** 41-51.

Jacobsen, M. and Beroza, M. (1964). Insect Attractants. *Sci. Am.* **211,** 20-27.

Jakobsen, H. B. (1997). The Preisolation Phase of in Situ Headspace Analysis: Methods and Perspectives. In *Plant Volatile Analysis.* (ed. H. Linskens and J. Jackson), pp. 1-22. Berlin: Springer Verlag.

Kaissling, K. E. and Thorson, J. (1980). Insect Olfactory Sensilla: Structural, Chemical and Electrical Aspects of the Functional Organization. In *Receptors for Neurotransmitters, Hormones and Pheromones in Insects.* (ed. D. B. Satelle, L. M. Hall and J. G. Hildebrand), pp. 261-282. New York: Elsevier/North- Holland Biomedical Press.

Kaissling, K. (1996). Peripheral Mechanisms of Pheromone Reception in Moths. *Chem. Senses* **21,** 257-268.

Kaissling, K. -. (2004). Physiology of Pheromone Reception in Insects (an Example of Moths). *Anir* **6,** 73-91.

Kanaujia, S. and Kaissling, K. E. (1985). Interactions of Pheromone with Moth Antennae: Adsorption, Desorption and Transport. *J. Insect Physiol.* **31,** 71-81.

Karlson, P. and Luscher, M. (1959). Pheromones - New Term for a Class of Biologically Active Substances. *Nature* **183,** 55-56.

Keil, T. A. (1997). Comparative Morphogenesis of Sensilla: A Review. *Int. J. Insect Morphol. Embryol.* **26,** 151-160.

Keil, T. A. (1999). Morphology and Development of the Peripheral Olfactory Organs. In *Insect Olfaction.* (ed. B. S. Hansson), pp. 5-47. New York: Springer.

Kendall, M. D. (1972). Glandular Epidermis on the Tarsi of the Desert Locust, *Schistocerca gregaria*. *Acrida* **1,** 121-147.

Kenis, M. and Hilszczanski, J. (2007). Natural Enemies of Cerambycidae and Buprestidae Infesting Living Trees. In *Bark and Wood Boring Insects in Living Trees in Europe: A Synthesis.* (ed. F. Lieutier, K. R. Day, A. Battisti, J. C. Grégoire and H. F. Evans), pp. 475-497. Netherlands: Springer.

Kesselmeier, J. and Staudt, M. (1999). Biogenic Volatile Organic Compounds (VOC): An Overview on Emission, Physiology and Ecology. *J. Atmos. Chem.* **33,** 23-88.

Kim, J. L. and Yamasaki, T. (1996). Sensilla of *Carabus* (Isiocarabus) *Fiducarius saishutoicus* Csiki (Coleoptera: Carabidae). *Int. J. Insect Morphol. Embryol.* **25,** 153-172.

Knipling, E. F. and McGuire, J. U. J. (1966). Population Models to Test Theoretical Effects of Sex Attractants used for Insect Control. *USDA Inf. Bull.* **308,** 2-4.

Koontz, M. A. and Schneider, D. (1987). Sexual Dimorphism in Neuronal Projections from the Antennae of Silk Moths (*Bombyx mori*, *Antheraea polyphemus*) and the Gypsy Moth (*Lymantria dispar*). *Cell Tissue Res.* **249,** 39-50.

Lánsky, P. (2001). Receptor Heterogeneity and its Effect on Sensivity and Coding Range in Olfactory Sensory Neurons. *Bull. Math. Biol.* **63,** 885-908.

Latchininsky, A. V. (1991). Forecasting of the Moroccan Locust *Dociostaurus maroccanus* (Thunb.) Outbreaks in the USSR. *XIII International Plant Protection Congress.*

Latchininsky, A. V. (1993). Survival Strategies of Moroccan Locust *Dociostaurus maroccanus* (Thunberg, 1815) in Different Parts of its Range: An Ecotonal Aspect. *Metaleptea* **14,** 12.

Latchinisky, A. V. (1998). Moroccan Locust *Dociostaurus maroccanus* (Thunberg, 1815): A Faunistic Rarity or an Important Economic Pest? *J. Insect Conserv.* **2,** 167-178.

Laufer, H. and Biggers, W. J. (2001). Unifying Concepts Learned from Methyl Farnesoate for Invertebrate Reproduction and Post-Embryonic Development, *Amer. Zool.* **41,** 442-457.

Leal, W. S. (2005). Pheromone Reception. *Top. Curr. Chem.* **240,** 1-36.

Lecoq, M. (2010). Integrated Pest Management for Locusts and Grasshoppers: Are Alternatives to Chemical Pesticides Credible? *J. Orthop. Res.* **19,** 131-132.

Lelito, J. P., Fraser, I., Mastro, V. C., Tumlinson, J. H., Boeroeczky, K. and Baker, T. C. (2007). Visually Mediated 'Paratrooper Copulations' in the Mating Behavior of *Agrilus planipennis* (Coleoptera: Buprestidae), a Highly Destructive Invasive Pest of North American Ash Trees. *J. Insect Behav.* **20,** 537-552.

Lelito, J. P., Fraser, I., Mastro, V. C., Tumlinson, J. H. and Baker, T. C. (2008). Novel Visual-Cue-Based Sticky Traps for Monitoring of Emerald Ash Borers, *Agrilus planipennis* (Col., Buprestidae). *J. Appl. Entomol.* **132,** 668-674.

Lelito, J. P., Boeroczky, K., Jones, T. H., Fraser, I., Mastro, V. C., Tumlinson, J. H. and Baker, T. C. (2009). Behavioral Evidence for a Contact Sex Pheromone Component of the Emerald Ash Borer, *Agrilus planipennis* Fairmaire. *J. Chem. Ecol.* **35,** 104-110.

Lieutier, F., Day, K. R., Battisti, A., Grégoire, J. C. and Evans, H. F. (2004). In *Bark and Wood Boring Insects in Living Trees in Europe, a Synthesis.*, pp. 569. Netherlands: Springer.

Lockey, K. H. (1988). Lipids of the Insect Cuticle - Origin, Composition and Function. *Comp. Biochem. Physiol. B* **89,** 595-645.

Lockey, K. H. (1991). Insect Hydrocarbon Classes - Implications for Chemotaxonomy. *Insect Biochem.* **21,** 91-97.

Lockey, K. H. and Oraha, V. S. (1990). Cuticular Lipids of Adult *Locusta migratoria migratoriodes* (r and f), *Schistocerca gregaria* (Forskål) (Acrididae) and Other Orthopteran Species .2. Hydrocarbons. *Comp. Biochem. Physiol. B* **95,** 721-744.

Loher, W. (1960). The Chemical Acceleration of the Maturation Process and its Hormonal Control in the Male of the Desert Locust. *P. Roy. Soc. London, B* **153,** 380-397.

Loher, W. (1990). Pheromones and Phase Transformation in Locusts. In *Biology of Grashoppers*. (ed. F. Chapman, R.F. and Joern, A.), pp. 337-355. Wiley, New York.

Lombardero, M. J. and Fernández de Ana Magán, F.J. (1996). Distribución y Daños de *Coroebus florentinus* (HERBST) (COL., BUPRESTIDAE) en Galícia (No de la Península Ibérica). *Ecología* **10,** 499-508.

Lorelle, V. (1989). Casques Verts Contre Criquets Pélerins. *Phytoma* **404,** 22-30.

Loreto, F. (2002). Distribution of Isoprenoid Emitters in the *Quercus* Genus Around the World: Chemo-Taxonomical Implications and Evolutionary Considerations Based on the Ecological Function of the Trait. *Perspect. Plant Ecol.* **5,** 185-192.

Magor, J. (1989). Joining Battle with the Desert Locust. *Shell Agric..* **3,** 12-15.

Mahamat, H., Hassanali, A., Odongo, H., Torto, B. and El Bashir, S. (1993). Studies on the Maturation-Accelerating Pheromone of the Desert Locust *Schistocerca gregaria* (Orthoptera:Acrididae). *Chemoecology* **4,** 159-164.

Mahamat, H., Hassanali, A. and Odongo, H. (2000). The Role of Different Components of the Pheromone Emission of Mature Males of the Desert Locust, *Schistocerca gregaria* (Forskål) (Orthoptera: Acrididae) in Accelerating Maturation of Immature Adults. *Insect Sci. Appl.* **20,** 1-5.

Malual, A. G., Hassanali, A., Torto, B., Assad, Y. O. H. and Njagi, P. G. N. (2001). The Nature of the Gregarizing Signal Responsible for Maternal Transfer of Phase to the Offspring in the Desert Locust *Schistocerca gregaria*. *J. Chem. Ecol.* **27,** 1423-1435.

Manos, P. S., Doyle, J. J. and Nixon, K. C. (1999). Phylogeny, Biogeography and Processes of Molecular Differentiation in *Quercus* subgenus *Quercus* (Fagaceae). *Mol. Phylogenet. Evol.* **12,** 333-349.

Marchal, E., Zhang, J., Badisco, L., Verlinden, H., Hult, E. F., Van Wielendaele, P., Yagi, K. J., Tobe, S. S. and Vanden Broeck, J. 2011. Final Steps in Juvenile Hormone Biosynthesis in the Desert Locust, *Schistocerca gregaria*. *Insect Biochem. Mol. Biol.* in press.

Martikainen, P., Viiri, H. and Raty, M. (2001). Beetles (Coleoptera) Caught with Pheromones of *Gnathotrichus retusus* and *G. sulcatus* (Col., Scolytidae) in Southern Finland. *J. Pest Sci.* **74,** 7-10.

Martín-Bernal, E., Cobos-Suárez, J. M., Arias-Giralda, A., Morales-Agacino, E. and Sopeña-Mañas, J. M. (1993). La Langosta Mediterránea *Dociostaurus maroccanus* (Thunberg): Adenda. *Bol. San. Veg. Pl.* **19,** 1001-1011.

Mayer, M. S. and McLaughlin, J. R. (1991). In *Handbook of Insect Pheromones and Sex Attractants.*, pp.1085. Boca Raton: CRC Press.

McCaffery, A. R., Simpson, S. J., Islam, M. S. and Roessingh, P. (1998). A Gregarizing Factor Present in the Egg Pod Foam of the Desert Locust *Schistocerca gregaria. J. Exp. Biol.* **201,** 347-363.

McCullough, D. G. and Roberts, D. L. (2002). Emerald Ash Borer. *USDA Forest Service Pest Alert*.

McGarvey, D. J. and Croteau, R. (1995). Terpenoid Metabolism. *Plant Cell* **7,** 1015-1026.

McIntosh, R. L., Katinic, P. J., Allison, J. D., Borden, J. H. and Downey, D. L. (2001). Comparative Efficacy of Five Types of Trap for Woodborers in the Cerambycidae, Buprestidae and Siricidae. *Agr. Forest Entomol.* **3,** 113-120.

Mendizabal, M. (1943). Datos sobre la Plaga de Langosta en la Provincia de Almeria. *Bol. Pat. Veg. Entomol. Agr.* **12,** 285-293.

Merivee, E., Rahi, M. and Luik, A. (1999). Antennal Sensilla of the Click Beetle, *Melanotus villosus* (Geoffroy) (Coleoptera: Elateridae). *Int. J. Insect Morphol. Embryol.* **28,** 41-51.

Merivee, E., Ploomi, A., Rahi, M., Luik, A. and Sammelselg, V. (2000). Antennal Sensilla of the Ground Beetle *Bembidion lampros* Hbst (Coleoptera, Carabidae). *Acta Zool.* **81,** 339-350.

Merivee, E., Ploomi, A., Rahi, M., Bresciani, J., Ravn, H. P., Luik, A. and Sammelselg, V. (2002). Antennal Sensilla of the Ground Beetle *Bembidion properans* Steph. (Coleoptera, Carabidae). *Micron* **33,** 429-440.

Miller, D. R. (2006). Ethanol and (–)-a-Pinene: Attractant Kairomones for some Large Wood-Boring Beetles in Southeastern USA. *J. Chem. Ecol.* **32,** 779-794.

Miller, G. A., Islam, M. S., Claridge, T. D. W., Dodgson, T. and Simpson, S. J. (2008). Swarm Formation in the Desert Locust *Schistocerca gregaria*: Isolation and NMR Analysis of the Primary Maternal Gregarizing Agent. *J. Exp. Biol.* **211,** 370-376.

Mitchell, E. R., Sugie, H. and Tumlinson, J. H. (1983). *Spodoptera exigua*: Capture of Feral Males in Traps Baited with Blends of Pheromone Components. *J. Chem. Ecol.* **9,** 95-104.

Montgomery, M. E. and Wargo, P. M. (1983). Ethanol and Other Host-Derived Volatiles as Attractants to Beetels that Bore into Hardwoods. *J. Chem. Ecol.* **9,** 181-190.

Montoya, J. M. (1989). *Los Alcornocales.*, pp. 155. Madrid: Instituto Nacional de Investigaciones Agrarias.

Moorhouse, J. E., Yeadon, R., Beevor, P. S. and Nesbitt, B. F. (1969). Method for Use in Studies of Insect Chemical Communication. *Nature* **223,** 1174-1175.

Moraal, L. G. and Hilszezanski, J. (2000). The Oak Buprestid Beetle, *Agrilus biguttatus* (F.) (Col., Buprestidae), a Recent Factor in Oak Decline in Europe. *J. Pest Sci.* **73,** 134-138.

Moreno-Márquez, V. (1944). Zonas Permanentes de Langosta en España: Boceto Ecológico de la Serena. *Bol. Pat. Veg. Ent. Agr.* **13,** 335-376.

Morgan, D. (2010). In *Biosynthesis in Insects, Advanced Edition.*, pp. 344. Cambridge: RSC Publishing.

Mori, K. (2010). Pheromones in Chemical Communication. In *The Chemistry and Biology of Volatiles* (ed. A. Herrmann), pp. 123-147. West Sussex, UK.: John Wiley and Sons Ltd.

Murugan, K. (2006). Bio-Diversity of Insects. *Curr. Sci.* **91,** 1602-1603.

Myers, J. H., Simberloff, D., Kuris, A. M. and Carey, J. R. (2000). Eradication Revisited: Dealing with Exotic Species. *Trends Ecol. Evol.* **15,** 316-320.

Narasimhan, B., Mourya, V. and Dhake, A. (2006). Design, Synthesis, Antibacterial, and QSAR Studies of Myristic Acid Derivatives. *Bioorgan. Med. Chem. Lett.* **16,** 3023-3029.

Narayanan, U. S. and Nadarajan, L. (2005). Evidence for a Male-Produced Sex Pheromone in Sesame Leaf Webber, *Antigastra catalaunalis* Duponchel (Pyraustidae, Lepidoptera). *Curr. Sci. (Bangalore)* **88,** 631-634.

Njagi, P. G. N. and Torto, B. (1998). Sex Pheromone Studies in the Desert Locust *Schistocerca gregaria* (Forskål) (Orthoptera: Acrididae). *Chemoecology* **7,** 172-178.

Njagi, P. G. N., Torto, B., Obeng-Ofori, D. and Hassanali, A. (1996). Phase-Independent Responses to Phase-Specific Aggregation Pheromone in Adult Desert Locusts, *Schistocerca gregaria* (Orthoptera: Acrididae). *Physiol. Entomol.* **21,** 131-137.

Norris, M. J. (1954). (Mrs. O. W. Richards). Sexual Maturation in the Desert Locust (*Schistocerca gregaria* Forskål) with Special Reference to the Effects of Grouping. *Anti-Locust Bull. London* **18,** 1-44.

Norris, M. J. (1964). Accelerating and Inhibiting Effects of Crowding on Sexual Maturation in Two Species of Locusts. *Nature* **203,** 784-785.

Norris, M. J. (1968). Laboratory Experiments on Oviposition Responses of the Desert Locust, *Schistocerca gregaria* (Forsk.). *Anti-Locust Bull.*, *London*. **43,** 1-47.

Norris, M. J. (1970). Aggregation Response in Ovipositing Females of the Desert Locust, with Special Reference to the Chemical Factor. *J. Insect Physiol.* **16,** 1493-1515.

Nuñez, A. J., Gonzalez, L. F. and Janak, J. (1984). Pre–concentration of Headspace Volatilesfrom Trace Organic Analysis by Gas Chromatography. *J. Chromatogr.* **300,** 127-162.

Obeng-Ofori, D., Torto, B. and Hassanali, A. (1993). Evidence for Mediation of Two Releaser Pheromones in the Aggregation Behavior of the Gregarious Desert Locust, *Schistocerca gregaria*. *J. Chem. Ecol.* **19,** 1665-1676.

Obeng-Ofori, D., Torto, B., Njagi, P. G. N., Hassanali, A. and Amiani, H. (1994a). Faecal Volatiles as Part of the Aggregation Pheromone Complex of the Desert Locust, *Schistocerca gregaria* (Forskål) (Orthoptera: Acrididae). *J. Chem. Ecol.* **20,** 2077-2087.

Obeng-Ofori, D., Njagi, P. G. N., Torto, B., Hassanali, A. and Amiani, H. (1994b). Sex Differentiation Studies Relating to Releaser Aggregation Pheromones of the Desert Locust, *Schistocerca gregaria*. *Entomol. Exp. Appl.* **73,** 85-91.

Ochieng, S. A., Hallberg, E. and Hansson, B. S. (1998). Fine Structure and Distribution of Antennal Sensilla of the Desert Locust, *Schistocerca gregaria* (Orthoptera: Acrididae). *Cell Tissue Res.* **291,** 525-536.

Ochieng, S. A. and Hansson, B. S. (1999). Responses of Olfactory Receptor Neurones to Behaviourally Important Odours in Gregarious and Solitarious Desert Locust, *Schistocerca gregaria*. *Physiol. Entomol.* **24,** 28-36.

Oehlschlager, A. C., Chinchilla, C., Castillo, G. and Gonzalez, L. (2002). Control of Red Ring Disease by Mass Trapping of *Rhynchophorus palmarum* (Coleoptera: Curculionidae). *Fla. Entomol.* **85,** 507-513.

Okada, K., Mori, M., Shimazaki, K. and Chuman, T. (1992). Morphological Studies on the Antennal Sensilla of the Cigarette Beetle, *Lasioderma serricorne* (F.) (Coleoptera: Anobiidae). *Appl. Entomol. Zool.* **27,** 269-276.

Oraha, V. S. and Lockey, K. H. (1990). Cuticular Lipids of *Locusta migratoria migratoriodes* (R and F), *Schistocerca gregaria* (Forskål) (Acrididae) and other Orthopteran Species I. Polar Components. *Comp. Biochem. Physiol. B:* **95,** 603-608.

Paré, P. W. and Tumlinson, J. H. (1996). Plant Volatile Signals in Response to Herbivore Feeding. *Fla. Entomol.* **79,** 93-103.

Pawliszyn, J. (1997). In *Solid Phase Microextraction: Theory and Practice.*, pp. 264. Weinheim: Wiley-VCH.

Pawliszyn, J. (1999). In *Applications of Solid Phase Microextraction.*, pp. 655. London: RSC Publishing.

Pener, M. P. (1991). Locust Phase Polymorphism and its Endocrine Relations. *Adv. Insect Physiol.* **23,** 1-79.

Pener, M. P. and Yerushalmi, Y. (1998). The Physiology of Locust Phase Polymorphism: An Update. *J. Insect Physiol.* **44,** 365-377.

Petroski, R. J., Tellez, M. R. and Behle, R. W. (2005). In *Semiochemicals in Pest and Weed Control.*, pp. 184. Oxford: ACS Publication.

Pophof, B. (1997). Olfactory Responses Recorded from Sensilla Coeloconica of the Silkmoth *Bombyx mori*. *Physiol. Entomol.* **22,** 239-248.

Prestwich, G. D., Graham, S. M., Handley, M., Latli, B., Streinz, L. and Tasayco J., M. L. (1989). Enzymatic Processing of Pheromones and Pheromone Analogs. *Experientia* (Basel) **45,** 263-270.

Quesada-Moraga, E. and Santiago-Alvarez, C. (1999). Inducción de la Salida de la Diapausa en la Langosta Mediterránea, *Dociostaurus maroccanus* (Thunberg). *J. Orthoptera Res.* **8,** 13-16.

Quesada-Moraga, E. and Santiago-Alvarez, C. (2000). Temperature Related Effects on Embryonic Development of the Mediterranean Locust, *Dociostaurus maroccanus*. *Physiol. Entomol.* **25,** 191-195. m

Quesada-Moraga, E. and Santiago-Alvarez, C. (2001a). Histopathological Effects of *Bacillus thuringiensis* on the Midgut of the Mediterranean Locust, *Dociostaurus maroccanus*. *J. Invertebr. Pathol.* **78,** 183-186.

Quesada-Moraga, E. and Santiago-Alvarez, C. (2001b). Rearing and Breeding of the Moroccan Locust, *Dociostaurus maroccanus* (Thunberg) (Orthop., Acrididae) Under Laboratory Conditions. *J. Appl. Entomol.* **125,** 121-124.

Rai, M. M., Hassanali, A., Saini, R. K., Odongo, H. and Kahoro, H. (1997). Identification of Components of the Oviposition Aggregation Pheromone of the Gregarious Desert Locust, *Schistocerca gregaria* (Forskål). *J. Insect Physiol.* **43,** 83-87.

Raming, K., Krieger, J. and Breer, H. (1990). Primary Structure of a Pheromone-Binding Protein from *Antheraea pernyi*: Homologies with Other Ligand-Carrying Proteins. *J. Comp. Physiol. B* **160,** 502-509.

Recalde, J. I. and San Martín, A. F. (2003). Coleópteros Xilófagos Asociados a Ramas de *Quercus* Muertas por la Acción del Bupréstido *Coraebus florentinus* (Herbst, 1801) en la Navarra Media. *Heteropt. Rev. Ent.* **3,** 43-50.

Reigart, J. and Roberts, J. (1999). In *Recognition and Management of Pesticide Poisonings.*, pp. 238. Washington: EPA.

Reinecke, A., Ruther, J. and Hilker, M. (2002a). The Scent of Food and Defence: Green Leaf Volatiles and Toluquinone as Sex Attractant Mediate Mate Finding in the European Cockchafer *Melolontha melolontha*. *Ecol. Lett.* **5,** 257-263.

Reinecke, A., Ruther, J., Tolasch, T., Francke, W. and Hilker, M. (2002b). Alcoholism in Cockchafers: Orientation of Male *Melolontha melolontha* Towards Green Leaf Alcohols. *Naturwissenschaften* **89,** 265-269.

Renou, M. and Guerrero, A. 2. (2000). Insect Parapheromones in Olfaction Research and Semiochemical-Based Pest Control Strategies. *Annu. Rev. Entomol.* **45,** 605-630.

Ridgway, R. L., Silverstein, R. M. and Inscoe, M. N. (1990). In *Behavior-Modifying Chemicals for Insect Management*., pp.780. New York: CRC Press.

Rocha, S., Delgadillo, I. and Ferrer-Correia, A. J. (1996). GC-MS Study of Volatiles of Normal and Microbiologically Attacked Cork from *Quercus suber* L. *J. Agr. Food Chem.* **44,** 865-871.

Rodriguez-Saona, C., Poland, T. M., Miller, J. R., Stelinski, L. L., Grant, G. G., de Groot, P., Buchan, L. and MacDonald, L. (2006). Behavioral and Electrophysiological Responses of the Emerald Ash Borer, *Agrilus planipennis*, to Induced Volatiles of Manchurian Ash, *Fraxinus manchurica*. *Chemoecology* **16,** 75-86.

Roelofs, W. L. (1984). Electroantennogram Assays: Rapid and Convenient Screening Procedure for Pheromones. In *Techniques in Pheromone Research.* (ed. H. E. Hummel and T. A. Miller), pp. 131-160. New York: Springer.

Roessingh, P. and Simpson, S. J. (1994). The Time-Course of Behavioral Phase Change in Nymphs of the Desert Locust, *Schistocerca gregaria*. *Physiol. Entomol.* **19,** 191-197.

Roessingh, P., Bouaichi, A. and Simpson, S. J. (1998). Effects of Sensory Stimuli on the Behavioural Phase State of the Desert Locust, *Schistocerca gregaria*. *J. Insect Physiol.* **44,** 883-893.

Roffey, J. and Popov, G. (1968). Environmental and Behavioral Processes in a Desert Locust Outbreak. *Nature* **219,** 446-450.

Romanyk, N. and Cadahia, D. (1992). In *Colección Técnica ICONA.* Plagas de Insectos en las Masas Forestales Españolas., Madrid: Ministerio de Agricultura, Pesca y Alimentación.

Rono, E., Njagi, P. G. N., Bashir, M. O. and Hassanali, A. (2008). Concentration-Dependent Parsimonious Releaser Roles of Gregarious Male Pheromone of the Desert Locust, *Schistocerca gregaria*. *J. Insect Physiol.* **54,** 162-168.

Rudinsky, J. A. (1962). Ecology of Scolytidae. *Ann.Rev.Ent.* **7,** 327-348.

Ruther, J., Reinecke, A., Thiemann, K., Tolasch, T., Francke, W. and Hilker, M. (2000). Mate Finding in the Forest Cockchafer, *Melolontha hippocastani*, Mediated by Volatiles from Plants and Females. *Physiol. Entomol.* **25,** 172-179.

Ruther, J., Meiners, T. and Steidle, J. L. M. (2002). Rich in Phenomena-Lacking in Terms. A Classification of Kairomones. *Chemoecology* **12,** 161-167.

Sabelis, M. W. and van de Baan, H. E. (1983). Location of Distant Spider Mite Colonies by Phytoseid Predators: Demonstration of Specific Kairomones Emitted by *Tetranychus urticae* and *Panonychus ulmi*. *Entomol. Exp. Appl.* **33,** 303-314.

Saini, R. K., Rai, M. M., Hassanali, A., Wawiye, J. and Odongo, H. (1995). Semiochemicals from Froth of Egg Pods Attract Ovipositing Female *Schistocerca gregaria*. *J. Insect. Physiol.* **41,** 711-716.

Sanna, V., Mariani, A., Caria, G. and Sechi, M. (2009). Synthesis and Evaluation of Different Fatty Acid Esters Formulated into Precirol® ATO-Based Lipid Nanoparticles as Vehicles for Topical Delivery. *Chem. Pharm. Bull.* **57,** 680-684.

Santiago-Alvarez, C., Quesada-Moraga, E. and Hernandez-Crespo, P. (2003). Diapause Termination and Post-Diapause Development in the Mediterranean Locust, *Dociostaurus maroccanus* (Orth., Acrididae) Under Field Conditions. *J. Appl. Entomol.* **127,** 369-373.

Schmidt, G. H. and Albütz, R. (2002). Sexual Maturation and Yellow Coloration of Adult Males in the Gregarious Desert Locust, *Schistocerca gregaria*, in Relation to Volatile Emitted (Caelifera: Acrididae). *Entomol. Gen.* **26,** 121-141.

Schneider, D. (1957). Elektrophysiologische Untersuchungen von Chemo- und Mechanorezeptoren der Antenne des Seidenspinners *Bombyx mori*. *Z. Vergl. Physiol.* **40,** 8-41.

Schneider, D. (1969). Insect Olfaction: Deciphering System for Chemical Messages. *Science* **163,** 1031-1037.

Schneider, D. (1999). Insect Pheromone Research: Some History and 45 Years of Personal Recollections. *West Palaearctic Regional Section Bull.* **22,**.

Schoonhoven, L. (1990). Insects in a Chemical World. In *Handbook of Natural Pesticides, Volume VI: Insect Attractants and Repellents.* (ed. E. D. Morgan and N. B. Mandava), pp. 1-22. Boca Raton, Florida.: CRC Press.

Seidelmann, K. and Ferenz, H. M. (2002). Courtship Inhibition Pheromone in Desert Locusts, *Schistocerca gregaria*. *J. Insect Physiol.* **48,** 991-996.

Seidelmann, K., Weinert, H. and Ferenz, H. J. (2003). Wings and Legs are Production Sites for the Desert Locust Courtship-Inhibition Pheromone, Phenylacetonitrile. *J. Insect Physiol.* **49,** 1125-1133.

Seidelmann, K., Warnstorff, K. and Ferenz, H. J. (2005). Phenylacetonitrile is a Male Specific Repellent in Gregarious Desert Locusts, *Schistocerca gregaria*. *Chemoecology* **15,** 37-43.

Seidelmann, K. (2009). The Courtship-Inhibiting Pheromone is Ignored by Female-Deprived Gregarious Desert Locust Males. *Biol. Lett.* **2,** 525-527.

Showler, A. T. and Potter, C. S. (1991). Synopsis of the 1986–89 Desert Locust (Othoptera: Acrididae) Plague and the Concept of Strategic Control. *Am. Entomol.* **37,** 106-110.

Showler, A. T. (1995). Locust (Orthoptera: Acrididae) Outbreak in Africa and Asia,1992–94: An Overview. *Am. Entomol.* **41,** 179-185.

Showler, A. T. (2002). A Summary of Control Strategies for the Desert Locust, *Schistocerca gregaria* (Forskål). *Agr. Ecosyst. Environ.* **90,** 97-103.

Siljander, E., Gries, R., Khaskin, G. and Gries, G. (2008). Identification of the Airborne Aggregation Pheromone of the Common Bed Bug, *Cimex lectularius*. *J. Chem. Ecol.* **34,** 708-718.

Silk, P. J., Ryall, K., Lyons, D. B., Sweeney, J. and Wu, J. (2009). A Contact Sex Pheromone Component of the Emerald Ash Borer *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae). *Naturwissenschaften* **96,** 601-608.

Silverstein, R. M. (1981). Pheromones: Background and Potential for use in Insect Pest Control. *Science* **213,** 1326-1332.

Simpson, S. J., Despland, E., Hägele, B. F. and Dodgson, T. (2001). Gregarious Behavior in Desert Locusts is Evoked by Touching their Back Legs. *P. Natl. Acad. Sci. USA.* **98,** 3895-3897.

Simpson, S. J., Sword, G. A. and De Loof, A. (2005). Advances, Controversies and Consensus in Locust Phase Polyphenism Research. *J. Orthoptera Res.* **14,** 213-222.

Simpson, S. J. and Sword, G. A. (2007). Phase Polyphenism in Locusts: Mechanisms, Population Consequences, Adaptive Significance and Evolution. In *Phenotypic Plasticity in Insects* (ed. T. Ananthakrishnan and D. Whitman), pp. 93-135. Enfield, NH: Science Publishers.

Simpson, S. J. and Sword, G. A. (2008). Locust. *Curr. Biol.* **18,** 364-366.

Solinas, M. (1974). *Coroebus florentinus* (Herbst) (Coleoptera, Buprestidae). *Biol. Danni Lotta Entomol.* **10,** 141-193.

Soria, F. J. (1990). Estudios Biologicos sobre *Coreobus undatus* (Fabricius) y *Coroebus florentinus* (Herbst) (Coleoptera, Buprestida) en Alconorcales de Andalucia Occidental. *PhD thesis.*, pp.1-326.

Soria, F. J., Villagrán, M. and Ocete, M. E. (1992). Estudios Poblacionales sobre *Coroebus undatus* (Fabricius) (Coleoptera, Buprestidae) en Alcornocales de Andalucía Occidental. I: Relación Infestación-Bosque. *Bol. San. Veg. Pl.* **18,** 377-383.

Soria, F. J. and Ocete, M. E. (1993). Estudios Poblacionales sobre *Coroebus florentinus* (Herbst) (Col. Buprestidae) en Alcornocales Andaluces. *Bol. San. Veg. Pl.* **19,** 27-35.

Soria, F. J., Villagrán, M., Del Tío, R. and Ocete, M. E. (1994). Estudios Prospectivos de los Principales Perforadores del Alcornoque en la Sierra Norte de Sevilla. *Bol. San. Veg. Pl.* **20,** 643-651.

Staudt, M., Mir, C., Joffre, R., Rambal, S., Bonin, A., Landais, D. and Lumaret, R. (2004). Isoprenoid Emissions of *Quercus spp.* (*Q. suber* and *Q. ilex*) in Mixed Stands Contrasting in Interspecific Genetic Introgression. *New Phytol.* **163,** 573-584.

Steinbrecht, R. A. (1964). Freinstruktur und Histochemie der Sexualduftdrüse des Seidenspinners *Bombyx mori*. *Z. Zellforsch.* **64,** 227-261.

Steinbrecht, R. A. (1973). Der Feinbau Olfactorischer Sensillen des Seidenspinners (Insecta, Lepidoptera). *Z. Zellforsch.* **139,** 533-565.

Steinbrecht, R. A. (1987). Functional Morphology of Pheromone Sensitive Sensilla. In *Pheromone Biochemistry*. (ed. G. D. Prestwich and G. J. Blomquist), pp. 353-383. Orlando, Florida: Academic Press.

Steinbrecht, R. A., Laue, M., Maida, R. and Ziegelberger, G. (1996). Odorant-Binding Proteins and their Role in the Detection of Plant Odours. *Entomol. Exp. Appl.* **80,** 15-18.

Steinbrecht, R. A. (1996). Structure and Function of Insect Olfactory Sensilla. *Ciba Found. Symp.* **200,** 158-174.

Steinbrecht, R. A. (1998). Odorant-Binding Proteins: Expression and Function. *Olfaction and Taste XII. , Ann. N. Y. Acad. Sci.* **855,** 323-332.

Stengl, M., Ziegelberger, G., Boekhoff, I. and Krieger, J. (1999). Perireceptor Events and Transduction Mechanisms in Insect Olfaction. In *Insect Olfaction* (ed. B. S. Hansson), pp. 50-66. Berlin: Springer.

Streett, D. A. and Henry, J. E. (1990). Microbial Control of Locusts and Grasshoppers in the Semi-Arid Tropics. *Bol. San. Veg. Pl.* **20,** 21-27.

Stride, B., Shah, A. and Sadeed, S. M. (2003). Recent History of Moroccan Locust Control and Implementation of Mechanical Control Methods in Northern Afghanistan. *Int. J. Pest Manag.* **49,** 265.

Suñer, D. and Abós, L. (1994). Estudio de la Infestación de *Coroebus undatus* (FABRICIUS) (COL., BUPRESTIDAE) en los Alcornocales Catalanes. *Scientia gerund.* **20,** 45-53.

Takabayashi, J., Dicke, M. and Posthumus, M. A. (1996). Volatile Herbivore-Induced Terpenoids in Plant-Mite Interactions: Variation Caused by Biotic and Abiotic Factors. *J. Chem. Ecol.* **20,** 1329-1354.

Tanaka, S. (2005). Hormonal Control of Phase Polyphenism in Locusts. *Formosan Entomol.* **25,** 131-143.

Timms, L. L., Smith, S. M. and de Groot, P. (2006). Patterns in the Within-Tree Distribution of the Emerald Ash Borer *Agrilus planipennis* (Fairmaire) in Young, Green-Ash Plantations of South-Western Ontario, Canada. *Agr. For. Entomol.* **8,** 313-321.

Tingle, F. C. and Mitchell, E. R. (1982). Disruption of Pheromone Communication of the Tobacco Budworm in Tobacco Fields Treated with Pheromone Components. *J. Econ. Entomol.* **75,** 50-56.

Torto, B., Obeng-Ofori, D., Njagi, P. G. N., Hassanali, A. and Amiani, H. (1994). Aggregation Pheromone System of Adult Gregarious Desert Locust *Schistocerca gregaria* (Forskål). *J. Chem. Ecol.* **20,** 1749-1762.

Torto, B., Njagi, P. G. N., Hassanali, A. and Amiani, H. (1996). Aggregation Pheromone System of Nymphal Gregarious Desert Locust, *Schistocerca gregaria* (Forskål) (Orthoptera: Acrididae). *J. Chem. Ecol.* **22,** 2273-2281.

Torto, B., Assad, Y. O. H., Njagi, P. G. N. and Hassanali, A. (1999). Evidence for Additional Pheromonal Components Mediating Oviposition Aggregation in *Schistocerca gregaria*. *J. Chem. Ecol.* **25,** 835-845.

Torto, B., Suazo, A., Alborn, H., Tumlinson, J. H. and Teal, P. E. A. (2005). Response of the Small Hive Beetle (*Aethina tumida*) to a Blend of Chemicals Identified from Honeybee (*Apis mellifera*) Volatiles. *Apidologie* **36,** 523-532.

Tumlinson, J. H. and Teal, P. E. A. (1987). Relationship of Structure and Function to Biochemistry in Insect Pheromone Systems. In *Pheromone Biochemistry.* (ed. G. D. Prestwich and G. J. Blomquist), pp. 3-26. Orlando, Florida: Academic Press.

Tumlinson, J. H., Turlings, T. C. J. and Lewis, W. J. (1993). Semiochemically Mediated Foraging Behavior in Beneficial Parasitic Insects. *Arch. Insect Biochem. Physiol.* **22,** 385-391.

Turlings, T. C. J., Tumlinson, J. H. and Lewis, W. J. (1990). Exploitation of Herbivore-Induced Plant Odors by Host-Seeking Parasitic Wasps. *Science* **250,** 1251-1253.

Turlings, T. C. J. and Tumlinson, J. H. (1991). Do Parasitoids use Herbivore-Induced Plant Chemical Defenses to Locate Hosts? *Fla. Entomol.* **74,** 42-50.

Turlings, T. C. J., Tumlinson, J. H., Eller, F. J. and Lewis, W. J. (1991). Larval-Damaged Plants: Source of Volatile Synomones that Guide the Parasitoid *Cotesia marginiventris* to the Micro-Habitat of its Host. *Entomol. Exp. Appl.* **58,** 75-82.

Turlings, T. C. J., Loughrin, J. H., McCall, P. J., Rose, U. S. R., Lewis, W. J. and Tumlinson, J. H. (1995). How Caterpillar-Damaged Plants Protect Themselves by Attracting Parasitic Wasps. *P. Natl. Acad. Sci. USA.* **92,** 4169-4174.

Uvarov, B. P. (1957). The Aridity Factor in the Ecology of Locusts and Grasshoppers of the Old World. In *Arid Zone Research VIII. Human and Animal Ecology. Reviews of Research,* pp. 164-198. Paris: UNESCO.

Uvarov, B. (1966). In *Grasshoppers and Locusts. A Handbook of General Acridology. Volume 1. Anatomy, Development Phase Polymorphism, Introduction to Taxonomy.*, pp. 481. London: CUP.

Uvarov, B. P. (1977). In *Grasshoppers and Locusts. A Handbook of General Acridology. Vol. II: Behaviour, Ecology, Biogeography Population Dynamics.*, pp.614. London: Centre for Overseas Pest Research.

Van den Dool J. and Kratz, P. D. (1963). A Generalization of the Retention Index System Including Linear Programmed Gas-Liquid Partition Chromatography. *J. Chromatogr.* **11,** 463-463.

Van der Pers, J. N. C. and den Otter, C. J. (1978). Single Cell Responses from Olfactory Receptors of Small Ermine Moths to Sex-Attractants. *J. Insect Physiol.* **24,** 337-343.

Verlinden, H., Badisco, L., Marchal, E., Van Wielendaele, P. and Vanden Broeck, J. (2009). Endocrinology of Reproduction and Phase Transition in Locusts. *Gen. Comp. Endocrinol.* **162,** 79-92.

Visser, J. H. (1986). Host Odor Perception in Phytophagous Insects. *Annu. Rev. Entomol.* **31,** 121-144.

Visser, J. H. and de Jong, R. (1988). Olfactory Coding in the Perception of Semiochemicals. *J. Chem. Ecol.* **14,** 2005-2018.

Vogiatzakis, I. N., Griffiths, G. H. and Bacchetta, G. (2005). Human Impacts on *Quercus suber* Habitats in Sardinia: Past and Present. *Botan. Chron.* **18,** 293-300.

Vogt, R. G. and Riddiford, L. M. (1981). Pheromone Binding and Inactivation by Moth Antennae. *Nature* **293,** 161-163.

Vogt, R. G., Riddiford, L. M. and Prestwich, G. D. (1985). Kinetic Properties of a Sex Pheromone-Degrading Enzyme: The Sensillar Esterase of *Antheraea polyphemus*. *P. Nat. Acad. Sci. USA* **82,** 8827-8831.

Vogt, R. G. (2005). Molecular Basis of Pheromone Detection in Insects. In *Comprehensive Insect Physiology, Biochemistry, Pharmacology and Molecular Biology.* (ed. L. I. Gilbert, K. Latro and S. Gill), pp. 753-804. London: Elsevier.

Volkovitsh, M. G. (2001). The Comparative Morphology of Antennal Structures in Buprestidae (Coleoptera): Evolutionary Trends, Taxonomic and Phylogenetic Implications. *Acta Musei Morav. Sci. Biol.* **86,** 43-169.

Vosshall, L. B., Wong, A. M. and Axel, R. (2000). An Olfactory Sensory Map in the Fly Brain. *Cell* **102,** 147-159.

Wadhams, L. J. (1992). The Perception of Semiochemicals. In *Insect Molecular Science. Proceedings, 16th Symposium of the Royal Entomological Society London. Imperial College London, September 1991.* (ed. J. M. Crampton and P. Eggleston), pp. 152-162. London: Academic Press.

Wall, C. (1989). Monitoring and Spray Timin. In *Insect Pheromones in Plant Protection.* (ed. A. R. Jutsum and R. F. S. Gordon), pp. 39-66. Chichester: Wiley.

Wang, Q. and Millar, J. G. (2000). Mating Behavior and Evidence for Male-Produced Sex Pheromones in *Leptoglossus clypealis* (Heteroptera: Coreidae). *Ann. Entomol. Soc. Am.* **93,** 972-976.

Welter, S. C., Pickel, C., Millar, J. G., Cave, F., Van Steenwyk, R. A. and Dunley, J. (2005). Pheromone Mating Disruption Offers Selective Management Options for Key Pests. *Cal. Agr.* **59,** 16-22.

Wheeler, C. M. and Aus, C. (1946). Control of Typhus in Italy 1943-1944 by use of DDT. *Am. J. Pub. Health* **36,** 119-129.

Yarsan, E. and Cakir, O. (2006). Effects of Dichlorvos on Lipid Peroxidation in Mice on Subacute and Subchronic Periods. *Pestic. Biochem. Physiol.* **86,** 106-109.

Zacharuk, R. Y. (1980). Ultrastructure and Function of Insect Chemosensilla. *Annu. Rev. Entomol.* **25,** 27-47.

Zar, J. H. (1999). In *Biostatistical Analysis.*, pp. 663. New Jersey: Prentice Hall.

Zhang, A., Robbins, P. S., Averill, A. L., Weber, D. C., Linn, J., C.E., Roelofs, W. L. and Villani, M. G. (2003). Identification of the Female-Produced Sex Pheromone of the Scarab Beetle, *Hoplia equina*. *J. Chem. Ecol.* **29,** 1635-1642.

Zhang, Q. and Schlyter, F. (2004). Olfactory Recognition and Behavioral Avoidance of Angiosperm Nonhost Volatiles by Conifer-Inhabiting Bark Beetles. *Agr. For. Entomol.* **6,** 1-19.

Zhang, Q., Liu, G., Schlyter, F., Birgersson, G., Anderson, P. and Valeur, P. (2001). Olfactory Responses of *Ips duplicatus* from Inner Mongolia, China to Nonhost Leaf and Bark Volatiles. *J. Chem. Ecol.* **27,** 995-1009.

RESUMEN

INTRODUCCIÓN GENERAL

Control biorracional de insectos plaga

Los insectos representan el grupo más diverso de los animales en el planeta. Por un lado están involucrados en la conservación de varios procesos de ecosistemas vitales, como por ejemplo la polinización o la descomposición y, además, contribuyen directamente a la economía de los seres humanos, por ejemplo mediante la producción de seda o miel (Murugan, 2006). Por otro lado, los insectos constituyen la mayor parte de los organismos herbívoros y ejercen una gran presión selectiva sobre las plantas. A lo largo de la evolución, las plantas han desarrollado numerosos mecanismos de defensa contra sus enemigos (Howe and Westley, 1993; Harborne, 1995), pero debido al gran incremento de la población humana y la necesidad de aprovechar una flora (agricultura) “intacta” tanto para alimentarnos como por intereses económicos, la propia defensa de las plantas ya no es suficiente. Además, ha aumentado de una manera drástica la variedad y el número de dichos insectos-plaga así como el daño y la pérdida de alimentos que producen. Otro gran problema que presentan algunos insectos es que pueden ser usados como vectores de varias enfermedades graves, como son la malaria o el dengue, por lo que, su excesiva proliferación ha suscitado el uso de tratamientos químicos para su control.

En los últimos 60 años la utilización de pesticidas ha representado una pieza angular en la producción de alimentos en países avanzados y en vías de desarrollo (Barr and Needham, 2002), para reducir el daño causado por insectos, enfermedades y malas hierbas. Sin embargo, aunque eficaces a corto plazo, estos tratamientos poco específicos son agresivos con el entorno, provocando pérdida de la biodiversidad circundante y deterioro ambiental. Además, representan un riesgo evidente sobre la salud de los potenciales consumidores de los cultivos (Byers, 1991) por posibles intoxicaciones agudas y crónicas (Yarsan and Cakir, 2006). Finalmente, existe el problema que los insectos disponen de una gran capacidad de adaptación y de crear resistencias contra los pesticidas. Por todas esas razones, es imprescindible encontrar alternativas menos contaminantes que permitan el manejo selectivo de estas plagas para un desarrollo agrícola sostenible. Un enfoque efectivo y sensible con el medioambiente es el control integrado de plagas (*Integrated Pest Management*, IPM), que es una estrategia basada en una combinación de varios métodos mecánicas, químicas, biológicas y de cultivo para controlar las plagas a niveles aceptables con el medio ambiente (Dreistadt et al., 2004).

Entre los métodos alternativos a la utilización de pesticidas ocupa un espacio importante el uso de los semioquímicos, compuestos volátiles muy específicos que conllevan mensajes y regulan la comunicación química y las interacciones intra-/interespecíficas entre individuos y su entorno (Isman, 2004) (Fig. 1, p.6). Los compuestos semioquímicos son sustancias orgánicas que, a través de receptores olfativos y gustativos generalmente encontrados en las antenas de los insectos, actúan sobre el comportamiento de los mismos. Dependiendo de su función y del organismo beneficiado los semioquímicos se dividen en *feromonas* (nombre propuesto por Peter Karlson and Martin Lüscher, 1959), si el compuesto producido por un insecto afecta a individuos de la misma especie (Mori, 2010), y *allelomonas*, si el receptor es de otra especie distinta del emisor (Petroski et al., 2005). Dado que la olfacción es de crucial importancia para la supervivencia de los insectos, el aprovechamiento o la interrupción de este proceso representan una alternativa prometedora en el control de plagas.

El descubrimiento de la primera feromona, denominada bombykol (Butenandt et al., 1959), ha posibilitado la identificación de un gran número de composiciones feromonales de diferentes insectos (Hardie and Minks, 1999). Estos conocimientos permitieron la detección de nuevos atrayentes y repelentes de varios insectos plaga (Schneider, 1999). En numerosas investigaciones las feromonas a solas o en combinación con cairomonas, un subgrupo de allelomonas que benefician al receptor, han mostrado su utilidad en el control integrado de una gran variedad de plagas (Ridgway et al., 1990), tanto en lepidópteros (Mitchell et al., 1983) como en coleópteros (Brockerhoff et al., 2006a) y langostas (Hunter, 2004).

Para la estimación de la presencia, densidad de población, expansión y grado de infestación de una especie plaga se aplican semioquímicos sintéticos (generalmente feromonas de atracción) en trampas para atraer individuos de esa especie (Wall, 1989; Bordon, 1993) (**monitorización**). En la lucha directa contra el insecto se utiliza atrayentes altamente específicos (feromonas sexuales) en un gran número de trampas. El objetivo es capturar el mayor número posible de insectos y de esa manera reducir la población de la próxima generación hasta un nivel económicamente aceptable (Oehlschlager et al., 2002) (**trampeo masivo**). Otra alternativa es colocar una serie de emisores de feromona sintética de la especie a controlar para saturar el ambiente y provocar que los individuos no puedan localizar la emisión feromonal del sexo opuesto (Welter et al., 2005) (**confusión sexual**).

Sin embargo, es imprescindible conocer la morfología, fisiología y el comportamiento específico del insecto antes de empezar un programa de control

integrado de la plaga. Para interferir en su comunicación química es necesario tener, en primer lugar un conocimiento profundo de su percepción de los olores.

Percepción olfativa

Los insectos perciben su entorno a través de moléculas pequeñas (semioquímicos), las cuales les llevan información sobre potenciales parejas, posibles depredadores y características específicas del medio ambiente, como fuentes de alimento, sitios para la oviposición, etc. (Visser, 1986). En la mayoría de animales y para valorar el entorno las señales olfativas son más esenciales que los estímulos visuales o acústicos (Schoonhoven, 1990).

El principal órgano por el cual los insectos detectan los semioquímicos son las antenas, las cuales presentan unas unidades a modo de pelos denominadas *sensilas*, responsables de la detección y reconocimiento de los olores (Fig. 2, p.11) (Keil, 1999). Una sensila es una estructura cuticular en cuyo interior se hallan una o varias células receptoras inervadas por dendritas más o menos ramificadas, responsables de la sensibilidad del insecto hacia feromonas o otros atrayentes (Steinbrecht, 1973). La cutícula posee una serie de poros y túbulos por los que penetran las moléculas de olor hacia la linfa sensilar (Kanaujia and Kaissling, 1985), donde se unen a proteínas transportadoras llamadas *odorant binding proteins* (OBPs) (Steinbrecht, 1998). Dependiendo de su capacidad de unión, éstas se dividen en dos grupos, las proteínas de unión a feromona (*pheromone binding proteins,* PBPs) (Vogt et al., 1985) y las que se unen a moléculas odoríferas generales, como volátiles de la planta huésped *(general odorant binding proteins,* GOBPs) (Breer et al., 1990). Ambas clases están encargadas de transportar las moléculas hasta la membrana dendrítica donde células receptoras interaccionan con el estímulo y produciéndose una cascada de eventos dentro de las dendritas. Estos eventos se resumen en un proceso de transducción que transforma el estímulo en una respuesta bioeléctrica (potencial receptor), la cual refleja el cambio de potencial de la membrana (Stengl et al., 1999). Varios potenciales receptores simultáneos pueden combinarse y desencadenar potenciales de acción que discurren por los axones de las neuronas hasta el cerebro del insecto (lóbulos antenales) (Anderbrant et al., 1995). A través de interneuronas y neuronas de proyección la señal olfativa/informativa es transmitida a centros superiores del protocerebro, lo que provoca una acción/movimiento del sistema motor (Fig. 3, p.12) (Hildebrand, 1997). Para evitar la acumulación de moléculas en los receptores y su consiguiente saturación, la molécula de feromona se inactiva por la acción de enzimas

degradadores de olores (*odorant degrading enzymes,* ODEs) presentes en la linfa sensilar (Ishida and Leal, 2005; Leal, 2005). De este modo, las sensilas se mantienen sensibles a la captación de nuevos olores entrantes del entorno.

Existe una gran variedad de tipos morfológicos entre las sensilas sensoriales de insectos con diferentes funciones, como por ejemplo sensibilidad a estímulos gustativos, olfativos, mecánicos, etc. (Keil, 1999). La mayoría de sensilas olfativas, responsables de la detección de olores, se encuentra en las antenas y los palpos maxilares. Se ha demostrado la percepción de semioquímicos por parte de las *sensillas tricoideas*, *basicónicas*, *coelocónicas* y *auricílicas* (Steinbrecht, 1996; Pophof, 1997; Anderson et al., 2000). En ellas existe una gran variabilidad entre sexos, especies y también en su localización antenal. Un típico mecanorreceptor está representado por *sensilas caéticas* (van der Pers and den Otter, 1978).

En el presente trabajo se ha investigado la morfología, comunicación química, fisiología y comportamiento de cuatro especies de insectos plaga. El objetivo principal es la detección e identificación de posibles componentes feromonales o cairomonales y la posible aplicación de estos compuestos en pruebas de campo en el marco de un control integrado. Diferentes estudios en bupréstidos y acrídidos citados en los siguientes capítulos han sostenido el fundamento de nuestras hipótesis y objetivos de trabajo.

OBJETIVOS

Capítulo 1 Estudios analíticos y de comportamiento de *Coroebus florentinus* (Coleoptera: Buprestidae)

- Análisis morfológico de la antena de machos y hembras e identificación de las sensilas antenales.
- Identificación de compuestos orgánicos volátiles producidos y emitidos por adultos de ambos sexos y por la planta huésped *Q. suber*.
- Determinación de la actividad de estos compuestos en ensayos electrofisiológicos y de comportamiento.

Capítulo 2 Estudios analíticos y pruebas de campo para la captura de la culebra del corcho, *Coroebus undatus* (Coleoptera: Buprestidae)

- Análisis morfológico de la antena de machos y hembras e identificación de las sensilas antenales.
- Análisis e identificación de compuestos orgánicos volátiles producidos y emitidos por adultos de ambos sexos.
- Confirmación de la actividad biológica de diferentes compuestos e influencia del diseño de trampas en pruebas de campo.

Cápitulo 3 Estudios analíticos y electrofisiológicos de la langosta marroquí *Dociostaurus maroccanus* (Orthoptera: Acrididae), plaga de la Península Ibérica

- Análisis morfológicos de la antena de machos y hembras e identificación de las sensilas antenales.
- Identificación de compuestos orgánicos volátiles producidos y emitidos por ninfas y adultos de ambos sexos, volátiles de huevos así como de la espuma de las ootecas.
- Determinación de la actividad de estos compuestos en ensayos electrofisiológicos y de oviposición.

Capítulo 4 Estudios de maduración en ninfas gregarias de la langosta del desierto *Schistocerca gregaria* (Orthoptera: Acrididae), una plaga destructiva en África

- Determinación del efecto de agrupar ninfas con adultos conespecíficos en la maduración de ninfas.
- Influencia de la feromona producida por los machos en el proceso de maduración de ninfas.

MÉTODOS Y MATERIALES GENERALES

Insectos

En la presente tesis doctoral se han estudiado diferentes aspectos de la comunicación química y del comportamiento de cuatro especies de insectos plaga. Se ha trabajado en la bioecología de dos bupréstidos, *Coroebus florentinus* y *C. undatus* (Coleoptera: Buprestidae) (Fig. 4, p.25), plagas del alcornoque *Quercus suber* en la región Mediterránea (Soria et al., 1992; Soria and Ocete, 1993; Evans et al., 2007).

Además, se ha investigado la comunicación química de dos especies de langostas. La langosta marroquí, *Dociostaurus maroccanus* (Orthoptera: Acrididae), es plaga de la Península Ibérica (Uvarov, 1966; Latchinisky, 1998), mientras la langosta del desierto, *Schistocerca gregaria* (Orthoptera: Acrididae), es una de las plagas agrícolas más importantes de África, Oriente Medio y Asia (Enserink, 2004) (Fig. 7, p.26). En el estudio de las cuatro especies se utilizaron las siguientes técnicas:

Microscopía electrónica de barrido

Se utiliza la microscopía de barrido *(scanning electron microscope, SEM),* entre otras aplicaciones, para obtener un mejor conocimiento de la morfología y la superficie de las antenas de insectos. Mediante el escaneo de las mismas utilizando transmisión de electrones de alta energías se puede determinar la presencia de diferentes tipos de sensilas y detectar diferencias entre especies y/o sexos.

Se cortan las antenas por debajo del primer artejo más cercano a la cabeza y se cubren las antenas, depositadas en un soporte metálico, con una solución de oro para la obtención de imágenes con una alta resolución y un buen contraste. La preparación de las muestras se ha realizado en el Servicio de Microscopía Electrónica de la Universidad de Barcelona. El modelo de microscopio utilizado para la captación de imágenes fue un Stereoscan S-360 (Leica) at 15 kV.

Recogida de volátiles e identificación de los compuestos

En primer lugar, la investigación de la ecología química de insectos plaga requiere la recogida e identificación de los volátiles emitidos (traducción de la información específica). Para el enriquecimiento y recogida de los volátiles producidos por

Coroebus spp. y también por su planta huésped *Q. suber* se han llevado a cabo dos métodos diferentes.

El método "d*ynamic headspace*" se basa en el principio del "c*losed-loop stripping*", descrito por Grob y colaboradores (1975) y Boland y colaboradores (1984). El método radica en la recogida de productos volátiles emitidos por un organismo dentro de una cámara de aireación dinámica. Una bomba insufla aire purificado sobre los insectos, mientras una segunda bomba aspira el aire acumulado con los volátiles emitidos y lo conduce a través de un filtro de adsorción, colocado en la salida de la cámara, donde los volátiles quedan retenidos (Fig. 8, p.28). Dependiendo del organismo estudiado y de los compuestos esperados, se utilizan diferentes tipos de filtros adsorbentes. A continuación, se extraen los volátiles del adsorbente con hexano y/o diclorometano y se guardan a -20ºC para sucesivos análisis y ensayos biológicos.

El segundo método utilizado es por microextracción en fase sólida (s*olid phase microextraction*", SPME) sobre una fibra de sílice activada, que absorbe las sustancias volátiles que emiten los insectos colocados en el interior de un recipiente de vidrio (Fig. 10, p.29). Este método, que había sido desarrollado por Pawliszyn (1997), es una técnica que permite la extracción de compuestos sin el uso de disolventes. La adsorción de los hidrocarburos cuticulares se obtiene al frotar el abdomen, tórax o los élitros con la punta de la fibra. Para analizar los compuestos recogidos se inyecta la fibra directamente en un cromatógrafo de gases (CG) o un cromatógrafo de gases acoplado a un espectrómetro de masas (CG-EM) (Pawliszyn, 1999).

Caracterización estructural de los compuestos

La caracterización estructural de compuestos que volátiles se ha llevado a cabo por CG y/o CG-EM. Estas técnicas permiten la separación de mezclas complejas de olores en compuestos individuales. Se identifican estos compuestos por comparación de sus espectros de masas, tiempos de retención y/o índices de retención relativos (RI) con los de patrones auténticos. En algunos casos la identificación se ha realizado por comparación con espectros de masas de bibliotecas comerciales o presentes en bases de datos como la publicada por Adams (2007). Los RI relativos se han calculado por aplicación de la fórmula de van den Dool and Kratz (1963) en la que se relaciona el tiempo de retención del compuesto a determinar con el de los dos alcanos más próximos (Fig. 11, p.30). La aplicación del procedimiento requiere conocer el tiempo de retención de una mezcla de hidrocarburos lineales, que en nuestro caso fue de 8 a 25 átomos de carbono.

Electrofisiología

En general, la electrofisiología abarca el estudio de las propiedades eléctricas de las células y tejidos biológicos (Gullan and Cranston, 2004). Esto incluye la medida de cambios de voltaje o potencial eléctrico que puede ocurrir en el interior de los mismos (Kaissling and Thorson, 1980). Las técnicas utilizadas en este trabajo han sido la electroantenografía (EAG) y la cromatografía de gases acoplada a la electroantenografia (GC-EAD).

El electroantenograma es una técnica empírica que forma una parte básica de la ecología química y en especial de la investigación de la percepción olfactiva en insectos (Roelofs, 1984). El EAG permite medir el "output" de la antena al cerebro producido por un cierto olor o estímulo olfativo (feromona o cairomona). El EAG representa la suma de las despolarizaciones eléctricas producidas por un determinado estímulo sobre los receptores antenales de los insectos (Kaissling and Thorson, 1980). Generalmente, la amplitud de la respuesta en EAG crece gradualmente con el aumento de la concentración del estímulo hasta llegar a un nivel de saturación (Schneider, 1969). Hay que tener en cuenta que una respuesta electrofisiológica positiva puede ser debida tanto a un efecto atrayente (feromona) como a un efecto repelente (inhibidor).

En el presente trabajo se ha estudiado la actividad electrofisiológica de extractos naturales y de compuestos sintéticos sobre antenas de *Coroebus spp*., y de *D. maroccanus*. Se extrae una de las dos antenas del insecto y se secciona una mínima parte del extremo distal de la misma. Se dispone la antena cortada sobre un electrodo ajustado a un amplificador (x 100) de alta impedancia, conectado a una interfase (Syntech, Hilversum, The Netherlands) que está conectada a su vez a un ordenador. La antena se mantiene bajo una corriente continua de aire purificado y humedecido a una distancia fija del estímulo (0.5 cm). Este estímulo proviene de una fuente de olor aplicada previamente en un papel de filtro dentro de una pipeta Pasteur (Fig. 12, p.32). La duración del impulso del estímulo en todos los casos es de 0.4 s, con un margen de tiempo de 1 min entre cada estímulo para obtener la debida recuperación de los receptores antenales. Como blanco se utiliza un papel de filtro impregnado con el disolvente utilizado antes y después de cada serie de estímulos con el extracto a ensayar. La diferencia de la media de las respuestas al test (3 insufladas) con la de las respuestas al blanco (2 insufladas) determina la potencia electrofisiológica del estímulo en cuestión. La adquisición y el análisis de datos se han efectuado con el programa EAG-Pro (Syntech).

La cromatografía de gases es una técnica potente que permite la separación de mezclas complejas de productos en sus componentes individuales. En combinación con el electroantenograma ("*cromatografía de gases con detector electroantenográfico*", GC-EAD) permite investigar los compuestos individuales que forman parte de un extracto natural y que desencadenan una determinada respuesta electrofisiológica sobre la antena (Moorhouse et al., 1969). El tiempo de retención y el espectro de masas del pico activo pueden conducir a la identificación de la feromona. Se utilizan antenas preparadas siguiendo el mismo protocolo que en el apartado anterior (EAG). El CG empleado en este estudio es un Focus GC (Thermo Instruments, Barcelona, Spain) equipado con un detector de ionización de llama (FID) y una columna HP-5MS (30 m x 0,25 mm ID x 0,25 μm) (Agilent Technologies, Madrid) con helio como gas transportador y nitrógeno como make up. La adquisición y análisis simultáneos del EAD y FID se lleva a cabo con el programa GC-EAD v4.4 (Fig. 13, p.33) (Syntech, Kirchzarten, Germany).

Ensayos biológicos de comportamiento

Para comprobar la actividad atrayente o repelente de diferentes fuentes de olores (posibles feromonas) sobre los insectos se requiere en muchos casos desarrollar bioensayos con individuos vivos. En este trabajo se ha investigado la atracción o el rechazo de adultos de *C. florentinus* hacia diferentes estímulos con un olfactómetro de vidrio de doble elección (Fig. 14, p.34). El olfactómetro permite cuantificar el número de individuos que son atraídos por la fuente de volátiles frente al control. El número de insectos que responden se ha analizado con el test estadístico χ^2 *goodness-of-fit test* ($P \leq 0,05$).

CAPÍTULO 1

Respuestas electrofisiológicas y de comportamiento de Coroebus florentinus (Herbst) (Coleoptera: Buprestidae) a semioquímicos conespecíficos y volátiles de la planta huésped

El bupréstido *Coroebus florentinus* es un coleóptero plaga del alcornoque *Q. suber* L., un árbol valioso y endémico de la región mediterránea, principalmente distribuido por Portugal y España. El interés económico proviene de la producción anual de corcho (la

corteza del árbol) con más de 340.000 toneladas con un valor de 1.5 billones de dólares. Entre las variadas utilidades del corcho en la industria de productos manufacturados se destaca el uso en tapones para productos vinícolas con una producción estimada de más de 13 billones por año. A pesar del impacto económico y social del corcho no se sabe nada de la biología y ecología química de una de las plagas del alcornoque *C. florentinus* (Soria and Ocete, 1993; Soria et al., 1994).

En junio-julio, las hembras de este insecto invaden el árbol y ponen los huevos en el interior de la corteza de ramas jóvenes, sobre todo las más exteriores y soleadas. La larva al nacer comienza a alimentarse del interior de la rama, elaborando una larga galería descendente de hasta más de 1 metro de largo, y termina por hacer un anillo interior que la seca al cortarse la savia. Por ello las hojas aparecen amarillas y secas, aisladas en la parte exterior de la copa del árbol. Si bien en general, los daños producidos son escasos (Lombardero and Fernández de Ana Magán, F.J., 1996), la reducción del volumen de la copa puede disminuir la producción de madera, corcho o bellotas. Sin embargo, ataques repetitivos pueden debilitar el árbol allanando el camino a otras plagas (Dajoz, 2000).

De momento no se han establecido tratamientos efectivos para controlar la plaga por su largo ciclo de vida (puede ser de algunos años) y por la protección que ofrece la corteza a las larvas. Además, el conocimiento de depredadores del insecto es casi nulo (Kenis and Hilszczanski, 2007). Por lo tanto, en el presente trabajo se presentan por primera vez estudios dirigidos a la investigación de una posible feromona sexual o de agregación de *C. florentinus* para el desarrollo de un control biorracional de este insecto plaga.

Análisis morfológicos de antenas de adultos

Los análisis morfológicos de las antenas de *C. florentinus* mediante microscopía electrónica de barrido revelan que las antenas de machos y hembras, formadas por 11 artejos, son casi idénticas en su estructura pero varían en su tamaño (Figs. 1.4 A, E, p.53). Adicionalmente, la microscopía electrónica de bárrido (SEM) ha permitido la detección de cuatro tipos de sensilas diferentes, cuya terminología está basada en los trabajos de Zacharuk (1980) y Crook y colaboradores (2008a). Por comparación con sensilas descritas en otros coleópteros se han identificado *sensilas caéticas* (mecanorreceptoras), dos tipos de *s. basicónicas* con función olfativa y *sensilas de un solo poro* en ambos sexos (Figs. 1.4 B-D, F-H, p.53). Estos tipos de sensilas, muy

comunes en la familia de los bupréstidos, fueron utilizados en la clasificación de ese grupo de coleópteros (Bellamy, 1985).

Se ha observado que las antenas de machos de *C. florentinus* son hasta dos veces más largas y con más sensilas olfativas de los dos tipos de *s. basicónica* que las hembras. Esto podría indicar una posible percepción de diferentes olores más pronunciada en machos que en hembras. Además, se reveló que la morfología y la disposición de los diferentes tipos de sensilas son muy similares a las de las antenas del bupréstido *A. planipennis*. En esta especie se han encontrado compuestos, producidos por hembras, responsables de la atracción sexual hacia los machos (Lelito et al., 2009; Silk et al., 2009).

En *C. florentinus* se demuestra que los machos tienen significativamente más sensilas gustativas de un solo poro que las hembras, por lo cual suponemos que los volátiles de contacto (de corto alcance) podrían estar implicados en el reconocimiento de la pareja, especialmente por los machos.

Recogida y análisis de volátiles y extractos

Para obtener volátiles de *C. florentinus* se colocaron 20 machos y 20 hembras por separado en una cámara de aireación (15 cm×3 cm) por donde circulaba una corriente de aire purificado con un flujo de aire de 350 mL min^{-1} durante 24 h a temperatura ambiente. Los volátiles se recogieron mediante filtros de adsorción adecuados (Super Q, Ars Inc., Gainesville, FL, USA), situados en la salida de la cámara. A continuación se extrajeron los volátiles del adsorbente con 2 mL de hexano, se concentraron 100 μL de los extractos con nitrógeno hasta aprox. 2 μL y se analizaron por CG-EM.

Los extractos de abdómenes de ambos sexos (tres de cada uno de ellos) se obtuvieron por inmersión de los mismos en 500 μL de hexano durante 3 h. Se llevaron a cabo 3 repeticiones y los extractos así obtenidos se conservaron a -20ºC hasta su utilización. Para los análisis en CG-EM se inyectó 1 μL de cada extracto (sin concentrar).

Se han identificado un total de 28 compuestos en los extractos de abdomen y en los volátiles recogidos por “dynamic headspace” de ambos sexos (Tabla 1.3, p.54). En general no se observan diferencias significativas entre los extractos de machos y de hembras de *C. florentinus*. La mayoría de ellos son hidrocarburos saturados, lineales o ramificados con uno o dos metilos, aldehidos y ácidos carboxílicos. Entre ellos se

encuentran varios compuestos con actividad feromonal en otros grupos de insectos. Por ejemplo, los aldehidos de nueve y diez carbonos, nonanal y decanal, y la geranilcetona están descritos como parte de la feromona de agregación de *Cimex lectularius* (Siljander et al., 2008). En los volátiles de machos sexualmente maduros de *Nicrophorus* (Coleoptera: Silphidae) se ha encontrado que la geranilcetona presenta actividad feromonal (Haberer et al., 2008).

En volátiles recogidos de planta huésped *Q. suber* se han identificado 33 compuestos (Tabla 1.4, p.55). Entre ellos se encuentran típicos volátiles de árboles y plantas. La mayoría son los denominados "*green leaf volátiles*" (GLVs), alcoholes, aldehídos y esteres de 6 a 10 carbonos emitidos en grandes cantidades como respuesta a daños mecánicos y ataques de herbívoros (Takabayashi et al., 1996). Además, se han detectado mono-, homo- y sesquiterpenos. Los GLVs de 6 carbonos son los que están presentes en mayores cantidades, 92% de la cantidad total de extracto producida por las ramas (36,5 g de peso fresco) de *Q. suber*. Destaca la presencia del homoterpeno (3*E*)-4,7-dimethyl-1,3,7-nonatriene (2% de la cantidad total), un compuesto generalmente inducido en plantas después de un ataque de herbívoros y muy atrayente para muchos insectos.

Estudios electrofisiológicos

Mediante GC-EAD se ha investigado los compuestos que forman parte de los volátiles de adultos de *C. florentinus* y de *Q. suber* y que desencadenan una determinada respuesta electrofisiológica sobre la antena de machos y hembras. Se ha demostrado que nonanal, decanal y la geranilcetona encontrados en volátiles de ambos sexos así como una mezcla sintética de estos tres compuestos provocan respuestas electrofisiológicas significativas en antenas de machos (Fig. 1.5, p.57), mientras en hembras solo se obtuvieron pequeñas respuestas.

También se observa que 5 de los compuestos contenidos en volátiles de la planta huésped provocan respuestas electrofisiológicas en antenas de hembras y en parte también en machos (Fig. 1.6, p.57). Entre ellos se encuentran los usuales GLVs (*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, acetato de (*Z*)-3-hexenilo y acetato de n-hexilo (Hatanaka et al., 1987).

En electroantenograma (EAG) se ha comprobado la actividad de los 3 compuestos activos en GC-EAD del extracto de volátiles del insecto. Se han estimulado 12 antenas de ambos sexos con diferentes cantidades de los compuestos

sintéticos y los resultados indican que todos han provocado respuestas en EAG (Tabla 1.5, p.58). En los casos de la mezcla de los 3 compuestos y de la geranilcetona se observa una respuesta dosis-dependiente. La mayor respuesta de antenas de machos se ha obtenido con la menor dosis de decanal (100 ng). En antenas de hembras se han observado resultados parecidos, pero en general las respuestas de hembras son más bajas que las de machos. Se supone que la mezcla de estos tres compuestos y en especial el decanal podrían tener importancia en el sistema olfativo de machos, mientras ese papel en las hembras podría atribuirse a la geranilcetona.

También se ha confirmado la actividad de los 5 GLVs. Se ha estimulado un mínimo de 10 antenas de hembras de *C. florentinus* con 5 µg de los GLVs (*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, acetato de (*Z*)-3-hexenilo y acetato de n-hexilo. Los resultados indican que todos los compuestos son activos en EAG (Fig. 1.7, p.59). El alcohol saturado 1-hexanol ha generado significativamente la mayor respuesta respecto al aldehído y a los dos acetatos. Además se observan diferencias significativas entre los alcoholes y aldehídos comparados con los acetatos, provocando estos últimos las menores respuestas.

Bioensayo de atracción en un olfactómetro de doble elección

En el bioensayo de comportamiento con un olfactómetro de doble elección se ha estudiado la atracción de machos y hembras hacia individuos del otro sexo, extractos de cuerpo entero de machos sobre hembras y de hembras sobre machos, extractos naturales de la planta huésped *Q. suber* y diferentes cantidades de compuestos sintéticos (Tabla 1.2, p.50). Los ensayos en blanco han demostrado que la elección de los insectos no estaba influida por factores externos, como preferencia por uno de los dos brazos o por incidencia de la luz etc. (Fig. 1.8, p.60). Se ha constatado que los machos se dirigen de manera significativa hacia hembras vivas y extracto de cuerpo entero de hembras, mientras no están atraídos por machos vivos. Las hembras por su parte no han demostrado ninguna atracción hacía machos y hembras vivas así como hacia extractos de cuerpo entero de machos. Aunque no se han encontrado diferencias en la composición de volátiles entre machos y hembras, es posible que las hembras emitan compuestos que atraen a los machos en cantidades muy pequeñas que no se detectaron con las técnicas utilizadas.

En los ensayos con compuestos sintéticos se ha probado la actividad de los tres compuestos encontrados en volátiles de ambos sexos y activos en GC-EAD y EAG (nonanal, decanal, geranilcetona). Los machos se dirigen de manera significativa hacia

la mezcla de los tres compuestos y a decanal. Por otro lado no demuestran preferencia hacia la geranilcetona y nonanal (Fig. 1.9, p.61). Se ha probado también la mezcla de los 3 compuestos en un brazo del olfactómetro frente a decanal en el otro y también decanal frente a nonanal (1 µg). Entre la mezcla y el decanal no se ha observado una preferencia significativa, pero comparando el efecto de los dos aldehídos los insectos se han dirigido de manera significativa hacia el decanal (Fig. 1.10, p.61). Estos resultados son concordantes con la actividad demostrada en EAG. Las hembras por su parte se sienten atraídas significativamente tanto por la mezcla como por la geranilcetona (Fig. 1.11, p.62). Dado que se han encontrado estos compuestos en volátiles de ambos sexos se puede excluir que formen parte de una feromona sexual, si bien podrían mediar en la agregación de los adultos. Para confirmar esta hipótesis se están preparando bioensayos más específicos.

En los ensayos con volátiles, extractos de hojas de *Q. suber* y con 1 µg de una mezcla de los 5 GLVs sintéticos activos en las pruebas electrofisiológicas no se observa ningún efecto sobre los machos (Fig. 1.12, p.63). Las hembras, por su parte, se dirigen de manera significativa hacia todos los estímulos de la planta huésped *Q. suber* ofrecidos. Estos resultados pueden asociarse al hecho de que las hembras quizás tengan mayor necesidad que los machos de alimentarse y encontrar un árbol adecuado para la puesta de huevos, y de esa manera garantizar el avituallamiento conveniente de su descendencia. Debido a los resultados significativos obtenidos con las hembras, se han realizado nuevos ensayos con los 5 GLVs de la mezcla por separado. En todos los casos prefieren el estímulo frente al control, pero únicamente la preferencia hacia los dos alcoholes y acetato de (*Z*)-3-hexenilo fueron significativos (Fig. 1.13, p.64). Los insectos presentan una mayor respuesta hacia el alcohol saturado 1-hexanol, acorde con la respuesta electrofisiológica en EAG. Se sugiere una posible función de los GLVs, especialmente de los alcoholes, como cairomona de oviposición o de búsqueda de alimento.

CAPÍTULO 2

Estudios analíticos para la caracterización de feromonas en Coroebus undatus. *Pruebas de campo*

La culebra o culebrilla del corcho, *Coroebus undatus,* de la familia de los bupréstidos, es uno de los insectos plaga más dañinos del alcornoque, *Q. suber* (Soria et al., 1992;

Suñer and Abós, 1994), una especie endémica del mediterráneo occidental con una importancia económica enorme por su efecto en la producción y calidad del corcho.

Al contrario de su especie hermana *Coroebus florentinus*, las hembras de *C. undatus* ponen unos 8-10 huevos blancuzcos aislados o en pequeños grupos, pegados en el fondo de las grietas del corcho y no en las ramas (Evans, et al., 2007). La larva nace a los 10-20 días, atravesando las sucesivas capas de corcho y estableciéndose en las inmediaciones de la capa generatriz. Las larvas se alimentan de las capas vivas del tejido existente bajo el corcho, originando largas galerías (hasta 1,8 m de longitud y 3-4 mm de anchura media) alrededor del tronco (Fig. 2.1, p.75) (Romanyk and Cadahia, 1992). Estas galerías están llenas de excrementos, inhabilitando al corcho para su uso en fabricación de tapones por su mal aspecto y peor calidad, por lo que se ha de utilizar únicamente en forma triturada. Además se producen manchas amarillas y podredumbre en el entorno de las galerías, las cuales van quedando, al crecer la corteza del árbol, en el interior del corcho (Bernal and Cardillo, 2004). Aunque el efecto fisiológico de este insecto sobre el árbol es escaso, el daño provocado en los tejidos productores de corcho causa grandes pérdidas económicas en la cantidad y calidad del material. No se conoce ningún tratamiento efectivo contra *C. undatus* y la necesidad de controlar la plaga es enorme. Por consiguiente, este trabajo presenta el primer estudio sobre la biología del bupréstido *C. undatus*, con especial énfasis en su comunicación química para el desarrollo de un posible control biorracional del mismo.

Para realizar estudios en el laboratorio se consideró la necesidad de capturar insectos vivos y para ello se han colocado trampas de tela blanca (mosquitera) alrededor de troncos de *Q. suber* (Fig. 2.2, p.78). Con los adultos vivos capturados se ha iniciado la investigación de los volátiles emitidos y de la morfología de las antenas mediante SEM. Además, se ha estudiado la influencia de diferentes parámetros de trampa (forma, posición, color y tipo de atrayente) para monitorear y/o establecer una captura masiva de la plaga. En estudios anteriores se había demostrado que la optimización de estos parámetros tiene efectos considerables en la atracción del bupréstido *A. planipennis* (Crook et al., 2008b; Francese et al., 2008).

Estudios analíticos de volátiles abdominals de C. undatus

Desde mediados de junio hasta finales de julio se ha recogido sólo un total de 12 adultos vivos (4 machos y 8 hembras) en las 90 trampas-tela colocadas en Santa Coloma de Farners, en un bosque que muestra graves síntomas de infestación de

Coroebus spp. En los extractos de cutícula de ambos sexos obtenidos por SPME y analizados por CG-EM se observan diferencias cualitativas y cuantitativas entre los 18 compuestos detectados (Fig. 2.5, p.84). La gran mayoría de los compuestos son hidrocarburos de cadena larga (>C_{20}) saturados, lineales o ramificados, y monoinsaturados. Además hay que destacar que dos de estos compuestos sólo están presentes en las hembras. Estos dos compuestos, aún por identificar, parecen ser hidrocarburos ramificados de 26 a 28 carbonos. Son necesarios estudios adicionales para determinar la estructura completa, la posición correcta de las ramificaciones y para averiguar su significado real en los extractos. En general, se asume que los hidrocarburos de la cutícula de los insectos contienen señales olfativas para el reconocimiento sexual de la pareja (Howard and Blomquist, 2005). En coleópteros, y en concreto en los cerambícidos, se ha encontrado la primera prueba de reconocimiento sexual de la pareja que se establece por señales de contacto. Se demostró que los machos se sentían atraídos por las hembras conespecíficas después de tocar la cutícula de ellas con las antenas (Wang and Millar, 2000; Ginzel and Hanks, 2003).

Análisis morfológicos de antenas de adultos

Los análisis para determinar la morfología y estructura antenal de machos y hembras de *C. undatus* mediante microscopía electrónica de barrido (SEM) revelaron que los adultos poseen *sensilas de un solo poro*, *s. caéticas* y dos tipos de *s. basicónicas* (Fig. 2.6, p.87). Las *s. basicónicas* de ambos tipos son muy comunes en el flagelo antenal de la especie hermana *C. florentinus* y de otros coleópteros (Merivee et al., 2002; Crook et al., 2008a). Por ejemplo, en los *Anobiidae* y *Carabidae* se demostró que la pared de estas sensilas está perforada por numerosos poros minúsculos, al igual que en *Coroebus spp*., y que el lumen de la base está llena de dendritas ramificadas derivadas de dos neuronas (Okada et al., 1992; Kim and Yamasaki, 1996). El número de poros y dendritas ramificadas se consideran como prueba de que las *s basicónicas* actuan como receptores olfativos (Zacharuk, 1980). Los machos de *C. undatus* presentan notablemente más sensilas de ese tipo y más *sensilas de un solo poro* con función gustativa que las hembras. Por el contrario no hay dimorfismo sexual evidente con respecto a la presencia y distribución de *s. caéticas*.

En machos del bupréstido *Agrilus planipennis*, la presencia abundante de *sensilas de un solo poro* en sus antenas sugiere que las señales de contacto (a corto plazo) son importantes para el reconocimiento de las hembras. En este contexto, machos de

A. planipennis pasaron significativamente más tiempo intentando copular con hembras muertas que con machos o hembras en los que los hidrocarburos cuticulares habían sido eliminados. Dos hidrocarburos ramificados con un grupo metilo han sido identificados como feromonas de contacto en esta especie (Lelito et al., 2009; Silk et al., 2009), estructuralmente muy similares a los dos compuestos detectados exclusivamente en la cutícula de las hembras de *C. undatus*. Por lo tanto, se supone también que, debido a la gran cantidad de sensilas gustativas de un solo poro, los machos de *C. undatus* podrían utilizar los hidrocarburos producidos por las hembras como señales químicas de contacto para el reconocimiento de su pareja. Pero todavía es necesario aclarar a qué tipo de estímulos olfativos responden las *s. basicónicas* y las de *un solo poro*.

Estos resultados mejoran nuestra comprensión sobre la ecología química de *C. undatus* y podrían tener aplicaciones potenciales para el control de esta especie plaga. El siguiente punto por investigar, que se debe confirmar en bioensayos de comportamiento, es averiguar si la quimiorrecepción de contacto juega un papel crucial en la comunicación de esta especie.

Pruebas de campo para capturar C. undatus

La clave para un programa de control de insectos plaga eficaz contra *C. undatus* es un programa de registro combinado con herramientas para la detección y delimitación de las poblaciones. De acuerdo con los resultados obtenidos con *A. planipennis* (Francese et al., 2005), en pruebas de campo hemos observado que las trampas de color púrpura parecen ser las preferidas por los adultos en comparación con otras trampas de colores distintos o transparentes. Aparte del efecto del color, se probaron diferentes diseños de trampas: a) trampa de embudo Lindgren, b) trampa prisma de tres caras y c) trampa panel de dos caras (Fig. 2.4, p.82). La mayoría de los adultos de *C. undatus* fueron capturados con las trampas prisma en comparación con trampas panel y Lindgren (Figs. 2.10–2.14, p.91-93). Las trampas panel a su vez capturaron significativamente más adultos que las trampas Lindgren, lo que indica que las trampas de embudo Lindgren, generalmente utilizadas para la captura de una variedad de coleópteros perforadores/barrenadores de la madera y de la corteza, no son apropiados para la captura de *C. undatus*.

Además, se han colocado dispensadores cargados con tres formulaciones de composición química diferente y un control con disolvente (blanco) para la captura de la plaga (Tabla 2.1, p.83). Se probaron las siguientes formulaciones: a) un atrayente

comercial de coleópteros (Pherotech), b) una mezcla de tres compuestos sintéticos presentes en los volátiles de machos y hembras de *C. florentinus* (nonanal, decanal y geranilcetona), y c) una mezcla de cinco GLVs ((*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, acetato de (*Z*)-3-hexenilo y acetato de n-hexilo), detectados en volátiles de la planta huésped, *Q. suber* y que demostraron actividad en bioensayos de comportamiento y electrofisiológicos *C. florentinus*. Sorprendentemente, no se capturó ni un solo individuo de esa última especie y, en cambio, se atrapó un número relativamente elevado de hembras de *C. undatus* con los volátiles de la planta y con la mezcla de los tres componentes de los volátiles del insecto (Fig. 2.15, p.94). La mezcla de los tres componentes y los GLVs de la planta huésped (véase capítulo 1) han atraído un mayor número de adultos en trampas prisma en comparación con el etanol (control) (Fig. 2.18, p.96), a pesar de que a éste se le había atribuido actividad como cairomona en algunos coleópteros xilófagos (Chenier and Philogene, 1989; Miller, 2006), y el bupréstido *Agrilus nilineatus* (Montgomery and Wargo, 1983).

Estos resultados preliminares requieren ser confirmados y optimizados, ya que los 3 compuestos (nonanal, decanal y geranilcetona) son producidos por varias especies de insectos (Dickens, 2006) y plantas, por lo que es necesario esclarecer su función en la ecología química de nuestra especie, *C. undatus*. En el presente estudio se ha obtenido una primera visión de la biología y el comportamiento de esta plaga y nuestros resultados servirán de base para desarrollar nuevos métodos de control biorracional contra la misma.

CAPÍTULO 3

Diferencias en la emisión de volátiles entre machos y hembras adultos y ninfas de la langosta marroquí, Dociostaurus maroccanus (Orthoptera: Acrididae): Identificación, respuestas electrofisiológicas y de comportamiento

La langosta marroquí o mediterránea, *Dociostaurus maroccanus*, Thunberg 1815 (Orthoptera: Acrididae) es una especie polífaga plaga de los cultivos y pastizales (El Ghadraoui et al., 2002), que se encuentra predominantemente en las estepas semiáridas o zonas semi-desérticas con una vegetación primaveral efímera (Uvarov, 1957).

Aproximadamente un mes después de la aparición de adultos (a partir de mayo) las hembras ponen 2-4 tubos alargados (canutos), que contienen unos 30 huevos, los

cuales están unidos por una espuma secretada en el momento de la puesta. En general, depositan los canutos en suelos rocosos, justo debajo de la superficie o hasta 4 cm de profundidad en tierra firme. Dependiendo de las condiciones climáticas y ecológicas las hembras ovipositan en grupos, lo que puede provocar una densidad de huevos muy alta (Martín-Bernal et al., 1993). Después de la diapausa invernal emergen las ninfas, cuya fase consta de cinco estadios. Su desarrollo dura de 25 a 40 días, antes de que los individuos empiecen a ser voladores (adultos inmaduros). Los adultos necesitan entre 1 y 2 semanas para alcanzar la madurez sexual, empezar la cópula y poner huevos.

D. maroccanus puede aparecer en una fase solitaria o en una fase gregaria, al igual que la langosta del desierto, *Schistocerca gregaria*. Este cambio de solitario a gregario se produce como consecuencia de un drástico aumento de la densidad de la población local por circunstancias climáticas y ambientales (Pener and Yerushalmi, 1998; Simpson and Sword, 2007). Una característica notable de la langosta marroquí es su estrecha asociación con las actividades agrícolas de los humanos, que crean hábitos adecuados para la plaga, como la deforestación, la destrucción de los matorrales y el pastoreo excesivo de animales domésticos. Esto suele proporcionar las condiciones necesarias para la concentración y gregarización de langostas, favoreciendo posteriores brotes de la plaga (Latchininsky, 1993). Los daños, principalmente ejercidos por las ninfas saltonas o los adultos que aparecen en enjambres de numerosos individuos, tienen un impacto económico muy alto en muchos países de la cuenca mediterránea, incluido el sur de Francia, el norte de África y el Oriente Medio, como Afganistán y el sur de Kazajstán (Santiago-Alvarez et al., 2003).

Los tratamientos químicos contra plagas de insectos, especialmente contra los que tienen una densidad de población muy alta como las langostas, son eficaces a corto plazo, pero también muy agresivos para el medio ambiente ya que pueden provocar la contaminación del suelo, agua y aire. Además, representan un grave riesgo para la salud de los potenciales consumidores de los cultivos (véase la introducción). La aplicación masiva de insecticidas y su impacto ambiental provocado por la excesiva proliferación de la langosta marroquí podría producir la extinción de numerosas especies de flora y fauna, y de ese modo una pérdida irreversible de la biodiversidad. Por lo tanto, es esencial la elaboración de estrategias alternativas de control que permitan un manejo selectivo de la plaga y garanticen un desarrollo sostenible de la agricultura.

La falta de métodos biorracionales y la certeza de que las señales químicas juegan un papel importante en la comunicación de acrídidos, especialmente la langosta del desierto tanto en ninfas y adultos como en fase solitaria y gregaria, nos ha llevado a investigar por primera vez la posible presencia de feromonas y su papel en la comunicación química de la langosta marroquí, *D. maroccanus*. Para determinar las posibles fuentes de feromonas se han recogido volátiles de machos y hembras adultos y de ninfas, y se han preparado extractos de heces, espuma y de huevos para analizarlos por CG-EM y GC-EAD. Por otra parte, se han estudiado la morfología y la estructura de las antenas de los insectos y su sensilas mediante SEM. Se ha estudiado también, mediante bioensayos de oviposición, si los extractos naturales de la espuma o compuestos sintéticos tienen alguna influencia sobre el comportamiento en la oviposición de hembras grávidas.

Análisis morfológicos de antenas de adultos

La estructura y distribución de sensilas en las antenas de la langosta marroquí se ajustan bastante a los tipos de sensilas ya descritos en otras especies de acrídidos (Greenwood and Chapman, 1984). Se han identificado cuatro tipos de sensilas diferentes, similares a los encontrados en *Locusta migratoria* L. y *S. gregaria* (Ochieng et al., 1998), las sensilas olfativas *s. basicónicas* (Fig. 3.5, p.120), *s. tricoideas* (Fig. 3.7, p.121) y *s. coelocónicas* (Fig. 3.6, p.120) y las mecanorreceptoras *s. caéticas* y no se observan diferencias entre ambos sexos.

En *S. gregaria* se demostró que las *s. basicónicas* y *s. coelocónicas* responden a compuestos feromonales, mientras que las *s. caéticas*, principalmente encontradas en el último artejo de las antenas, actúan como sensilas gustativas para el reconocimiento de lugares adecuados para la oviposición (Fig. 3.8, p.121). Se supone que las sensilas de la langosta marroquí pueden tener la misma función, y que aparte de las señales olfativas, las señales de contacto pueden jugar un papel importante en la comunicación química de las langostas. Sin embargo, son necesarias detalladas investigaciones fisiológicas para demostrar si los cuatro tipos de sensilas de *D. maroccanus* responden a estímulos químicos y/o mecánicos.

Estudios analíticos y de comportamiento de volátiles y extractos naturales de D. maroccanus

En volátiles de ambos sexos y extractos de heces, huevos y espuma de *D. maroccanus* se ha detectado un total de 58 compuestos químicamente diferentes (Tabla 3.2, p.122). Se han identificado varios ácidos carboxílicos (C_5-C_{16}) y aldehídos monoinsaturados en espuma y heces de hembras. De ellos, los ácidos hexanoico y octanoico se han descrito como inductores del comportamiento de agregación en ninfas de la langosta del desierto (Torto et al., 1999). Además, dos de los aldehídos insaturados encontrados en la espuma de la langosta marroquí (*E,E*)-2,4-decadienal y (*E,E*)-2,4-heptadienal, se habían identificado como semioquímicos de una chinche depredadora, y como feromona de agregación del crisomélido *Diorhabda alargado*, respectivamente (Ho et al., 2003); Cossé et al., 2005).

Nuestros resultados en EAG han mostrado que ambos compuestos ((*E,E*)-2,4-decadienal y (*E,E*)-2,4-heptadienal) provocan respuestas más intensas que los otros aldehídos y cetonas insaturados en antenas de ambos sexos (Fig. 3.20, p.133). Además, los extractos de espuma promovieron respuestas antenales significativamente más intensas que los extractos fecales y los volátiles de hembras (Fig. 3.19, p.132). En bioensayos de oviposición se ha investigado la influencia de la espuma y de algunos compuestos aislados identificados en espuma y huevos sobre el comportamiento de la langosta. Se supone que los huevos y la espuma de hembras de *D. maroccanus* contienen compuestos que atraen a otras hembras grávidas y/o provocan agregación durante la oviposición. Además, se había observado que las hembras de la langosta marroquí depositan sus huevos en lugares adecuados donde otras hembras habían ovipositado en años anteriores. Saini y colaboradores (1995) demostraron que algunos compuestos, obtenidos de la espuma de la langosta del desierto atraían a otras hembras de la misma especie a los mismos lugares de oviposición. Por lo tanto, se decidió ensayar si los extractos naturales y sintéticos mezclados con arena húmeda tenáin dicho efecto sobre el comportamiento de oviposición de hembras de *D. maroccanus*.

En general, las hembras pusieron pocos canutos durante los dos períodos de estudio y no se observaron diferencias significativas entre los diferentes tratamientos (Tabla 3.3, p.135). Estos resultados son sorprendentes, porque en la naturaleza se ha observado que hembras grávidas son capaces de ovipositar 2-4 canutos por individuo. Por lo tanto, son esenciales nuevos bioensayos para confirmar la actividad de los

extractos naturales y de los compuestos identificados, y también para entender su papel en la comunicación química y el comportamiento de la langosta marroquí.

Por microextracción en fase sólida (SPME) se han identificado varios hidrocarburos saturados lineales o ramificados tanto en machos como en hembras (Fig. 3.11, p.125). En general, se considera que estos compuestos pueden actuar como feromonas de contacto, atrayentes sexuales, afrodisíacos y feromonas de agregación en la mayoría de los insectos (Lockey, 1988). Es posible que los compuestos cuticulares de la langosta marroquí estén involucrados en el reconocimiento de su especie durante la fase gregaria, cuando varios miles de individuos están agrupados. Este supuesto tiene que ser confirmado en bioensayos especiales.

En volátiles recogidos con el método “dynamic headspace” se han revelado diferencias significativas entre las fases ninfa o adulto y los sexos (Fig. 3.9, p.123). Machos adultos maduros emiten nueve compuestos, ausentes en las ninfas de ambos sexos y en hembras adultas. Se ha podido identificar siete de estos compuestos: tetradecanal, 1-tetradecanol, pentadecanal, hexadecanal, 2-hexadecanona, 1-hexadecanol y 2-octadecanona. Por otra parte hay dos compuestos desconocidos hasta el momento, denominados 01BP84 y 02BP84, con sus espectros de masas casi idénticas y ambos con un pico base de *m/z* 84, por lo que suponemos que son 2 isómeros (Fig. 3.10, p.124), cuya identificación está en proceso. La especificidad masculina de los compuestos se ha confirmado por SPME (Fig. 3.11, p.125). El análisis y la cuantificación de los volátiles emitidos indican que los machos adultos inician la producción de algunos de estos compuestos (los aldehídos y los dos compuestos desconocidos) 1-4 días después de la última muda y llegan a la emisión máxima 12-20 días después. En cambio, las mayores cantidades de los dos alcoholes producidos por machos se han registrado una semana más tarde (Fig. 3.13, p.127). Es posible que estos compuestos sean productos de metabolismo o precursores de los aldehídos correspondientes.

Estos resultados pueden ayudar a esclarecer el proceso de maduración en los machos de la langosta marroquí. Asumimos que los compuestos producidos por machos y detectados en los volátiles de los mismos podrían estar involucrados en el sistema de feromonas de esa especie de langostas, similar a la langosta del desierto, cuya principal feromona es el fenilacetonitrilo (PAN) (Hassanali et al., 2005; Seidelmann et al., 2005).

Después de descubrir que los machos de *D. maroccanus* producen unos volátiles concretos al convertirse en adultos maduros, se ha iniciado la investigación de su origen. Supusimos que podían ser biosintetizados y emitidos por los machos maduros, aunque hasta ahora no se han descrito las glándulas productoras de feromonas en esta especie de langosta. Se han detectado los dos posibles isómeros de BP84 también en los extractos de alas y patas, aunque que los otros compuestos producidos por los machos estaban ausentes (Fig. 3.14, p.128). Las patas producen hasta tres veces más de los dos compuestos que las alas (Fig. 3.16, p.129). Estos resultados son similares a los obtenidos en la langosta del desierto por Seidelmann y colaboradores (2003), que demostraron que las alas y las patas de los machos maduros gregarios constituyen los lugares principales de la emisión del PAN, mientras que el abdomen y la cabeza sólo emiten pequeñas cantidades de esta feromona. Adicionalmente, se han comparado las cantidades de BP84 encontradas en diferentes partes de las patas de la langosta marroquí. Las mayores cantidades de los dos isómeros se producen en el fémur mientras que en el tarso, la parte exterior de las patas, sólo se han detectado pequeñas cantidades del compuesto (Fig. 3.17, p.130).

En GC-EAD y EAG se ha evaluado la actividad electrofisiológica de los volátiles emitidos por machos maduros, de los extractos de fémur de machos y de diferentes compuestos identificados en la espuma. En GC-EAD se ha probado también la actividad de volátiles de machos sobre antenas de ambos sexos y 4 de los compuestos identificados (1-tetradecanol, hexadecanal, 1-hexadecanol y 2-octadecanona) más el compuesto desconocido 02BP84 han suscitado respuestas antenales en las hembras. Por el contrario, en las antenas de los machos sólo el 02BP84 ha demostrado actividad electrofisiológica (Fig. 3.18, p.131).

En EAG los volátiles de machos han provocado las respuestas más intensas en antenas de ambos sexos, seguido por el extracto de fémur de machos adultos, que contiene el BP84 como componente principal (Fig. 3.20, p.133). Por lo tanto, consideramos que el compuesto BP84, exclusivamente producido por machos sexualmente maduros, podría ser uno de los principales componentes de la feromona de la langosta marroquí, al igual que el PAN producido por machos maduros de la langosta del desierto. Para confirmar esta hipótesis es necesario caracterizar estructuralmente a los compuestos BP84 y determinar su actividad biológica.

CAPÍTULO 4

Estudios de maduración en ninfas gregarias agrupadas con adultos conespecíficos de la langosta del desierto, Schistocerca gregaria (Orthoptera: Acrididae)

En este capítulo se describe un resumen de las actividades desarroladas por el autor en el lab de Dr. Torto (ICIPE; Nairobi, Kenya) con ocasión de una estancia predoctotal durante el periodo enero-abríl en 2010.

La langosta del desierto, *Schistocerca gregaria* (Forskål), es un acrídido plaga que causa graves daños a la agricultura en muchas partes de África y Asia (Anton et al., 2002), como ya se había descrito incluso en el Antiguo Testamento. Las langostas pueden llegar a ocupar un área enorme de unos 29 millones de km^2, esto es más del 20% de la superficie terrestre total del mundo. Un enjambre puede contener miles de millones de insectos y viajar cientos de kilómetros cada día (Simpson and Sword, 2008).

S. gregaria vive un total de entre tres y cinco meses, dependiendo principalmente de las condiciones climáticas y ecológicas. El ciclo de vida (desarrollo por metamorfosis incompleta) se compone de tres etapas: huevo, ninfa y adulto (Fig. 4.1, p.146). Después de la copulación las hembras ponen los huevos (80-150), unidos por una secreción espumosa que forma un canuto (ooteca) en suelos arenosos a una profundidad de 5-10 cm bajo la superficie. Después de 10-20 días, los huevos eclosionan en ninfas que presentan varios estados de desarrollo separados por mudas hasta convertirse en adultos (Roffey and Popov, 1968). En cada muda hasta llegar a 5, la ninfa desarrolla más aspectos de adulto hasta que después del quinto estadio se transforma en un adulto con alas completamente formado. Estos adultos, aún inmaduros y de color rosa, necesitan 1-2 semanas para madurar (color amarillo), de acuerdo con las circunstancias ambientales.

Dependiendo de la densidad de población, la langosta del desierto cambia de una fase gregaria (alta densidad) a una solitaria (baja densidad), que se evidencia al cambiar sus características morfológicas, fisiológicas y de comportamiento (Uvarov, 1966; Deng et al., 1996; Anstey et al., 2009). Estos cambios de fase son provocados por una combinación de señales olfativas, visuales y táctiles (Byers, 1991). Se supone que la agregación (formación de enjambres), que es fundamental para el cambio de fases, se provoca principalmente por señales olfativas (Torto et al., 1994; 1996). Entre los efectos mediados por feromonas en la fase gregaria se pueden destacar la cohesión social, la maduración sincronizada y la oviposición colectiva (McCaffery et

al., 1998; Hagele and Simpson, 2000; Malual et al., 2001). La maduración sincronizada en la langosta del desierto es de gran importancia para garantizar el apareamiento sincronizado de los sexos y las oviposiciónes comunales, y se supone que están involucradas diferentes señales químicas. Entre otros aspectos, la maduración se muestra al cambiar el color del rosa al amarillo y que está asociada a la emisión de la feromona (fenilacetonitrilo, PAN), exclusivamente por machos (Pener, 1991; Seidelmann et al., 2003).

Estudios previos habían demostrado que la maduración, en la que intervienen estímulos visuales, táctiles y químicos se retrasa en adultos inmaduros y agrupados con ninfas de la misma especie (Assad et al., 1997), mientras que se acelera en adultos jóvenes agrupados con machos adultos maduros (Mahamat et al., 1993; 2000). Por lo tanto, se supone que los efectos de aceleración (provocados por adultos maduros) y de retraso (provocados por las ninfas) podrían promover la maduración sincronizada en la langosta del desierto (Hassanali et al., 2005). Sin embargo, el efecto inverso provocado por agrupar ninfas con adultos conespecíficos en la maduración de las ninfas era desconocido. El presente trabajo se ha llevado a cabo para estudiar este aspecto. Se ha investigado el efecto de agrupamiento evaluando el desarrollo y la maduración de las ninfas con respecto a: (a) peso corporal, (b) longitud corporal, (c) duración del desarrollo, (d) cantidad de PAN en volátiles de machos adultos, (e) número de ooetcas puestas por hembras adultas y (f) nivel de farnesoato de metilo (MF) y de hormona juvenil III (JH III) en la hemolinfa de ninfas de los diferentes estadios. Suponiendo que el agrupamiento de ninfas con adultos tiene un efecto notable sobre el proceso de maduración de las ninfas, se ha considerado que la presencia de adultos de *S. gregaria* y la producción de sus compuestos volátiles pueden acelerar el desarrollo y la maduración de ninfas de la misma especie. Esto podría tener implicaciones importantes para entender la agregación y la formación de enjambres en la langosta del desierto.

Efecto de la presencia de adultos en la maduración y desarrollo de ninfas

Se colocaron 10 ninfas gregarias del primer estadio (receptor) con 10 machos y 10 hembras adultos (fuentes de señal) en jaulas de aluminio. Los individuos receptores tenían contacto visual, táctil y olfativo con los adultos. Se utilizaron dos lotes de insectos como control, en el primero 10 y en el segundo 70 ninfas de la de la misma edad, pero sin agruparlas con adultos (Tabla 4.1, p.150). Cada día se alimentaba a los insectos con plantas y salvado de trigo. Los individuos adultos (fuente de señal) que

morían en el transcurso del experimento, se reemplazaron por otros de la misma edad y del mismo sexo.

En primer lugar se ha demostrado un crecimiento más rápido (aumento de peso y longitud) de las ninfas agrupadas con adultos, en presencia de todos los estímulos, en comparación con las ninfas no agrupadas (Figs. 4.3; 4.4, p.153, 154). Por otra parte, las ninfas expuestas a los estímulos de adultos necesitaron menos tiempo para desarrollar alas y convertirse en adultos inmaduros de color rosa en comparación con los individuos control (Fig. 4.5; p.154; Tabla 4.2, p.155). Estos resultados indican que la presencia de adultos acelera el tiempo de desarrollo de las ninfas.

Para verificar la hipótesis de que la presencia de adultos afecta la maduración de los individuos receptores, se ha evaluado el proceso de maduración final por el color del cuerpo de los insectos y la cantidad de PAN en los volátiles de estos machos. El PAN es un indicador ideal de los estadios de desarrollo de la langosta del desierto, ya que este compuesto es el principal componente feromonal producido exclusivamente por machos maduros (Torto et al., 1994; Pener and Yerushalmi, 1998). Por otra parte, se ha revisado la maduración de las hembras (ninfas ex receptoras) por registro del inicio de la oviposición y por el número de ootecas puestas. Los resultados revelan que las ninfas macho que habían estado agrupadas con adultos cambian antes su color del rosa al amarillo, y también se adelanta la producción del PAN, en comparación con ninfas que no habían estado agrupadas (Fig. 4.6, p.156). En este contexto, se han observado cantidades notables de PAN a partir de 19 días después de la última muda. En estudios previos se había descrito que la emisión de feromonas de machos maduros en *S. gregaria* se producía generalmente a partir de 24 días después de la última muda (Deng et al., 1996).

En el presente trabajo se ha observado también una maduración acelerada en hembras agrupadas con adultos. Las hembras empiezan a ovipositar 1-2 semanas antes que las hembras no agrupadas, y el número de ootecas puestas es también superior (Fig. 4.7, p.156). Estos resultados coinciden con el periodo de emisión del PAN en machos, existiendo una semana de diferencia entre los individuos que fueron agrupados y los que no. El intervalo de tiempo entre la emisión máxima del PAN en machos y el mayor número de ootecas puestas por hembras es de 7 días, aproximadamente. Este período podría representar el momento de mayor actividad sexual en los adultos maduros y el tiempo desde la fecundación hasta la oviposición.

Además, se ha revelado que los niveles de la hormona juvenil III (JH III) en la hemolinfa de ninfas del 4º y 5º estadio que habían estado agrupadas con adultos

disminuyen en comparación con los niveles de ninfas no agrupadas (Fig. 4.9, p.158). Estos resultados están de acuerdo con la creencia de que altos niveles de JH podrían inhibir la transición a la etapa adulta (Uvarov, 1966). Posiblemente, el bajo nivel de JH III en la hemolinfa de las ninfas agrupadas con adultos podría ser responsable de su maduración acelerada.

El papel del farnesoato de metilo (MF) en la langosta del desierto se ha descrito sólo ligeramente (Marchal et al., in press). En general, se supone que esta hormona acelera la muda y el desarrollo en invertebrados (Laufer and Biggers, 2001). Por lo tanto, consideramos que las mayores cantidades de MF, detectadas en la hemolinfa de ninfas del 5º estadio de *S. gregaria,* que habían estado agrupadas con adultos (Fig. 4.8, p.157), pudieran ser un indicador de maduración acelerada.

En resumen, los resultados de este estudio proporcionan una ampliación del conocimiento del desarrollo (procesos de muda), gregarización y formación de enjambres de la langosta del desierto. Las feromonas pueden promover la maduración sincronizada en poblaciones gregarias y de este modo garantizan la cohesión y el mantenimiento de la fase gregaria. Por tanto, si la población de la langosta del desierto en fase gregaria está dominada por ninfas, el efecto retardante de sus señales feromonales puede evitar la maduración de adultos jóvenes, mientras que un predominio de adultos maduros podría inhibir la producción de JH lo que provoca una maduración acelerada en ninfas y adultos inmaduros.

Así pues, la maduración sincronizada garantiza la oviposición colectiva, lo que garantiza una alta densidad de ootecas en los lugares de oviposición y en consecuencia la cohesión territorial de la progenie. Esto es de gran importancia, porque la oviposición en acrídidos en fase gregaria constituye el principal medio de transmisión de rasgos gregarios a la descendencia (Roffey and Popov, 1968; Norris, 1970).

CONCLUSIONES

Capítulo 1 Estudios analíticos y de comportamiento de *Coroebus florentinus* (Coleoptera: Buprestidae)

- Los análisis mediante SEM de antenas de machos y hembras revelaron la presencia de cuatro tipos de sensilas. Se han identificado *s. basicónicas* tipo 1 y 2 (olfativas), *sensilas de un solo poro* (gustativas) y *s. caéticas* (mecanorreceptoras). Las antenas de machos son más largas, tienen una mayor superficie y disponen de más *sensilas de un solo poro* que las hembras, lo que indica una posible importancia de las señales de contacto para el reconocimiento de la pareja en *C. florentinus*, especialmente por los machos.
- En volátiles y en extractos de abdómenes de ambos sexos, se han identificado un total de 28 compuestos. La mayoría de estos compuestos son hidrocarburos saturados lineales o ramificados, generalmente utilizados como feromonas de contacto por otros insectos.
- No se han observado diferencias cualitativas en la composición de volátiles entre machos y hembras. Sin embargo, en bioensayos con un olfactómetro de doble elección los machos se sienten atraídos de manera significativa por volátiles y extracto de cuerpo entero de hembras, mientras los volátiles y extractos de machos no tienen ningún efecto sobre hembras. Al parecer, es posible la existencia de una feromona sexual producida por las hembras.
- En estudios electrofisiológicos tres de los compuestos identificados (nonanal, decanal y geranilcetona) provocan respuestas antenales en machos y hembras. En bioensayos de atracción los machos eligen la mezcla de tres componentes y decanal, mientras las hembras prefieren la mezcla de los tres componentes, la geranilcetona y en parte nonanal. Se asume que estos compuestos podrían ser componentes de una posible feromona de agregación.
- En volátiles de la planta huésped *Q. suber*, se han identificado 32 compuestos, principalmente volátiles de hojas verdes (green leaf volatiles, GLVs). Posiblemente, el daño que producen los adultos de *Coroebus spp.* al alimentarse de las hojas del árbol induce la producción de estos compuestos, o incrementa la emisión de los mismos.
- En hembras se han observado significativas respuestas electrofisiológicas y de atracción hacia los volátiles de la planta huésped y a 5 de los GLVs identificados ((*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, acetato de (*Z*)-3-

hexenilo y acetato de n-hexilo). Se han relevado preferencias significativas hacia los (*E*)-2-hexenol, 1-hexanol y acetato de (*Z*)-3-hexenilo. Estos resultados apuestan a que estos compuestos podrían jugar un papel importante en una posible cairomona de oviposición o de búsqueda de alimento

- En pruebas de campo con diferentes tipos de trampas y compuestos semioquímicos no se han capturado individuos de *C. florentinus.* En cambio, se ha atrapado un número relativamente elevado de hembras de *C. undatus*. La razón de estos resultados podría ser que las trampas se colocaron a la altura del tronco y no en la copa del árbol donde los adultos de *C. florentinus* suelen encontrarse.

Capítulo 2 Estudios analíticos y pruebas de campo para la captura de la culebra del corcho *Coroebus undatus* (Coleoptera: Buprestidae)

- Las antenas de ambos sexos de *C. undatus* son idénticas en forma y tamaño y disponen de los mismos cuatro tipos de sensilas como *C. florentinus* y *A. planipennis*. Como estas dos especies de Buprestidae, los machos poseen notablemente más *s. basicónicas* y *sensilas de un solo poro* que las hembras. Hay una creciente evidencia de la importancia de la percepción de olores, en especial de señales de contacto para el reconocimiento de su pareja por los machos en esta familia de escarabajos.
- Los datos espectroscópicos de extractos de cutícula del abdomen de ambos sexos revelan la presencia de 18 hidrocarburos lineales y ramificados, de entre los cuales dos se hallan exclusivamente en las hembras. Dado que, en *A. planipennis* se han identificado también dos hidrocarburos ramificados como feromonas de contacto, similares estructuralmente a los compuestos de *C. undatus*, es posible que los machos de *C. undatus* también podrían utilizar los hidrocarburos producidos por las hembras como señales químicas de contacto para el reconocimiento de la pareja.
- En pruebas de campo se han capturado significativamente más individuos (sólo hembras) en trampas prisma de color púrpura que en trampas de embudo Lindgren y trampas panel del mismo color. Por lo tanto, el diseño de la trampa y las señales visuales (por ejemplo, el color morado) juegan un papel importante en la atracción y captura de la plaga del alcornoque.

- Se ha constatado una actividad significativa como atrayente de *C. undatus* por las mezclas nonanal, decanal y geranilacetona (1:1:1) y la de los GLVs, (*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, acetato de (*Z*)-3-hexenilo y acetato de n-hexilo en trampas prisma.
- Se postula la hipótesis de que los tres compuestos detectados en volátiles de *C. florentinus* (nonanal, decanal y geranilacetona) podrían ser componentes de una posible feromona de agregación y que los GLVs de la planta huésped, particularmente (*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, acetato de (*Z*)-3-hexenilo y acetato de n-hexilo), están posiblemente involucrados en una cairomona de oviposición del género *Coroebus.*

Cápitulo 3 Estudios analíticos y electrofisiológicos de la langosta marroquí *Dociostaurus maroccanus* (Orthoptera: Acrididae), plaga de la Península Ibérica

- Las antenas de machos y hembras adultos de la langosta marroquí son filiformes, con 24 segmentos, y son idénticas en estructura y distribución de sus sensilas. Por comparación con antenas de la langosta del desierto y otras especies de acrídidos, se han identificado tres tipos de sensilas olfativas (*s. basicónicas*, *s. tricoideas* y *s. coelocónicas*) y una mecanorreceptora (*s. caéticas*). Estos resultados reflejan la importancia de la percepción olfativa de feromonas en el comportamiento y desarrollo de los acrídidos, tanto en machos como en hembras.
- Se han detectado nueve compuestos de cadena larga, más de 24 atomos de carbón (tres aldehídos, dos alcoholes, dos cetonas y dos compuestos desconocidos de momento) en volátiles de machos adultos y ausentes en hembras adultas y ninfas de ambos sexos. Las cantidades máximas de estos compuestos son emitidos 12-20 días después de la última muda por machos adultos. Estos compuestos podrían estar involucrados en la comunicación química de *D. maroccanus*.
- Los dos compuestos no identificados, denominados 01BP84 y 02BP84 también se encuentran en extractos de alas y patas de machos adultos. Las cantidades significativamente más altas se han detectado en las patas y sobre todo en los extractos de fémur, lo que indica una posible fuente de producción y/o emisión de estos compuestos.

- En EAG los volátiles y el extracto de fémur de machos adultos han provocado respuestas significativas en antenas de ambos sexos. En GC-EAD cinco de los compuestos producidos por machos, 1-tetradecanol, hexadecanal, 1-hexadecanol, 2-octadecanona y 02BP84 han sido electrofisiológicamente activos en antenas de hembras. Por el contrario, en machos adultos sólo el compuesto 02BP84 ha provocado respuestas antenales. Los volátiles producidos por machos y en particular el compuesto BP84 podrían ser componentes feromonales de la langosta marroquí.
- En la espuma de oviposición de hembras maduras se han identificado una gran cantidad de ácidos grasos saturados e insaturados, aldehídos y cetonas. Entre ellos hay dos compuestos, (*E*)-3-octen-2-ona y (*E,E*)-3,5-octadien-2-ona, que ya habían sido descritos como inductores de respuestas antenales en hembras grávidas de la langosta del desierto, y por su papel en el proceso de oviposición colectiva en. Sin embargo, en machos y hembras de la langosta marroquí estos compuestos no han mostrado ninguna actividad electrofisiológica. Por contra, los extractos de la espuma y los dos aldehídos insaturados (*E,E*)-2,4-heptadienal y (*E,E*)-2,4-decadienal han provocado respuestas antenales significativas, si bien no se han observado efectos de estos compuestos y de la espuma en ensayos de oviposición.

Capítulo 4 Estudios de maduración en ninfas gregarias de la langosta del desierto *Schistocerca gregaria* (Orthoptera: Acrididae), plaga importante en África

- Las ninfas de la langosta del desierto agrupadas con adultos conespecíficos presentan una maduración acelerada, en comparación con individuos que no habían estado agrupados. Ello se manifiesta por el crecimiento (peso y longitud), los niveles de la hormona juvenil III y del farnesoato metilo en la hemolinfa y la formación de alas. Una vez transformados en adultos, su maduración accelerada se evidencia por el cambio de color del rosa al amarillo, la cantidad de feromona mayoritaria producida por machos maduros (PAN) y el número de ootecas puestas por hembras grávidas.
- Se ha confirmado una de las teorías existentes acerca de la maduración sincronizada en la langosta del desierto, es decir, un retraso en la maduración de adultos inmaduros y la maduración acelerada de ninfas en una población mixta de ninfas y adultos inmaduros. Consideramos que las feromonas, principalmente el PAN, producidas por machos adultos y maduros promueven

la maduración sincronizada en poblaciones gregarias de la langosta del desierto. De esta manera garantizan la cohesión y mantienen la fase gregaria Por lo tanto, estos resultados tienen importantes implicaciones para la comprensión de la gregarización y de la formación de enjambres.