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IDENTIFICACIÓN DE FEROMONAS Y PROTEÍNAS IMPLICADAS EN LA PERCEPCIÓN FEROMONAL DE LEPIDÓPTEROS PLAGA

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10. SUMMARY

10.1. INTRODUCTION

Many species responsible for important damage to agriculture, forestry and stored products belong to the order Lepidoptera. Due to environmental and public health problems in addition to an incoming tolerance derived from the expanded application of pesticides, new alternatives have been developed in order to control these insect pests. One of these new tools which has attracted an enormous interest in the management of lepidopteran pests is the use of sex pheromones. Their high specificity, sensitivity and lack of toxicity make these substances a real strategy in pest control.

10.1.1. Pheromones

Pheromones are semiochemicals that when released by an organism elicit a specific response in another organism of the same species[1]. From the identification of the first sex pheromone, bombykol, by Butenandt et al.[2], the pheromone composition of several species has been determined.
Pheromones can be applied in different forms in lepidopteran pest control depending on the infestation level and the characteristics of the treatment area. They are the following:

Monitoring: Synthetic pheromones can be used as an indicator of the presence, abundance and propagation of a species. This tactic allows to weigh up the pest condition and decide the most adequate control method to be applied. Monitoring is largely used to control pests in greenhouses, forestry and stored products.

Mass trapping: This tool consists basically on the deployment of a high number of traps containing a pheromone lure in order to attract males and in some cases an insecticide to prevent them from escaping. The idea of this strategy relies on capturing a large amount of males to reduce mating and therefore the offspring.

Mating disruption: Saturation of the atmosphere of the attacked field by a large number of pheromone release points can avoid matings because males cannot follow the plume produced by female's pheromone emission.

10.1.2. Olfactory system in Lepidoptera

Olfactory stimuli can be divided into two groups, those produced by the own insect and involved in intraspecific communication and the odours coming from the host plant, which are utilized for the selection of an adequate place for feeding and oviposition. Olfaction is then fundamental to the survival of all insects. The antenna is the main organ by which the odour molecules are perceived and contain several hair-like structures called sensilla which are innervated by one or several receptor neurons[3]. In Lepidoptera several types of sensilla can be found, most of which possess an olfactory function. Trichoid sensilla comprise the most studied type, they are mainly involved in the perception of pheromone molecules, existing a great variation among sex and species. Odour perception has also been demonstrated in other sensillar types such as basiconica, coeloconica and auricillica. Other two additional types of sensilla are syloconica and chaetica, the first ones are considered to be humidity and temperature receptors and the second type mecanorreceptors.

10.1.3. The physiology of perception

The sensillar cuticle is perforated by numerous pores from which odour molecules pass through. Once in the sensillar lymph, odours are bound to proteins called odorant binding proteins (OBPs). These proteins are divided into two groups depending on the ligand nature, pheromone binding proteins (PBPs) if the molecule to be bound is a pheromone[4] or general odorant binding proteins (GOBPs) if the odour is a more general volatile such as the one released by host plants[5]. These two types of proteins are proposed to transport the odours to the olfactory receptors[6]. Binding of these molecules to the receptors provokes a change in the dendritic ion composition known as receptor potential. Simultaneous receptor potentials can combine and provoke a generator potential which elicits action potentials if a certain threshold is reached[7]. These potentials are subsequently propagated down the neuron axons until they reach the antennal lobes (AL)[8], the primary olfactory processing centre. AL are composed of a great number of spheroidal neuropils called glomeruli and local interneurons[9]. In the glomeruli synaptic interactions between odorant receptor neurons and AL interneurons occur. Axons of olfactory receptor neurons specifically stimulated by pheromone or antagonist compounds transmit the information to special glomeruli only found in males called macroglomerular complex (MGC)[10], whereas the non-pheromonal signal projects to ordinary isomorphic glomeruli, present in both sexes[11]. In several species a receptor neuron specific to a certain pheromone compound projects to a particular glomerulus. Nevertheless in other

species one glomerulus can contain axonal branches from different types of pheromone specific receptor neurons. Independently of the odour type, the olfactory information is led through output neurons to higher centres in the protocerebrum, the mushroom bodies[12], where the signal is finally interpreted and the corresponding action is decided. This signal is subsequently transmitted to descendent neurons (ND), whose axon passes through the ventral nerve cord until reaching several efector organs. ND carrying flight triggering signals will have branches in the thoratic ganglia, whereas the pheromone or oviposition mediating neurons will be headed to the terminal ganglia of the ventral nerve cord.

The final step in the olfactory process requires the inactivation of the signal. This role is performed by special enzymes, so-called odorant degrading enzymes (ODEs) that degrade the odorants making the receptor sites available for new molecules and avoiding therefore the saturation of the antenna[13]. Thanks to these enzymes the antenna remains sensitive to the perception of new incoming molecules, allowing the detection of fluctuating pheromone concentration changes in the pheromone plume.

10.1.4. Ditrysia

This division comprises the most evolved lepidopteran groups, consisting in three families of butterflies and 31 families of moths. Localization of the intraspecific congener is typically mediated by pheromones, with the exception of butterflies. These substances are emitted by females through a gland located in the intersegmental membrane, typically between the eighth and ninth abdominal segments. The female adopts a calling posture, while exposing the pheromone gland, and emits the pheromone blend, which is subsequently perceived by a conspecific male.

Butterflies and moths present different morphological and physiological adaptations depending on their daylight habits. The first ones are mostly diurnal and the second ones nocturnal. One of the main differences in both types of Lepidoptera relies on the antenna, well-developed in moths and less specialized and clubbed shape-ended in butterflies. The main reason of this distinct appearance is that the nocturnal species need a well-developed antennal surface to detect minimal amounts of the substances emitted by females at long distances for mating since vision is not very efficient at dusk. On the other hand, butterflies depend mostly on visual cues to locate the opposite sex, using quite often short range pheromones as a secondary system for mating. These pheromones are usually emitted by males and produced in modified scales called androconia located in abdomen, wings, thorax and legs.

10.2. OBJECTIVES

The present memory is focused on the study of the olfactory system in Lepidoptera pests with the main aim at controlling them afterwards by disruption of their odour perception. The objectives proposed in this thesis have been summarized as follows.

1. Identification of the pheromone blend in the Spanish strain of the beet armyworm *Spodoptera exigua* (Lepidoptera:Noctuidae) and subsequent evaluation of the pheromone compounds activity by electrophysiological techniques, wind tunnel and field tests to develop an optimum formulation for its management.

2. Analysis of the proteins present in males and females antennae of the Mediterranean corn borer *Sesamia nonagrioides* by proteomic techniques, followed by homology studies in the OBPs expression pattern of three species (*S. nonagrioides*, *S. exigua* and *Spodoptera littoralis)* within the same family (Noctuidae).

3. Study of the chemical communication system of the palm borer *Paysandisia archon* (Lepidoptera:Castniidae) by spectroscopic, electrophysiological and behavioural techniques.

10.3. TECHNIQUES USED IN THE STUDY OF OLFACTORY PROCESSES

10.3.1. Scanning electron microscope

The scanning electron microscope (SEM) obtains micrographs from a specific surface by scanning it under a high energy electrons transmission. These electrons interact with the sample atoms producing signals which contain information about the topography, composition and other properties of the sample surface. This technique has been mainly used to obtain micrographs from the insects antennae and therefore to determine the different types of sensilla present in them. The microscope employed for the images capture was a Stereoscan 360 model (Leica).

10.3.2. Electrophysiology

Electrophysiology is defined as the discipline that studies the electrical signals found in biological systems. In this work we have concentrated our efforts in the study of the response of the antennal receptors of insects to different odour molecules, and to do it, basically two electrophysiological techniques have been used: the electroantennogram (EAG) and the gas chromatography coupled to electroantennographic detection (GC-EAD).

10.3.2.1. Electroantennogram

EAG is a high-efficiency technique to study the insect antennal response to a certain chemical stimulus. The EAG is the sum of all receptor potentials elicited in all antennal sensilla tuned to the stimulating compound. In our case both antennal ends were placed in two electrodes, reference and registration connected to a high impedance amplifier, initially an IDAC-1 (Syntech) and subsequently changed to an IDAC-2. The amplifier was also connected to a stimulus controller (CS-01, Syntech) and to a computer. The acquisition and data analysis were performed by the EAG 2.3 software developed by Syntech, and later replaced by a more recent version, the EAGPro. Most of the assembly was protected by a Faraday cage which was connected to ground.

A glass tube with three openings was employed as a stimulus dispenser. One of these openings was connected to a continuous humid air current directed to the antenna, another opening was used as a complementary air current to keep a constant air flow, and finally the third one to deliver vapours of the stimulus to the antenna (Figure 3.3, page 25). The stimulus source consisted of a small (2x2 cm) folded Whatman paper impregnated with the testing sample, placed into a Pasteur pipette. Stimuli duration was 0.4 seconds with a 40 seconds interval to facilitate the complete recovery of the antennal receptors and, thus, avoiding their saturation.

Previously, the antenna was stimulated with hexane (the same solvent used to dissolve the pheromone compounds) as a blank. Three puffs were performed on each stimulus in an alternate manner with the blank, and the average of the three responses obtained with the blank was subtracted from the average of the three responses elicited by the stimulus. For statistical analysis a F test followed by the Sudent t test ($P<0.05$) were used.

10.3.2.2. Gas chromatography coupled to electroantennographic detection

This technique allows the rapid localization of the active compounds present in a pheromone blend by the combination of a GC with a flame ionization detector (FID) and an EAG system. The GC allows the efficient separation of the compounds present in a sample which are then immediately transferred to the EAG preparation for activity. The equipment used was composed basically of the EAG system explained above coupled to a Focus GC (Thermo-Instruments) with helium as carrier gas. The GC column effluent was diluted with a N_2 flow (make-up gas II) which was then split to the EAD preparation in 1:1 ratio. The column used in all cases was a HP-5 (Agilent Technologies, 30 m x 0.25 mm i.d.). The software used for data analysis was GC-EAD 3.2 by Syntech. A schematic representation of the GC-EAD used is shown in figure 3.4, page 28.

10.3.3. Wind tunnel

Pheromone compounds are emitted in a particular ratio that produces a specific behaviour in receptive moth males. This behaviour includes raising of the antennae followed by wing fanning, upwind flight to the source, approach to the lure and final contact with the source.

Upon orienting through the pheromone plume, males are able to distinguish different pheromone concentrations which are finally integrated in higher centres of the brain leading to the identification of the odour perceived. Wind tunnel is utilized to study the attraction capacity of the compounds found in a blend in laboratory conditions. The principal advantage of this system lies in the possibility of performing several assays in a continuous manner independently of the weather conditions. In addition temperature, air speed and humidity can be daily reproduced with no remarkable variation in the results in contrast to field tests.

The wind tunnel model used for the different assays was rectangular shaped (180 cm long x 55 cm wide x 50 cm high) and made of glass (Figure 3.5, page 31). The frame of the tunnel was made of aluminum and the access gained by two sliding doors, each one covering one half of the tunnel. The air was pushed through by a centrifugal fan and pulled out of the building with the aid of an exhaust blower. The fan and blower worked simultaneously and their potency was carefully regulated by a potentiometer to avoid undesired turbulence in the moving air. The air stream was cleansed through a 2 cm-thick glass woollen bed and conducted through two nylon screens to smooth the air flow and get a laminar regime throughout the tunnel. The lure was put in the end close to the fan and in

the other one the experimental males. A 58 W red light fluorescent was placed laterally outside the tunnel and wrapped in filter paper to reduce light intensity to 1-2 lux.

Female pheromone emission usually occurs at certain times of the scotophase, which is commonly coincident with the period of maximum response in males. If a male is subjected to an optimal blend composition out of this period, a decrease or even absence of response will be observed. For this reason, all assays were performed in coincidence with female calling behaviour.

10.3.4. Gas chromatography coupled to mass spectrometry

The identification of the different compounds found in the numerous extracts prepared (glands, wings, abdomen, etc) as well as in the emitted volatiles, was performed by a GC-MS MD 800 instrument (Fisons Instruments). In general a HP-5 (Agilent Technologies, 30 m x 0.22 mm i.d.) and/or a SPB-20 (Supelco, 30 m x 0.25 mm i.d.) capillary column were/was used for the analyses.

Among the several existing techniques for collecting volatiles two different systems were employed, solid phase microextraction (SPME) and adsorption on filter cartridges containing different adsorbents. The first technique allows the extraction of the compounds emitted by a species in the absence of a current of air. A syringe containing an adsorbent material was inserted in a glass vial containing the insects and the volatiles emitted adsorbed were subsequently analized by GC or GC-MS. This was applied to collect compounds emitted by females of *S. exigua.* On the other hand and for collecting emissions from males and females of *P. archon*, the air-entrapped volatiles emitted by this insect were adsorbed in filter cartridges of two types of absorbents charcoal and Porapak Q (Supelco) and extracted with hexane. Extracts were subsequently analyzed by GC-MS.

10.3.5. Analysis of the antennal proteins by proteomic techniques

Proteomics is the study of the proteome; group of proteins expressed in a cell, tissue, organism or biological fluid at a certain moment under specific conditions.
Since pheromones are becoming more popular as a biorational pest management tool, the study and understanding of the molecular basis of the insect olfactory system are gaining importance in future alternatives to conventional pesticides. In this context one of the main objectives proposed in this memory is the study of the olfactory proteins expressed in the antennae of males and females of different species. To accomplish this,

several proteomic techniques have been applied in order to separate, quantify, identify and characterize the proteins (Figure 3.6, page 33).

The procedure applied in all cases for the analysis of the antennal proteins was basically the same. Antennae from both sexes were excised and homogenized in lysis buffer (CHAPS, urea, thiourea, Tris and DTT) to facilitate the solubilization and denaturation of the proteins found in the sample. The proteins were then separated by two-dimensional electrophoresis (2DE), in the first dimension according to their isoelectric point by isoelectric focusing and in the second dimension by their molecular mass in denaturing polyacrylamide gels. To visualize the separated proteins silver staining was performed. The resulting gels were scanned, and the images obtained analyzed by a special software, in our case Image Master Platinum by which differential spots were determined. The spots with different expression were cut and digested with trypsin in an automatic digestor. Protein identification was performed by MS. Firstly peptide extracts were analyzed by matrix assisted laser desorption/ionization -time of flight (MALDI-TOF) MS that resulted in a peptide mass list named peptide mass fingerprinting (PMF). When protein identification was not possible for being absent in the database but ions were detected in MALDI–TOF spectra, a second analysis was performed by MS/MS. A electrospray (ESI-MS/MS) equipment was utilized for protein analysis of *S. nonagrioides* and a LC-MS/MS apparatus for the rest of the species. These two techniques allowed us to get some peptide sequence tags which were subsequently introduced into a program, MS-TAG or FASTA, when the ESI-MS/MS was the technique chosen and PEAKS in the case of LC-MS/MS. The sequences obtained from this last program were submitted in the basic linear alignment sequence tool (BLAST) for protein identification. All these programs allowed the identification of most of the analyzed proteins.

10.4. RESULTS AND DISCUSSION

10.4.1. Pheromone composition in the Spanish strain of *Spodoptera exigua*

The beet armyworm, *Spodoptera exigua*, is a polyphagous pest, worldwide distributed, that causes a great damage in numerous crops and ornamental plants both in nature and in greenhouses. There is a great disparity among all existing data relating to its pheromone composition. Initial studies on pheromone gland extracts from American strains identified (*Z*,*E*)-9,12-tetradecadienyl acetate (*Z*9,*E*12-14:Ac) as a pheromone compound[14] but the low efficiency of this acetate in field tests led to a reinvestigation of

the pheromone complex. In 1981, Persoons et al.[15] found five compounds in gland extracts: *Z*9,*E*12-14:Ac, (*Z*,*Z*)-9,12-tetradecadienyl acetate (*Z*9,*Z*12-14:Ac), (*Z*)-11-tetradecenyl acetate (*Z*11-14:Ac), (*Z*)-9-tetradecenyl acetate (*Z*9-14:Ac) and tetradecyl acetate (14:Ac). In 1990 Tumlinson et al.[16] identified in volatile collections in addition to *Z*9;*E*12-14:Ac, *Z*9,*Z*12-14:Ac and *Z*9-14:Ac, two other compounds: (*Z*)-11-hexadecenyl acetate (*Z*11-16:Ac) and (*Z*)-9-tetradecenol (*Z*9-14:OH) in a 40.2:47.9:6.5:1.7:4 ratio. Analysis of strains present in other countries showed also a disparity in the pheromone composition. In Japan, Mochizuki et al.[17] found a pheromone blend composed of *Z*9,*E*12-14:Ac, *Z*9-14:OH, (*Z*,*E*)-9,12-tetradecadienol *(Z*9,*E*12-14:OH) and *Z*9-14:Ac in 98:100:86:60 ratio, and more recently, in 2002, Dong et al.[18] discovered the same composition in China but in a different ratio 47:18:17:18. Howeever, none of these blends was significantly effective in trap catches in the field. Therefore, due to the absence of an optimum pheromone formulation for a proper management of this species in the field, a reinvestigation of the pheromone blend of the Spanish strain has been performed.

10.4.1.1. Analysis of the pheromone composition in gland extracts

A total of 28 gland extracts were analysed by GC coupled to mass spectrometry (GC-MS). Six pheromone compounds were identified by comparison of their retention time and mass spectra with those from synthetic standards: *Z*9-14:OH (**5**), *Z*9,*E*12-14:Ac (**1**), *Z*9,*E*12-14:OH (**4**), *Z*9-14:Ac (**2**), (*Z*)-11-hexadecenol (*Z*11-16:OH) (**6**) and *Z*11-16:Ac (**3**) (Figure 4.9, page 70) in 31:26:22:11:9:1 ratio. This composition differs from the pheromone blends found in other strains. When compared with other pheromone compositions, compound **1** is present in all species studied being the most abundant in strains like the one found in China[18]. In our strain, however, this acetate is the second component in abundance being compound **5** the first one. With regard to the other compounds, **2** and **4** have also been identified in China, Japan and USA[17-19] and compound **3** only in this last country[16]. In addition, Z11-16:OH has not been found in any of the strains studied so far.

10.4.1.2. Analysis of the pheromone composition in volatiles

It is well known that the pheromone compounds emitted into the air might differ from those found in gland extracts, both in their relative proportion as in their chemical structures[20]. To check this point in our strain of *Spodoptera exigua*, eight volatile collections from five females each were performed by SPME. In all extracts only four out of the six previously identified compounds were found: compounds **2**, **1**, **5** and **3** in a 40:34:22:4 ratio (Figure

4.13, page 74). Therefore, the blend emitted by the Spanish strain is also different from the one found in gland extracts due to the absence of compounds **4** and **6**. These results slightly differ from those obtained by Tumlinson et al.[16] who also found a blend composed of the same four components **1**, **2**, **3** and **5** in a different ratio (40.2:47.9:1.7:4), in addition to a fifth compound *Z*9,*Z*12-14:Ac, not observed by us in the Spanish strain. The lack of *Z*9,*E*12-14:OH (**4**) and *Z*11-16:OH (**6**) in volatile emissions suggests that they could act as biosynthetic precursors of the emitted acetates **1** and **3** and not as active pheromone components. In species of the same genera the same observation has been made. For example in *Spodoptera eridania*, the compound *Z*9-14:OH was detected in gland extracts but not among the emitted volatiles[21]. Likewise, in members of the Noctuidae family, such as *Heliothis subflexa*, the pheromone released is mainly composed of acetates and aldehydes, while the correspondent alcohols are only present in the pheromone gland[22].

10.4.1.3 Study of the male antennal response by electrophysiological techniques

The two pheromone blends found in gland extracts and volatile collections were used to stimulate male antennae in EAG at three different doses: 10, 100 and 1000 ng. In general, the responses elicited with the volatile blend were higher than those evoked with the pheromone gland mixture, except when 1000 ng were used where no significant differences were observed ($P<0.05$, Figure 4.16, page 77), probably because of the high amount of pheromone utilized as stimulus causing saturation of the olfactory receptors. Therefore the blend found in volatile collections was more active in EAG than the gland extracts.

To find out which compound from the six ones found in glands extracts elicited a higher EAG response in males, individual synthetic chemical were insuflated onto the antenna. As shown in figure 4.17, page 78, there was an enormous variation in the EAG activity of the single components. The highest antennal response was evoked by compound **1** at all doses. Since EAG is considered to be the summation of receptor potentials from the simultaneous firing of all olfactory receptor cells, the intensity of the EAG can be taken as a measure of the relative number of responding receptor cells[23]. Therefore, the highest response caused by compound **1** could mean a major abundance of receptor cells tuned to this compound in the antenna. Regarding the rest of the pheromone compounds, the response increased in accordance with the dose, reaching the maximum level at 1000 ng. Compounds **2** and **4** were more active than the rest, and the minor components **3** and **6** elicited the lowest response. Therefore, it can be deduced that the number of sensilla

responding to any of these two later compounds is relatively small. The same results were observed in GC-EAD (Figure 4.21, page 81) wherein all compounds elicited an antennal response, the highest being produced by compound **1**, followed by **4** and **5**. With regard to the two minor compounds, **3** and **6**, a certain small response was observed from compound **6** whereas the corresponding response to **3** was much smaller, probably due to the tiny amount present in the gland.

10.4.1.4 Behavioural studies in wind tunnel

As commented above, there may be a great diversity in the attractiveness of the pheromone blends used in the field tests performed. Therefore, it is advisable to make a previous behavioural study of the pheromone blends in a wind tunnel before testing them in the field.

In initial experiments, when one microgram of the two pheromone blends found in gland extracts and volatile emissions were used as lure, no significant differences were observed in the total number of males reaching the different parts of the tunnel compared to five virgin females used as control. However, the number of males making contact with the pheromone source was higher when five virgin females were used than with any of the other two synthetic blends. Regarding the activity of these two mixtures, the volatile blend elicited a slightly higher number of male contacts with the source than the gland extract (Figure 4. 25, page 85).

Nevertheless, when component **1**, the most active in electrophysiology, was used alone or in combination with the other three compounds found in volatiles (**2**, **3** and **5**), important differences were observed (Figure 4.26, page 87). Compound **1** was attractive to males by itself (71% of them reached the final of the tunnel) but none contacted with the source. This is in accordance with the observations made by other authors, who noticed the inefficiency of this compound by itself in field tests being necessary the combination with another compound[24]. From the results obtained in the wind tunnel experiments it turns out that this component is *Z*9-14:OH (**5**) whose addition to *Z*9,*E*12-14:Ac elicits in males contact attempts with the pheromone lure. This effect is not displayed by the other two components **2** and **3,** which when mixed with compound **1** induce also a cooperative effect but not sufficient to make insects contact with the source.

The same combination (**1** + **5**) but in different ratios was also found to be fairly attractive in field tests performed in USA[25] and Taiwan[26] (10:1 ratio) and in Japan and Korea[27, 28] (70:30 ratio). Furthermore, Tumlinson et al. proved that mixtures lacking both compounds

were completely ineffective in the field. According to our results and others' compound **1** acts as a long-range attractant whereas compound **5** displays its activity in the vicinity of the lure.

In order to see the effect of the other two compounds (**2** and **3**) on the most attractive blend so far (A: **1**+**5** (60:40)), new behavioural assays were performed. A total of four combinations were used: A, D: **1**+**5**+**2** (35:23:42 ratio), E: **1**+**5**+**3** (56:37:7 ratio) and F: **1**+**5**+**3** (87.2:2.5:10.3 ratio). This last formulation is one of the blends considered in the literature as highly attractive in the field[16]. As shown in figure 4.27, page 88, all combinations elicited taking flight in most of the males tested, although the number of males displaying the different behavioural parameters decreased with blend F. On the other hand, there were no significant differences between D and E combinations. In comparison with blend A males displayed lower number of contacts with the source when attracted to these two ternary blends but this effect was not significant.

A study focused on the effect of the two compounds (**4** and **6**) found in glands but not in volatiles was also carried out. A fixed amount of these two alcohols (10 and 50% of compound **1**) was added to the previous combination (A: **1**+**5** 60:40) and used as lure. As shown in figure 4.28, page 89, compound **4** did not have a clear effect in the assays performed. The addition of this alcohol to blend A did not provoke any effect in the number of males attracted to the pheromone source. Due to the lack of effect and its absence in volatile emissions this compound was not considered important for future field tests. However, compound **6** dimisnished the number of males that finally reached the lure when the amount of it was increased, indicating an inhibitory role when mixed with compounds **1** and **5**.

From all these results it can be deduced that the binary combination **1**+**5** in 60:40 ratio was quite attractive to males in wind tunnel assays. However, these results do not match with those observed in field tests. The two blends that captured the highest number of males in the field were those containing mixtures of **1**+**5**+**3** (mixtures F7 and F2), being particularly active the baits containing these compounds in 56:37:7 ratio (blend F7) (Figure 4.34, page 94). The binary blend, **1**+**5** (60:40) which was the most attractive in wind tunnel was not that efficient in the field. This discrepancy could be related to the wind tunnel characteristics since it does not fully mimic the pheromone plume dimensions and the natural conditions of the field.

10.4.2. Analysis of the antennal proteins in three Noctuid species

As commented above, olfaction is crucial for the survival of Lepidoptera. Moths and butterflies rely on both long and short-distance volatiles to locate conspecific partners and a suitable place for feeding and oviposition. Odour detection needs the presence of three types of proteins: Odorant receptor proteins (OR), OBPs and ODEs.

OR are expressed by odorant receptor neurons and are found to be located in the dendritic membrane of these cells. OBPs comprise a group of small hydrophilic proteins (~14KDa) and are expressed by the supporting cells found in the sensillum. The main function of these proteins is supposedly the transport of small hydrophobic ligands. Finally ODEs, also expressed by supporting cells, are involved in odour degradation.

Proteomics is a tool that let us know what is exactly happening in a cell or tissue at a certain moment. To study the proteome two aspects have been developed, what is known as expression proteomics and functional proteomics. The first one studies the global expression of the proteins at certain conditions, allowing the detection of those proteins presenting variations in those circumstances. On the other hand, functional proteomics studies the localization of protein complexes besides their cell movement, composition and protein interactions. One of the options proposed along this chapter has been the study of the antennal proteins involved in olfaction by expression proteomics, focusing mainly on the study of OBPs among different species and between sexes. In addition some attempts of localizing and identifying ODEs have been performed.

10.4.2.1. Morphology of the antennae

Sometimes a strong antennal dimorphism is noticed between male and female but in many cases differences are not that evident. In the three species studied: *S. nonagrioides*, *S. exigua* and *S. littoralis*, just in the first one the antennae differ between sexes, although at microscopic level this lack of variation is not that obvious. Males of the three species present more and longer sensilla trichodea type than females (Figure 10.1) in order to perceive a minimum amount of pheromone molecules emitted by conspecific females at long distances. In agreement with this observation, the quantity of total protein found in male antennae extracts was more than the one observed in females in all three species.

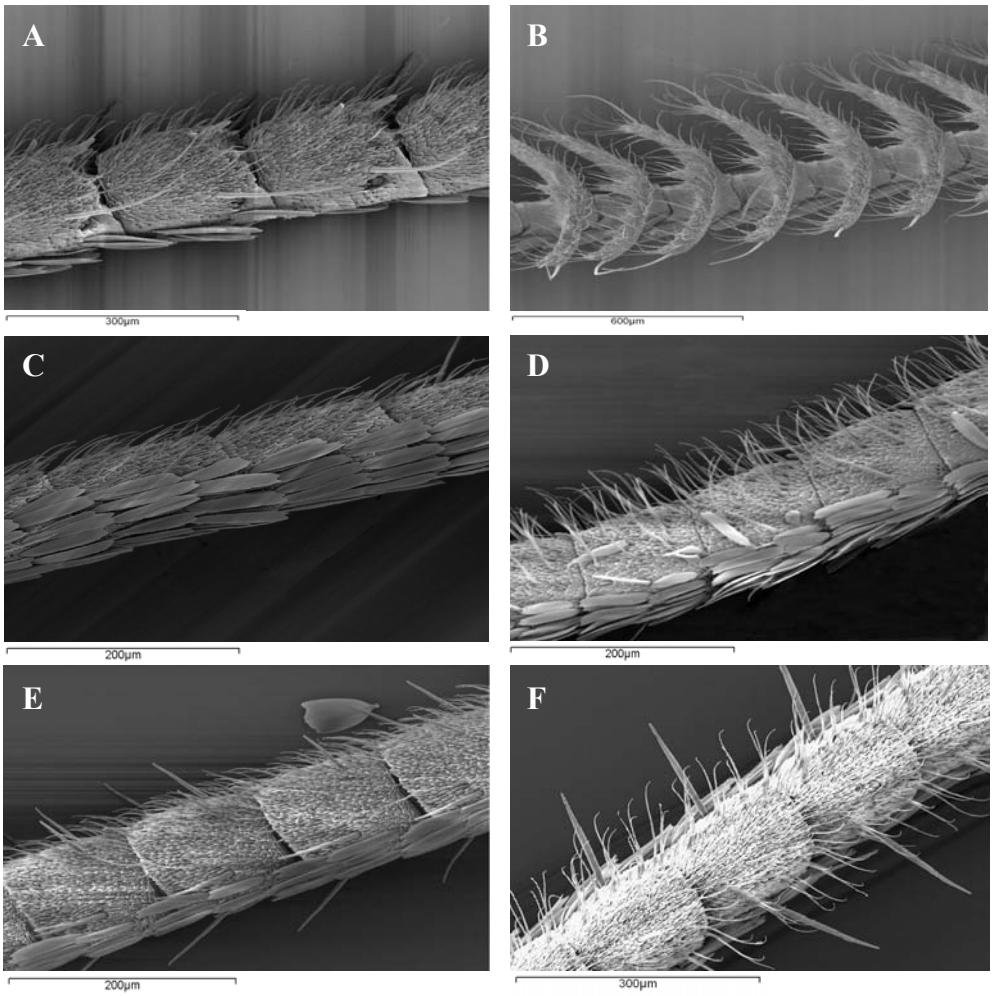

Figure 10.1. SEM micrographs from antennae of both sexes in the three studied species: A-B) *S. nonagrioides*, C-D) *S. exigua* and E-F) *S. littoralis*. Left column represents females and right column males.

10.4.2.2. Analysis and identification of the proteins found in the antenna

To see if there were also differences at the protein level, antennal proteins from males and females of the three species were separated by 2DE. *S. nonagrioides* was the model chosen to set up the technique with antennal extracts.

10.4.2.2.1. *Sesamia nonagrioides*

A total of 12 gels loaded with 100 µg of protein per sex were polymerized at 12.5% polyacrylamide. All the scanned gels images obtained were compared. An average of 800 spots was detected from which just 10 were differentially expressed in males and females. Nine of these spots were located in the low molecular weight region (< 25 KDa) and just one in the zone > 25 KDa. Five of the differential spots were found to be more expressed

in females (spots **7**, **9**, **10**, **11** and **12**) and the other five in males (spots **2**-**6**) (Figure 5.7, page 122).

In order to improve spot resolution and detection of the region of Mr > 50 KDa, the putative zone for possible odorant degrading enzymes (ODEs), the second dimension was run with 8% polyacrylamide to cover the 25–100 KDa region. Among the 850 spots detected, only six spots were differentially expressed. Five of them (spots **13**, **14**, **16**, **17** and **18**) showed a higher expression level in females and just one (spot **15**) in males (Figure 5.12, page 129).

The 18 spots selected for identification were cut from the gels and after trypsin digestion were subjected to MALDI-TOF MS. By this technique just two spots could be identified, **1** and **8** as cellular retinoic binding protein (CRABP) and GOBP2 respectively. CRABP belongs to the lipocalin family and is involved in retinoic acid transport whose function is related to cell growth and differentiation. This spot together with spot **8** showed a similar expression level in both sexes.
Regarding the other proteins, seven were identified by ESI-MS/MS. Spot **2**, which was more abundant in males than in females was identified as PBP2. Spots **4**, **5** and **6**, which showed identical PMFs and a higher expression in males than in females, were all identified as PBP1. Spot **7**, highly expressed in females, corresponded to another protein related to olfaction identified as GOBP1. On the other hand the other two proteins identified had not an olfactory role. Spot **12** corresponded to a glyceraldehyde-3-phosphate dehydrogenase and finally spot **13** to a ribosomal protein 60. Glyceraldehyde 3-phosphate dehydrogenase catalyzes the conversion of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate during glycolysis and 60S ribosomal protein participates in the translation of RNA to protein. It is not known the reason of a higher expression of these two proteins in females compared to males.

With the aim at determining the rest of unidentified proteins, two gels of both polyacrylamide percentages utilized previously were made, loading a double amount of protein (200 µg). In spite of this quantity, none of the differential proteins subjected to analysis could be identified since proteins previously found weakly expressed remained at these expression levels.

To see if this variation in the expression of the proteins identified as OBPs was constant between sexes along the photoperiod or was just dependent on the moment antennae were excised, the expression of these proteins was studied during the photo and scotophase in males and females. Among the six proteins identified previously as OBPs,

the three spots corresponding to PBP1 (spots **4**, **5** and **6**) were found to be more expressed in males than in females in both light and darkness, showing a slightly higher expression during the scotophase. In females, PBP1 expression, as represented by spot **4** (Figure 5.14, page 132), was very low during the light period, increasing slightly during the scotophase. Spots **4**-**5** were almost undetectable at all times assayed in all female extracts. Regarding the PBP2, it was also always expressed in a higher level in males in comparison to females. On the other hand, GOBPs were found to be mainly expressed in females than in males, being this difference especially evident in GOBP1. This protein showed a slight increase in the expression level at the end of the scotophase in females and almost constant in males. In addition, GOBP2 was also found to be more expressed in females although no significant differences were observed between sexes.

10.4.2.2.2. *Spodoptera exigua*

Antennal proteins expression was also studied in two other species of the same family, *S. exigua* and *S. littoralis* to check mainly if there was a similarity in the protein pattern. Therefore to have a general visualization of the proteins expressed in antennae of both sexes, 12.5% polyacrylamide gels were run.

Following the correspondent electrophoretic protein separation and staining, scanned gel images were analyzed. Only seven spots were determined as differentially expressed in *S. exigua* males and females, five more abundant in males (spots **2**, **3**, **4**, **9** and **10**) and two in females (spots **6** and **11**). As observed in the previous species the majority of these differential proteins, in particular six, were found in the low molecular weight region (< 25 KDa). All these seven spots were cut in addition to four more located near the region where OBPs of *S. nonagrioides* were identified (Figure 5.18, page 138).

Among the 11 spots digested and subjected to a first analysis by MALDI-TOF MS, five of them were identified. Spots **2**, **3**, and **4,** more abundant in males than in females**,** were all determined as PBP1, as found in *S. nonagrioides* these three spots presented the same isoelectric point but a slight mass difference probably due to a posttranslational modification. Spot **8**, equally expressed in both sexes, was identified as PBP2 and finally spot **5**, also expressed similarly in males and females was found to be a GOBP2. The rest of the unidentified proteins were determined by LC-MS/MS. By this technique two spots corresponding to numbers **1** and **7** could be identified as CRABP and ATP synthase, respectively. Also identification of spots **3** and **4** was confirmed by this technique.

10.4.2.2.3. *Spodoptera littoralis*

Following the same process, an average of 900 spots was detected in this species, from which just a spot, located in the OBP region, was found to be differentially expressed in males and females (spot **4**). A total number of eight spots, including the differential spot and seven more localized nearby were cut from the 2DE gels. After the proper digestion with trypsin, spots were firstly analyzed by MALDI-TOF MS. In this case five spots were identified (**2**, **4**, **5**, **6** and **7**) (Figure 5.22, page 144). Spot **2** was determined as a fatty acid binding protein (FABP), also a lipocalin, like the CRABP, whose function consists basically in amino acids transport. Spot **4**, more abundantly expressed in males than in females, was identified as PBP1. This identification also corresponded to spot **5**, this equal identification could also be about a posttranslational modification which unlike the other two species might affect the isoelectric point instead the relative weight. Finally, spot **6** was identified as PBP2 and spot **7** as GOBP2. Peptide extracts of the other spots (**1**, **3** and **8**) were identified by LC-MS/MS, spot **1** as CRABP, **3** as GOBP1 and **8** as PBP4. From the three PBPs sequenced by Jacquin-Joly (personal communication) only PBP1 and 2 were identified by MS. No PBP3 could be identified, although another PBP no previously reported in this species (PBP4) was identified by homology with PBP4 of *Spodoptera frugiperda*.

The first PBP described was found to be expressed only in male antennae of the species *Antheraea polyphemus*[29]. Due to this discovery and also to its ability to bind pheromone molecules, it was thought that this kind of proteins was solely expressed in this sex. Subsequent studies revealed an also existing PBPs expression in females, although in a lower level except for some species of the Noctuidae family, where high PBP expression levels were observed. In all three species studied, more than one PBP has been identified in agreement with other authors' findings. In general, many of the identified PBPs showed a differential expression level between sexes, with some exeptions. In the three species, PBP1 protein was more abundantly expressed in males compared to females. On the other hand, PBP2 that was more abundant in *S. nonagrioides* males, did not keep this difference between sexes in the two *Spodoptera* species, where it appeared similarly expressed in both males and females. The main PBPs function is supposedly the transport of pheromone molecules to the receptors located in the dendrites of the olfactory neurons, therefore a major expression of these proteins in males indicates a greater sensory activity as a result of an active pheromone carrying and the correspondent perception of these molecules. On the contrary, a similar PBP2 expression in females of the two Spodoptera species could be involved in the detection of their own pheromone

blend or at least of some compounds. In fact, it has been observed that females of many species are able to detect their own pheromone such as *Panaxia quadripunctata*[30], *Tricoplusia ni*[31], *Manduca sexta*[32] or *S. littoralis*[33] among others. This is in agreement with the results obtained in EAG, where females of both *Spodoptera* species showed a certain response to their gland extract, not observed in *S. nonagrioides* (Figure 10.2) which could explain the lower expression of this protein in females compared to males.

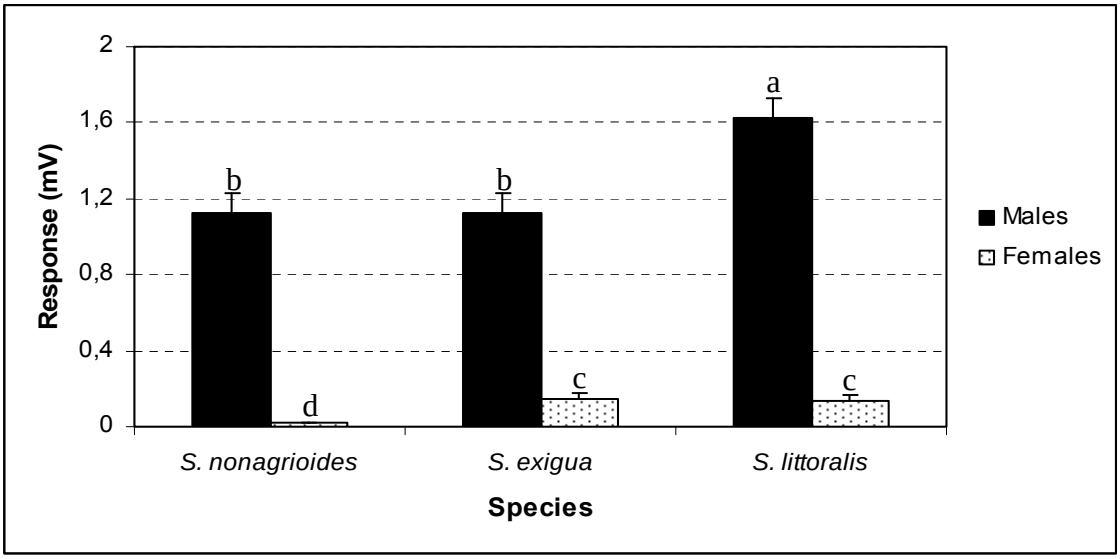

Figure 10.2. EAG responses of *Sesamia nonagrioides, Spodoptera exigua* and *Spodoptera. littoralis* male (N=10-15) and female (N=8-11) antennae to their own pheromone gland extract (equivalent to one female gland). Black bars indicate the responses of male antennae and dashed bars represent female antennae. Error bars represent the standard error. Equal letters above bars indicate an absence of significant differences (t Student, $P < 0.05$).

With regard to GOBPs, a similar expression was seen in both sexes of several species such as *Manduca sexta*[34], *Antheraea pernyi* and *A. polyphemus*[35]. However these proteins showed a higher expression in females of other species like *Bombyx mori*[35], *Epiphyas postvittana*[36] and *S. nonagrioides*[37]. In this last species, the GOBP that showed a differential expression level was GOBP2, but in our study the one seen as differential was GOBP1[38]. This disparity could be in relation to the identification procedure, since it was previously determined by western blot using an antibody raised against a recombinant GOBP2 of *M. sexta*[37] and in our case the novo sequency was employed. The high conservation of GOBPs among species and the use of an antibody against only a GOBP could be the reason of such different identification. So excluding the GOBP1 found in *S. nonagriodes*, which was more abundant in females, all GOBPs in the three studied species showed a similar expression in both sexes. These proteins are supposed to be involved in the transport of a more general type of odours such as host plant volatiles like alcohols and isoprenoids[39]. These odorants can be of a high relevancy for males and

females. Males of many species need the perception of host plant volatiles to get stimulated and locate the conspecific female which must be close to an adequate place for the offspring development. On the other hand, females also require the reception of this kind of molecules in order to find a suitable site for oviposition.

The study of the OBPs expression along the photoperiod confirmed the results commented above. Male PBPs were highly expressed in both photo and scotophase, with a light increase during the dark period. On the other hand PBPs were less abundantly expressed in females with no significant variations in the expression level. Pheromone emission occurs at a certain moment of the scotophase which is coincident with male receptivity. This slight increase in male PBPs expression during darkness could be related to this higher receptivity which also could explain the moderate increase in female GOBP1 expression, since females search an adequate place for oviposition at night.

Therefore a high similarity in OBPs localization has been observed in all three species, unlike ODEs which none of them could have been localized by 2DE. This was probably due to the high abundance of other proteins located in the same region difficulting their finding and also to the expression levels of some of these proteins like the esterases in two of the species under study; *S. nongrioides* and *S. littoralis* in which these proteins are expressed in a low similar level in both males and females[40].

10.4.3. Study of the chemical communication in *Paysandisia archon*

Paysandisia archon is a Castniid originally from South America (Argentina, Uruguay, Paraguay and Brazil) that has been introduced in Europe by palm trees imports and has become a severe pest in Spain. In general the larva feeds on palm trees of several genera such as *Phoenix*, *Trithrinax*, *Butia* and *Chamaerops*. There is not much data about the behaviour of this species in its native countries, all the information available in the literature is based on a collection of studies performed by other authors about specimens introduced in Europe. Males are usually found perched on leaves close to the palm crown until a flying specimen of a similar size is visualized, it is then when it starts chasing it. Once the individual is reached, if it is not of the same species, the male return to the origin place but if on the contrary the individual is a conspecific the pursuit is continued regardless of the sex. But in the case the member chased is a female, the two specimens fly at short distances, close to the palm crown until they alight. It is then when the male approaches the female and copulation takes place[41]. All the observations made seem to

point out a visual localization of the opposite sex at first sight. Nevertheless, all these data based on observation need to be complemented with experimental data. Therefore different experimental assays have been performed in order to find out the kind of communication used in this species.

10.4.3.1. Morphological study of the antenna by SEM

At first sight, the antennae are more alike the butterflies than the ones observed in moths since the final end is club shaped as a typical diurnal Lepidoptera. Also microscopically there is more similarity between *P. archon* antenna and another day flying species' such as *Pieris brassicae* (Lepidoptera:Pieridae), than the one found for example in the other two moths used previously like *S. nonagrioides* and *S. littoralis* (Figure 6.37, page 205). These last species with night-flying habits possess a well-developed trichoid sensillar surface to detect minimal amounts of pheromone at long distances but in both *P. archon* and *P. brassicae* the number of sensilla thrichoidea in males are reduced, not being much different from the ones found in females. The length reduction of this sensillar type could be related to a diurnal life adaptation in such a way that probably it has lost the ability to perceive pheromone compounds at long distances.

10.4.3.2. Analysis of the antennal response by EAG

Due to the lack of knowledge about the type of chemical communication existing in this species for intraspecific recognition, as well as the corporal region involved in the biosynthesis and emission of attractants, extracts of all the different body parts were prepared. In addition, volatile collections from males and females placed both individually and together were performed. Male and female extracts and volatiles were used to stimulate antennae of the opposite sex.

As the antenna is quite long, registrations were divided into two antennal parts, the club (distal part) and the one close to the head (basal part). Data from the basal part were not considered due to the unstable response observed which increased throughout the time independently from the stimulus used. The reason for this increase might probably be due to the abundance of mechanoreceptor sensilla in this area. Considering therefore the distal part, independently from the sex and stimulus employed, EAG response was very low (in general < 0.1 mV). Males showed even a lower depolaritation than the one observed in females, emphasizing the low response elicited by ovipositor extracts in comparison to the rest. On the other hand, females showed in general a higher response

than males, specially to thorax and wing male extracts, with a significant difference in the first one compared to the other two stimulus employed (abdomen and head male extracts) (Figure 6.15, page 180). Same results were obtained with antennae of *P. brassicae* where responses from males and females were also < 0.1 mV and where females also presented a higher response than males. Anyway, as a consequence of the low response observed in the previous assays with *P. archon*, although similar to the ones observed with *P. brassicae*, a complementary study on the antennal response of both sexes was performed using this time as stimuli the volatiles collected from males and females. As shown in figure 6.16, page 182, the response observed in females antennae was slightly higher than in males although no significantly different. In addition there was not an outstanding response to any of the stimuli tested in none of the two sexes. This low response of the male antenna to any of the female extracts and volatiles might point out the absence of an active pheromone compound emitted by the conspecific female. On the other hand, the higher EAG response seen in females, specifically to thorax and wing male extracts, could indicate the existence of a possible attractive compound in any of these two body parts. This section could be the thorax since the response elicited by this part was greater than the one provoked by the rest of extracts. Although the fact that female *P. brassicae* did not show a significant response to male wings (Figure 6.18, page 184), part responsible for attractants emission in Pieriids[42], could be due to a lower sensitivity of the perception system in species with daylight habits compared to the one existing in nocturnal species. Therefore there is a great difficulty in determining the EAG activity of the different stimuli in diurnal Lepidoptera.

10.4.3.3. Analysis of the antennal response and identification of the active compounds by GC-EAD and GC-MS

As no evident response was observed in EAG neither from males or females, a more sensitive technique, like GC-EAD was used. In order to inquire into the possible existence of a long distance pheromone emitted by females, males antennae were stimulated firstly with ovipositor extracts and volatiles collected from females. In both cases different peaks of the chromatogram elicited a supposedly active response (Figure 10.3). But when these extracts were analyzed by GC-MS the peaks in the ovipositor extract corresponded to fatty acids of 16-18 carbon atoms, and the ones belonging to the volatile extraction were mainly cyclopolisyloxanes, possibly due to air contamination.

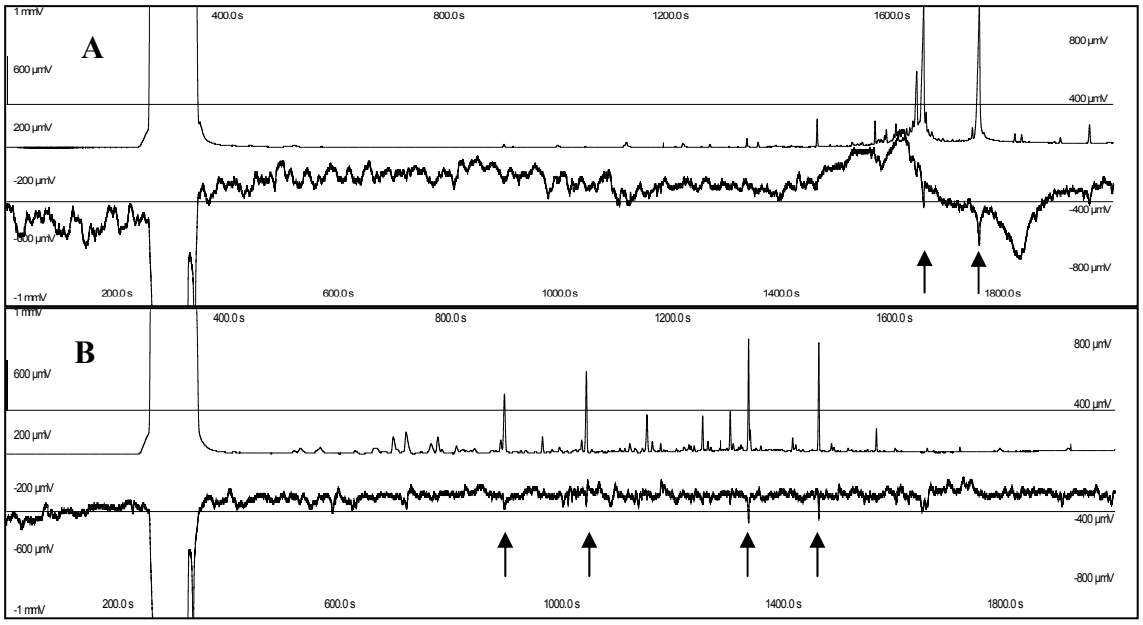

Figure 10.3. GC-EAD responses of two *P. archon* male antennae to: A) Ovipositor extract, B) Volatiles collected from a female. Black arrows indicate peaks in the chromatograms that elicit an active response in the antenna.

Therefore the next step was to study female antennal response to the volatiles emitted by males. In this case two additional peaks not found in the female volatiles chromatograms were observed (Figure 6.23, page 188), which after the following analysis by GC-MS surprisingly were identified as (Z,E)-3,7,11-trimethyl-2,6,10-dodecatrienal (Z,E-farnesal (**1**)) and E2,Z13-18:OH (**2**) comparing the mass spectra with the correspondent standards. Furthermore a third compound not visible in GC-EAD records was detected in the analysis of male volatiles, but not in females, this compound presented an equal mass spectrum and retention time to (E,E)-farnesal when compared to the correspondent standard (Figure 6.29, page 196).

These three compounds were also found in the volatiles collected when both male and female were placed together and also elicited an active response in male antennae showing an also existing intraspecific recognition in males. This phenomenon has also been observed in other species. One of the functions attributed to male perception among males of the same species is the inhibition of the sexual behaviour among congeners of the same sex[43, 44], of relevant importance in territorial species[45]. The few data found in the literature about *P. archon* confirm the territoriality in males as a typical feature[41].

To determine which body part was involved in the emission of these compounds, the antennal response of females to different male extracts corresponding to wings, thorax and abdomen, parts generally responsible for the emission of chemicals[46], was studied.

After the analysis of all the records, several responses to different peaks were observed. In the chromatogram corresponding to the wing extracts two of the three compounds found in male volatiles were visualized (Figure 6.24, page 190). This identification was confirmed by the proper analysis by GC-MS, where due to the higher sensitivity of the technique the compound (E,E)-farnesal could be observed (Figure 6.31, page 199). None of the three compounds were seen in female wing extracts (Figure 6.32, page 199). The pheromone synthesis is not much known in males in comparison to females. Many males of different Lepidoptera families accumulate substances from the host plant at the larval stage as a defence mechanism for predators[47], most of which can be used subsequently as pheromone precursors[48, 49]. None of the two compounds (E,E and Z,E)-farnesal have been found in the family Palmae, usual host plant of *P. archon*. Nevertheless in various species of this family have been identified the E,E-fanesene and Z,E-farnesene from which the correspondent alcohols could be synthesized being the precursors of the two aldehydes emitted by the males. These two compounds are emitted by other insects such as *Corcyra cephalonica* (Lepidoptera: Pyralidae) where males are responsible for this emission while females emit a long distance pheromone[50]. The last compound, E2,Z13-18:OH is a typical component in females of the Sesiidae family, one of the families phylogenetically close to Castniids. Nevertheless there are some examples in the literature reporting male pheromone compounds coincident with the females. in the species *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) males emit by the wings a compound also found in female emissions (Z,Z,Z)-3,6,9-heneicosatriene[51]. In the same way, the compound Z9-14:Ac, one of the components seen in the pheromone blend of female *Heliothis virescens* (Lepidoptera: Noctuidae), has also been discovered in male emissions[52].

To all these data that point to a high diurnal adaptation must be added the absence of an identified PBP. Analysis of 2DE antennal extracts gels of both sexes revealed the presence of only one OBP identified as GOBP2 (Table 6.1, page 202). This protein, more abundantly expressed in females in comparison to males could be involved not just in the transport of host plant volatiles but also of pheromones, since assays performed by other authors in other species showed in some cases a high affinity of GOBP2 for pheromone compounds[39].

Although no biological assays have been performed in order to confirm the pheromone nature of the three compounds found in male volatiles, it has been proved the lack of a long distance pheromone released by females.

10.5. CONCLUSIONS

10.5.1. Pheromone composition in the Spanish strain of *Spodoptera exigua*

1. The pheromone blend found in the Spanish strain is composed of four compounds: *Z*9-14:Ac, *Z*9,*E*12-14:Ac, *Z*9-14:OH and *Z*11-16:Ac in a 40:34:22:4 ratio. The two components *Z*9,*E*12-14:Ac and *Z*9-14:OH are necessary to attract males to the pheromone source

*2. Z*9,*E*12-14:Ac is the most attractive compound in electrophysiology. According to the results observed in wind tunnel, this compound is able to attract male at long distances but needs the addition of Z9-14:OH for the lure recognition.

3. The field tests performed have shown an increment of the attraction capacity of the previous pheromone blend made up of *Z*9,*E*12-14:Ac and *Z*9-14:OH when *Z*11-16:Ac was added. These three components in a proportion 56:37:7 captured the highest number of males.

4. The addition of *Z*9-14:Ac to the binary blend of *Z*9,*E*12-14:Ac + *Z*9-14:OH decreased the amount of males captured in traps. It is not known why this compound is emitted in such a high quantity.

5. Therefore, it has been found a pheromone blend efficient enough for attracting a high number of males in the field, preventing in this way, the localization of conspecific females and the consequent mating, maintaining the species in a controlled level with the correspondent reduction of damage.

10.5.2. Analysis of the antennal proteins in three Noctuid species

1. The differential study of the antennal proteins expression in both sexes of different species has been possible thanks to the optimization of 2DE gels of antennal extracts. In addition many of the proteins analyzed have been identified by different mass spectroscopy techniques like MALDI-TOF MS, ESI-MS/MS and LC-MS/MS.

2. In general, proteins that showed a different expression level in males and females and subsequently were identified as OBPs, were located in the low molecular weight region.

3. Proteins identified as PBPs were normally more abundantly expressed in males than in females except for some cases such as the two PBP2 in both *Spodoptera* species and the PBP4 in *S. littoralis*, which were equally expressed in both sexes.

4. EAG assays showed how females of both Spodoptera species were able to respond to their own pheromone blend, although less intensively than males. This could explain the equal expression of PBP2 in females.

5. In general GOBPs were similarly expressed in males and females with the exception of *S. nonagriodes* where the GOBP1 was more abundant in females. This higher expression was obvious during the scotophase which is coincident with a superior activity in females.

10.5.3. Study of the chemical communication in *Paysandisia archon*

1. Thanks to the application of diverse techniques such as SEM, electrophysiology and GC-MS, among others, different unknown aspects of this species like the morphology, behaviour and antennal protein expression have been discovered.

2. Studies on the antenna have revealed not just a disparity in the general structure, more similar to butterflies, but also in the reduction of sensilla thricoidea in males which are found to be well developed in moths.

3. EAG assays have also shown in males a lack of significant response to ovipositor extracts and female volatiles. On the other hand, volatiles just emitted by males could finally be visualized by both GC-EAD and GC-MS, these compounds have been identified as *Z*,*E*-farnesal, *E*,*E*-farnesal and *E*2,*Z*13-18:OH.

4. All these results in addition to the absence of an identified PBP, show a non-existing long distance pheromone emitted by females and therefore the proper adaptation of this species to diurnal conditions using visual cues firstly for the localization of a conspecific congener, and subsequently a possible emission of short range attractants by male wings for the intraspecific recognition.

5. Utilization of synthetic pheromones for the management of this species seems to be unviable, being necessary to employ new alternatives such as biological tools, less polluting than chemicals.

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