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ESCUELA TÉCNICA SUPERIOR DE INGENIERÍAS AGRARIAS

INSTITUTO UNIVERSITARIO DE INVESTIGACIÓN EN GESTIÓN FORESTAL SOSTENIBLE

# TESIS DOCTORAL:

## Semiochemical management of pine sawyer beetles *Monochamus galloprovincialis* (Olivier) and *M. sutor* (Linnaeus)

## Manejo de los escarabajos perforadores *Monochamus galloprovincialis* (Olivier) y *M. sutor* (Linnaeus) mediante compuestos semioquímicos

Presentada por **Gonzalo Álvarez Baz** para optar al grado de Doctor por la Universidad de Valladolid

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SUTAINABLE FOREST MANAGEMENT

**Research Institute**

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*A mi familia*

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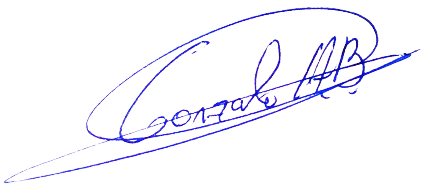

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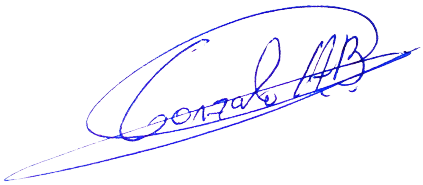

# Contents

# Abstract

Pine sawyer beetles *Monochamus galloprovincialis and M. sutor*, are secondary pests of pines in Europe and North Africa that have become important since the former was identified as the vector in Europe of the Pine wood nematode (PWN) *Bursaphelenchus xylophilus*. The latter species has been hypothesized as capable of transmitting *B. xylophilus* if the nematode ever became associated with it. PWN is the causal agent of pine wilt disease (PWD), a wilting disease that has caused dramatical economic and environmental loses in pine stands of several countries in Eastern Asia. The disease has been introduced and spread through Portugal in Europe, and recent infection foci are currently under eradication in Spain. Since PWN introduction in Asian countries, management of PWD through the insect vectors has been recommended as one of the most promising strategies.

Traps baited with specific lures have showed effective and preferable to insecticides due to the hazard they represents to non-target organisms and human health. Significant progress on the chemical ecology of *M. galloprovincialis* and *M. sutor* has been performed in recent years, resulting on highly attractive lures with practical applications in PWD management. The present thesis provides advances in the development of lures and traps especific for the semiochemical management of *Monochamus galloprovincialis* and *M. sutor*. Advances in *Monochamus* antenal physiology as a basis for further lure improvement are also presented. Finally, application of these tools to biological control by entomopathogenic fungi, by means of auto dissemination tactics, has been also researched.

An effective trapping system is needed, not only for monitoring the insect vector but also for direct control of its population. Trapping may also provide key information on the nematode load carried by the beetles, allowing early detection of infections, provided that captured beetles remain alive within the trap. Effective attractants have been developed in recent years that are commonly used in combination with diverse standard trap designs. In our experiments, several trap designs were developed and compared to commercial standard models in order to determine which designs maximized the number of attracted

insects actually caught and the proportion of them remaining alive. In total, 12 trap designs were evaluated in five field experiments carried out in France, Spain and Portugal. Teflon coating applied to the whole trap and extended, ventilated collecting cups resulted in a significant improvement of trap performance. These modifications led to significant increases of pine sawyer catches, up to 275%, when applied to multiple-funnel or black cross-vane traps, compared to standard designs. Furthermore, a significant proportion of the captured beetles remained alive within the trap. These findings have been used to develop new commercial traps (*Econex Multifunnel-12*® and *Crosstrap*®*;* Econex, Murcia, Spain) available to forest managers. A model for insect survival within the trap was also fitted. Elapsed time between consecutive samplings, mean relative humidity and maximum radiation were the three most significant variables. Thus, traps should provide a suitable sample of live insects if sun exposure of the trap is minimized and a reasonable sampling schedule is implemented.

Among field effective attractants identified in recent years are the specific *M. galloprovincialis* aggregation pheromone, host pine kairomones such as α-pinene and bark beetle kairomones like ipsenol and 2-methyl-3-buten-2-ol. The main objective of the study was to optimize the combination of these volatiles to improve lure attractiveness and specificity. Based on ten complementary field experiments, we found a pheromone dose-response of trap catches. The best combination of attractants associated the aggregation pheromone and two bark beetles kairomonal compounds, ipsenol and 2-methyl-3-buten-2-ol. By contrast the addition of pine terpenes, such as α-pinene, did not significantly improved *M. galloprovincialis* trap capture while increasing the capture of non target species, including natural enemies. The use of pine terpenes would be advisable only if priorizing to maximize removal of vectors. While this research has lead to the development a new, highly attractive commercial lure for mature pine sawyers, none of the tested blends were successful in attracting immature pine sawyer adults. Further investigation is needed to develop attractants for these beetles.

Studying *M. galloprovincialis* antennal physiology will provide clues for lure further improvement. The response of its olfactory receptor neurons (ORNs) to several odorants was tested using single sensillum electrophysiology. Behaviourally active pheromone and kairomone (host and sympatric bark beetle pheromones) odors were tested alongside smoke compounds released by burnt wood that are potentially attractive to the insect. The

antennae bore several types of sensillae. Two plate areas in the proximal and distal ends of each antennal segment were covered with basiconic sensillae that responded to the odor stimuli. Sensillae basiconica contained one or two cells of different spike amplitude. The 32 male and 38 female ORNs tested responded with excitations or inhibitions to the different plant odors. In general the response of male and female receptors was very similar so they were pooled to perform a cluster analysis on ORN responses. Six ORNs were clearly specialized for pheromone reception. Responses to kairomone and smoke odors were less specific than those of pheromone, but a group of 9 cells was clearly excited by smoke compounds (mainly eugenol and 4-methyl 2-methoxyphenol), a group of 8 cells was very responsive to α-pinene, β-pinene and *cis*-verbenol, and a group of 14 cells responded to a wider range of compounds. The rest of the cells (47%) were either non-responsive or slightly inhibited by smoke compounds. Dose-response curves were also obtained for several compounds..

Semiochemical management of *M. galloprovincialis* through biological control has been also approached. Three entomopathogenic fungal strains, *Isaria farinosa* (Holmsk.) Fr., *Lecanicillium attenuatum* (Zare & W. Games) and *Beauveria pseudobassiana* (Bals.) Vuill. were isolated in Spain from naturally infected *Monochamus galloprovincialis*. This is the first time that these entomopathogenic fungi have been isolated from *M. galloprovincialis* beetles. Assays showed the *B. pseudobassiana* EABps 11/01-Mg strain to be highly virulent against the pine sawyer. Horizontal and vertical transmission were assessed for both aqueous ($1\times10^8$ conidia/ml) and dry ($4.25\times10^9$ conidia/g) conidial formulations. Evidence of horizontal or vertical transmission was not found when insects were inoculated with the aqueous conidial suspension. However, when dry conidia were applied, 100% of the horizontally-infected insects died and their average survival times (AST) were significantly reduced (from 21.10 and 25.00 days in controls to 10.40 and 10.00 days in infected males and females, respectively). Compared to control females, numbers of egg-laying wounds, eggs laid, live larvae after 5 days and larvae entering the xylem after 6 months were significantly reduced in both inoculated females and clean females that had mated with inoculated males, pointing to horizontally-induced reduction of progeny. These results validate the potential of the isolated *B. pseudobassiana* strain as an important natural population regulator. Through auto-dissemination techniques (i.e “lure and infect”), this biological agent could be used for the integrated control of pine wood nematode vectors and constitute a new tool for Pine Wilt Disease management.

Recent reporting of 2-undecyloxy-1-ethanol as the *M. sutor* male-produced aggregation pheromone opened the possibility of developing an efficient lure for this species. It has also been reported that European *Ips* bark beetle pheromone compounds, ipsenol, ipsdienol, cis-verbenol and 2-methyl-3-buten-2-ol, alone or supplemented by host volatiles α-pinene and 3-carene, kairomonally attracted this species. Besides, smoke volatiles from burnt trees might play a role in *M. sutor* host location. Field trapping experiments during three years in three countries (Spain, Sweden and Austria), aimed to develop an efficient pheromone-kairomone lure operative for *M. sutor* management were carried out.. Electroantenographic responses by *M. sutor* to *Ips* pheromones and to the *Pityogenes chalcographus* pheromone chalcogran were also studied. GC-EAG recording showed that *M. sutor* males and females clearly responded to ipsenol and ipsdienol, and females also to 2-methyl-3-buten-2-ol. Chalcogran elicited a neat response to *M. sutor* female antennae. In field tests, ipsenol was the most attractive kairomone to both sexes of *M. sutor*, whereas ipsdienol, cis-verbenol and 2-methyl-3-buten-2-ol resulted very weakly attractive and chalcogran was unattractive. When combined with the pheromone, most *Ips* kairomones increased catches of both sexes, but it was significant only for ipsenol. On the contrary, chalcogran resulted completely ineffective Thus, ipsenol not only was shown the strongest individual kairomone for *M. sutor* but it resulted also the best single kairomone to be combined with the pheromone. Results, on the other hand, of a blend of smoke volatiles from burnt pines, tested in Spain and Austria, were negative, pointing to that smoke cues are not likely involved in host finding by this species.

# Resumen

Los perforadores *Monochamus galloprovincialis* y *M. sutor* son plagas secundarias de los pinos cuya distribución ocupa Europa y Norte de África. En los últimos años su importancia se ha visto grandemente incrementada desde que el primero de ellos fue identificado como el vector en Europa del nematodo de la madera del pino *Bursaphelenchus xylophilus*. Se planteado además la alta probabilidad de que el segundo se capaz de transmitir el nematodo si en algún momento ambos organismos entrasen en contacto. El nematodo de la madera del pino es el causante de la enfermedad del marchitamiento de los pinos. Esta patología ha causado graves pérdidas económicas y ambientales en países del Este asiático donde ha sido introducido. En Europa se ha extendido por gran parte de Portugal causando una alta mortalidad en las masas del pino resinero. Tres focos recientes de la enfermedad están actualmente bajo erradicación en España. Desde su introducción en los países asiáticos, el manejo de la enfermedad a través de sus insectos vectores ha sido considerado como una de las estrategias más prometedoras.

El uso de trampas cebadas con atrayentes específicos se ha mostrado una alternativa eficaz y preferible al uso de insecticidas, debido al peligro que éstos representan para otros organismos así como para la salud humana. En los últimos años se ha llevado a cabo un avance significativo en la ecología química de *M. galloprovincialis* y *M. sutor* dando como resultado cebos atrayentes con aplicaciones prácticas en el manejo de la enfermedad apoyado en compuestos semioquímicos. La presente tesis aporta también avances en el conocimiento de la fisiología antenal de *Monochamus*, como base para una posterior mejora de las combinaciones atrayentes. Finalmente, también se ha investigado sobre la aplicación de estas herramientas al control biológico con hongos entomopatógenos, mediante tácticas de autodiseminación (p.e. “atrae e infecta”).

Un sistema de trampeo eficaz puede ser útil no sólo para el monitoreo del insecto vector sino también para el control directo de su población. El trampeo, además, puede proporcionar información clave en la carga de nematodos transportados por los insectos

siempre que éstos permanezcan vivos en la trampa, permitiendo la detección temprana de focos de infección. En los últimos años se han desarrollado atrayentes eficaces que se usan frecuentemente con diversos diseños de trampas estándar. En este estudio se desarrollaron distintos diseños de trampas y se compararon con los modelos estándar comerciales para determinar cuáles maximizan el número de insectos atraídos que caen en su interior y la proporción de ellos que permanece viva. En total se evaluaron 12 diseños en cinco experimentos de campo realizados en Francia, España y Portugal. El recubrimiento de Teflón aplicado a toda la trampa y el uso de botes colectores extendidos y con ventilación mejoraron significativamente el rendimiento de las trampas. Estas modificaciones permitieron unos aumentos significativos del número de capturas de hasta el 275%, tanto en las trampas multiembudo como a las de intercepción, en comparación con los diseños estándar. Además, una proporción significativa de los insectos capturados permaneció viva en las trampas. Estos hallazgos han permitido el desarrollo de nuevos modelos de trampas comerciales, (*Econex Multifunnel-12*® y *Crosstrap*®*;* Econex, Murcia, España) disponibles para los gestores forestales. Con los datos recogidos se ajustó un modelo matemático de supervivencia de los insectos en el interior de la trampa. El tiempo transcurrido entre muestreos consecutivos, así como la humedad relativa y la radiación máxima fueron las tres variables más significativas. Por lo tanto, las trampas pueden proporcionar una muestra útil de insectos vivos si se minimiza su exposición al sol y se muestrean con una periodicidad razonable.

En los últimos años se han identificado atrayentes eficaces en campo para *M. galloprovincialis*, entre los que se encuentra su feromona de agregación, cairomonas de los pinos hospedantes como el α-pineno, y cairomonas de escolítidos como el ipsenol y el 2-metil-3-buten-2-ol. El principal objetivo de este estudio fue optimizar la combinación de estos volátiles para mejorar la atracción y especificidad de los cebos. Se realizaron diez experimentos de campo en los que se encontró una relación dosis-respuesta de la feromona con el número de capturas. La mejor combinación de atrayentes fue la de la feromona agregativa con las cairomonas de escolitidos ipsenol y 2-metil-3-buten-2-ol. Por el contrario, la adición de terpenos de pino, como el α-pineno, no mejoró significativamente las capturas de *M. galloprovincialis* y además aumentó las de especies no objetivo, incluyendo enemigos naturales. El uso de terpenos de los pinos sería aconsejable sólo si hubiese que priorizar la eliminación máxima de vectores. Aunque este trabajo ha dado lugar al desarrollo de un nuevo cebo comercial altamente atrayente para

los insectos maduros, ninguna de las mezclas ensayadas tuvieron éxito en la atracción de adultos inmaduros. Futuras investigaciones deberán buscar atrayentes para estos insectos.

Se ha estudiado la respuesta de las neuronas receptoras olfativas de *M. galloprovincialis* a diversos volátiles usando la técnica de electrofisiología de sensila única. Los compuestos utilizados fueron su feromona agregativa, cairomonas de los pinos hospedantes y de insectos escolítidos y volátiles de humo emitidos por la madera quemada, que podrían ser atractivos para este insecto. La antena de *M. galloprovincialis* alberga varios tipos de sensilas. Dos áreas aplanadas en los extremos próximal y distal de cada segmento antenal están recubiertos de sensilas basicónicas que contienen una o dos células distinguibles por la amplitud de su actividad espontánea. Se ensayaron 32 y 38 células de machos y hembras respectivamente que respondieron con excitaciones o inhibiciones a los diferentes volátiles. En general, la respuesta de ambos sexos fue muy similar, de manera que los datos se agruparon para el análisis. Seis neuronas resultaron estar claramente especializadas en la recepción de la feromona agregativa. Las respuestas a las cairomonas y a volátiles de humo fueron menos específicas pero un grupo de nueve células resultó claramente excitado por los volátiles de humo (principalmente eugenol y 4-methyl 2-metoxifenol). Un grupo de 8 células respondió muy bien al α-pineno, β-pineno y *cis*-verbenol y otro grupo de 14 células respondió a un amplio rango de compuestos. Se además obtuvieron curvas de dosis-respuesta para varios compuestos.

El manejo de *M. galloprovincialis* con compuestos semioquímicos aplicado al contrrol biológico se ha abordado en una serie de experimentos. A partir de ejemplares de este insecto infectados de forma natural se aislaron e identificaron tres cepas de hongos entomopatógenos pertenecientes a las especies *Isaria farinosa* (Holmsk.) Fr., *Lecanicillium attenuatum* (Zare & W. Games) y *Beauveria pseudobassiana* (Bals.) Vuill. Esta es la primera vez que estos hongos han sido aislados de *M. galloprovincialis*. Los ensayos mostraron que la cepa *B. pseudobassiana* EABps 11/01-Mg fue la más virulenta contra el perforador. Se estudió la transmisión horizontal y vertical, tanto con una suspensión de conidios en agua ($1\times10^8$ conidios/ml) como con una preparación seca en polvo de talco ($4.25\times10^9$ conidios/g). No se encontraron evidencias de transmisión horizontal o vertical cuando se utilizó la suspensión acuosa. Sin embargo, con la

preparación seca murieron el 100% de los insectos infectados por transmisión horizontal y su tiempo medio de vida se redujo significativamente (de 21.10 y 25.00 días en los controles a 10.40 y 10.00 en los machos y hembras infectados, respectivamente). En comparación con las hembras control, el número de mordeduras de oviposición, huevos puestos, larvas vivas 5 días después de la puesta y entradas de pupación en el xilema 6 meses después se redujeron significativamente en las hembras inoculadas y en aquellas limpias que se habían emparejado con machos inoculados, lo que sugiere un reducción de la progenie inducida horizontalmente. Estos resultados validan el potencial de la cepa de *B. pseudobassiana* como un importante regulador natural de la población que podría ser utlizado, aplicado a través de tácticas de autodiseminación (como “atrae e infecta”), en el control integrado de los vectores del nematodo de la madera del pino y, en definitiva, de la enfermedad del marchitamiento de los pinos.

Recientemente se ha identificado 2-undecyloxy-1-ethanol como una feromona agregativa de *M. sutor* liberada por los machos. Este hallazgo abre la posibilidad de desarrollar un cebo eficaz para este insecto. Es conocido también que combinaciones de los compuestos feromonales de las especies de Ips europeas, ipsenol, ipsdienol, cis-verbenol y metil-butenol, solas o junto con los volátiles de α-pineno y 3-careno, son atractivas para *M. sutor*. Los volátiles de humo también podrían jugar un papel relevante en la localización de hospedantes por parte de esta especie. Este trabajo aporta resultados de bioensayos electroantenográficos sobre cuatro compuestos feromonales de las especies de *Ips* y de la feromona de *Pityogenes chalcographus,* chalcogran. Se llevaron a cabo experimentos de atracción campo durante tres años en tres países (España, Suecia y Austria), con la finalidad de desarrollar un cebo feromonal-cairomonal operativo y eficaz para el manejo de *M. sutor*. Los registros de GC-EAG mostraron que tanto las hembras como los machos de *M. sutor* respondieron al ipsenol y al ipsdienol, y las hembras además al metil-butenol y chalcogran. En los ensayos de campo el ipsenol resultó ser la cairomona más atractiva para ambos sexos. Por el contrario, ipsdienol, cis-verbenol y metil-butenol resultaron débilmente atractivos y el chalcogran no lo fue en absoluto. Combinados con la feromona agregativa de *M. sutor*, la mayoría de las cairomonas de escolítidos aumentaron el número de capturas de ambos sexos, pero solamente el ipsenol lo hizo de forma significativa. El chalcogran no tuvo ningún efecto. A la vista de estos resultados, el ipsenol no solo se mostró como la cairomona más atractiva de forma individual para *M. sutor,* sino que también resultó ser la mejor para sinergizar

con la feromona. Por otro lado, los resultados de una combinación de volátiles de humo emitidos por los pinos quemados, ensayados en dos países (España y Austria), fueron negativos, lo que sugiere que estas señales probablemente no están implicadas en la búsqueda de árboles hospedantes por esta especie.

# Chapter 1

# Introduction

Human activity has been always linked to the movement, intentional or unintentional, of living organisms as the civilizations spread across the globe colonizing new territories. The start of trading came to intensify this effect. Clearly limited at first by the transport capacity, the pace of introductions of exotic organisms remained at low levels until the Industrial Revolution, when economic growth held the dual effect of increasing demand of products and the ability to transport them over long distances. In the last century, with the increase in capacity, speed, range and cheaper transport costs, especially of maritime and air media, the increase of goods movement throughout the world has grown exponentially, just as the pace of introduction of invasive species. Global timber transport for further manufacturing and wooden packaging materials handler (such as crates, dunnage and pallets) has made movement between continents of raw or slightly processed wood particularly increased. Inside this type of product, habitual and hardly desinfectable, xylophagous organisms have a particularly easy pathway of reaching new territories.

A particular case is the Pine Wood Nematode (PWN) *Bursaphelenchus xylophilus* (Steiner and Buhrer) (Nematoda: Parasitaphelenchidae). This is a saprophytic organism native to North America where it usually exploits dead or dying coniferous tree species without causing major damage, since they had co-evolved with the pathogen (Wingfield *et al*. 1982). However, during the last century, this organism has been accidentaly introduced in several countries where it has caused a rapid wilting, and ultimately death, of native conifers that has been described as Pine Wilt Disease (PWD).

## History and management of Pine Wilt Disease

The pine wilt disease appeared at the beginning of XX century in the Japanesse city of Nagasaki. Pine stands surrounding the city began to die and the disease spread north

along the main island, Honsu, during the following decades. At that time it was believed that boring insects that abounded in dead trees (longhorn beetles, weevils and bark beetles in particular) were the cause of the disease, but in 1971 Kiyohara y Tokushige found that the causal agent was not an insect, but a nematode: *Bursaphelenchus xylophilus* (Kiyohara T. y Tokushige Y.,1971). Later it was confirmed that PWN had been introduced into Japan during the first decade of the twentieth century and that a native longhorn insect, *Monochamus alternatus*, played the vector role in this country (Mamiya and Enda 1972). One of the first strategies adopted in Japan to deal with the PWD was spraying forest stands with an fenitrothion insecticides to prevent *M. alternatus* vectoring and inoculating nematodes. The use of this tactic seemed effective and reasonable but the application of the insecticide over vast areas of territory raised environmental and healthy concerns that limited their use. As a result of the disease, Japanese pine forests have lost more than 46 million cubic meters of timber in the last fifty years (Futai, 2008).

In the early eighties, when the disease had spread to almost all the Japanese territory and this country still was losing two million cubic meters of wood every year, China and Korea reported the presence of the nematode in their territories. In both countries, not only *M. alternatus* but also *M. saltuarius* acted as vectors (Zhao, 2008). In China, fumigation and trunk injections were applied as control methods. Although spraying got some success, it had to be suspended because of the negative effect showed on the populations of some birds as well as on natural insect predators and parasitoids of *Monochamus* (Zhao, 2008). Defense of some protected areas, such as Huangshan Mountains -World Heritage Site- was attempted by establishing a 4 kilometers wide and 100 km long pine tree-free strip around. Although this belt looked effective in containing the disease and forests inside seemed protected, it could not stop PWN resulting from human activity. Nowadays China has lost 80,000 hectares of pine forest and more than 50 million trees (Zhao, 2008). Korea also began wrestling with preventive spraying and burning the affected material. These methods showed effective, but stopping the cycle requires the treatment of every wood material bigger than 2 centimeters in diameter, which in the field represented a nearly insurmountable logistical problem (Shin, 2008).

In Europe, *Bursaphelenchus xylophilus* was first detected in 1984 in a shipment of wood from North America arriving in Finland (Rautapaa, 1986). After this episode, the European authorities launched a program to rigorously inspect wood products from North

**Figure 1.1.** Wilted maritime pines at Comporta (Portugal).

America in seaports, altough it was not adressed with the same rigor on those comming from East Asia. In 1999 *B. xylophilus* was declared the causal agent of the wilting that began to spread among pines of the Setúbal Peninsula (Portugal), 20 km from Lisbon (Mota *et al*. 1999) (Figure 1.1). The origin of this introduction is still unknown, although some studies indicate a direct origin from the Far East (Vieira *et al*. 2007). Early studies carried out showed that the only vector in Portugal was *Monochamus galloprovincialis* (Sousa *et al*. 2001) (Figure 1.2), a species that is also widely distributed through Europe and Northern Africa. It was initially aimed to restrict the spread of the disease within an area of 30 km radius from Setúbal. During the first years, the disease was contained within this demarcated area although actions to eradicate did not succeed. In 2006 the Portuguese authorities announced a new strategy to contain the disease that included the opening of a 3 km wide strip around the affected area where all *P. pinaster* trees were eliminated (Mota, 2008). In the spring of 2008, the disease situation in Portugal changed dramatically with the detection of new foci far away from the demarcated area what lead to

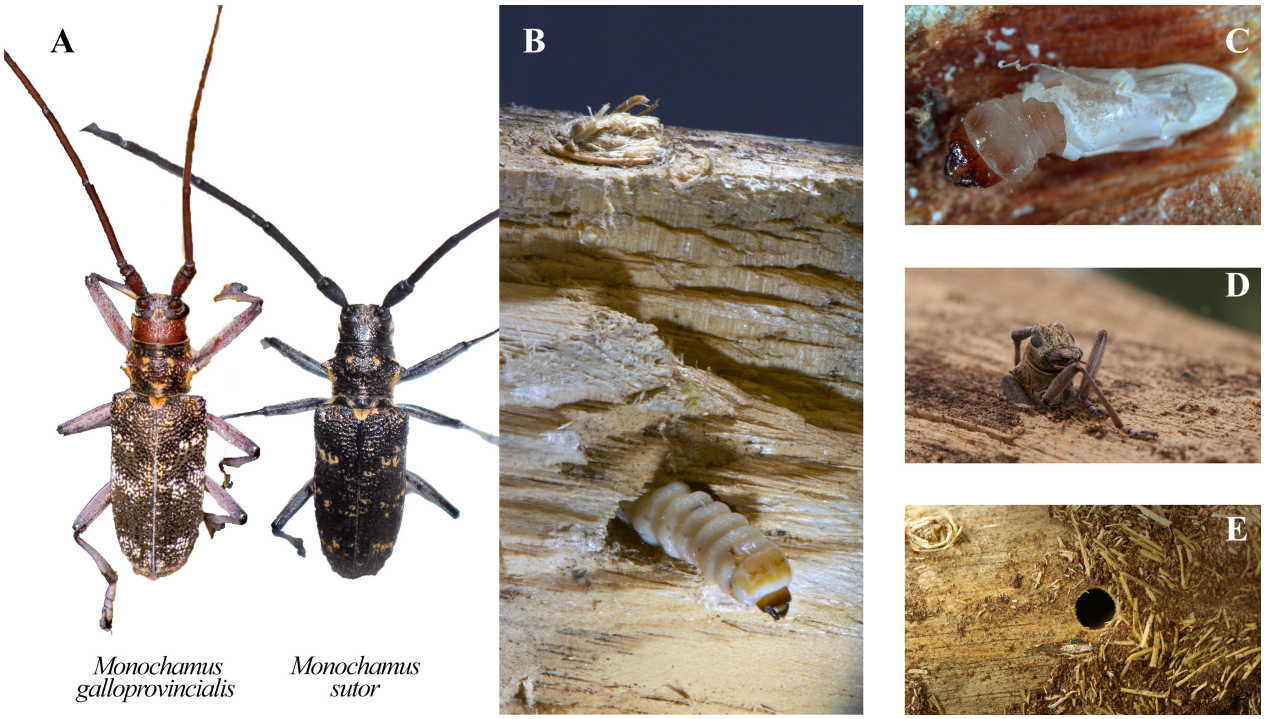


**Figure 1.2.** *Monochamus galloprovincialis* (Olivier) (A left) and *M. sutor* (Linnaeus) (A right). *M. galloprovincialis* larval gallery with entrance plugged (B). *M. galloprovincialis* 1st larvae (C). *M. galloprovincialis* adult emerging from a log (D). Exit hole after adult emergence (E).

declare all the country as demarcated area. The same year, PWN crossed into Spain for the first time (Zamora *et al*., 2015). Athough this infection was further eradicated, three new infestations of PWD have been later detected in Spain to which strong measures are being applied for eradication (Evans *et al*., 1996). As in Portugal, the introduction of the nematode in Spain put its pine forests at serious risk. At least three of the native pine species, *P. pinaster, P. nigra and P. sylvestris*, occurring in this country are very susceptible to PWN. Furthermore, warm Mediterranean weather prevalent on most of the territory provide the conditions required for the development of the disease (Rutherford and Webster 1987; Rutherford *et al*., 1990).

After these episodes, the risk of PWD further spreading to other European countries is obvious. *Monochamus saltuarius* and *M. galloprovincialis* are known vectors of *B. xylophilus* which are present in Europe (Schröder *et al*, 2009) (Figure 1.3). In addition, three other *Monochamus* species, *M. sutor, M. sartor* and *M. urussovi*, occur in Europe and it has been hypothesized that they would likely be capable of transmitting *B. xylophilus* if the nematode ever became associated with them (Evans *et al*., 1996). Two of these

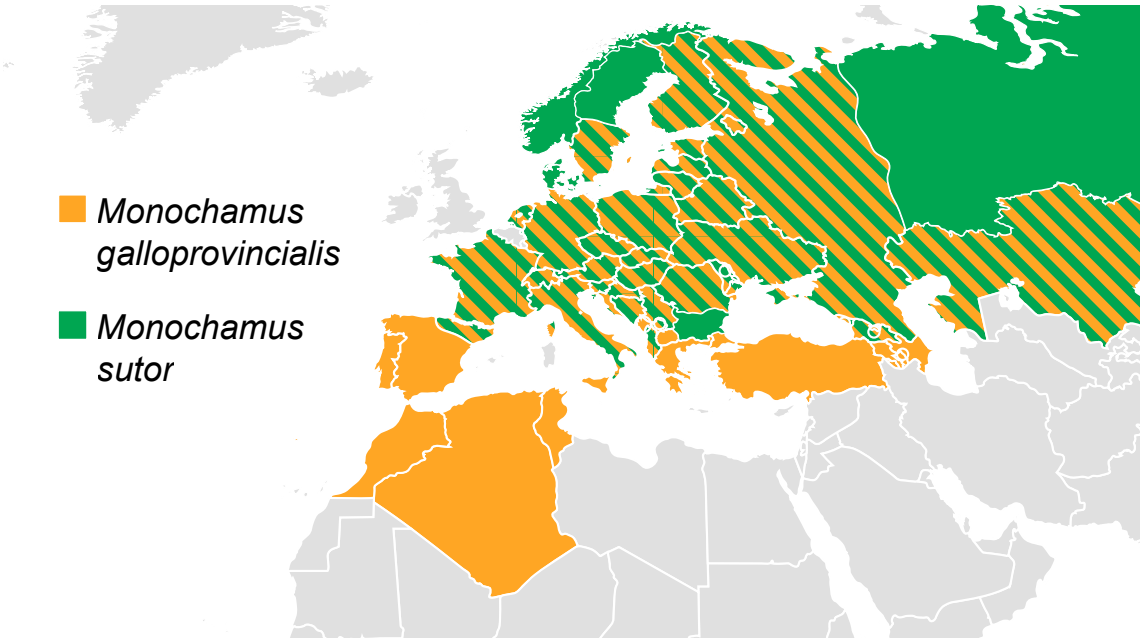


**Figure 1.3.** European and North African range of *M. galloprovincialis* and *M. sutor.* This figure is based on the Plantwise Knowledge Bank pests distribution maps (CABI, 2015).

species, *M. galloprovincialis* and *M. sutor*, are present in Spain. The former occurs all over the country, except in pure Stone pine stands, being abundant mils *P. pinatser* and *P. halepensis* stands. On the contrary, occurrence of the latter, widely distributed over Northern Europe, is restricted high elevation Black pine *P. uncinatta* stands in the Pyrennés mountains. Since in the lower limit ot these areas both species are sympatric, it opens the possibility that *M. sutor* gets in contact with the nematode that *M. galloprovincialis* might be already dispersing.

## The insect vector and PWD cycle

The genus *Monochamus* Dejean comprises about 130 species of long-horned beetles (Coleoptera: Cerambycidae) worldwide distributed. They are usually considered secondary forest pests that colonize conifers that are dead, dying or severely stressed by fire, drought or bark beetles, but they have becomed of first importance since it was determined that their life cycle and that of *B. xylophilus* were closely linked (Linit and Akbulut, 2008) (Figure 1.4). The cerambycid offspring develops in the phloem and xylem of stressed or recently dead trees, overwintering as larvae within the wood, to emerge as adults in late spring. Dauer larvae of the 3$^{st}$, a specialized stage of the nematode for survival under unfavourable conditions, occurring within these trees migrate into the pupation chambers of the beetle in spring and summer.

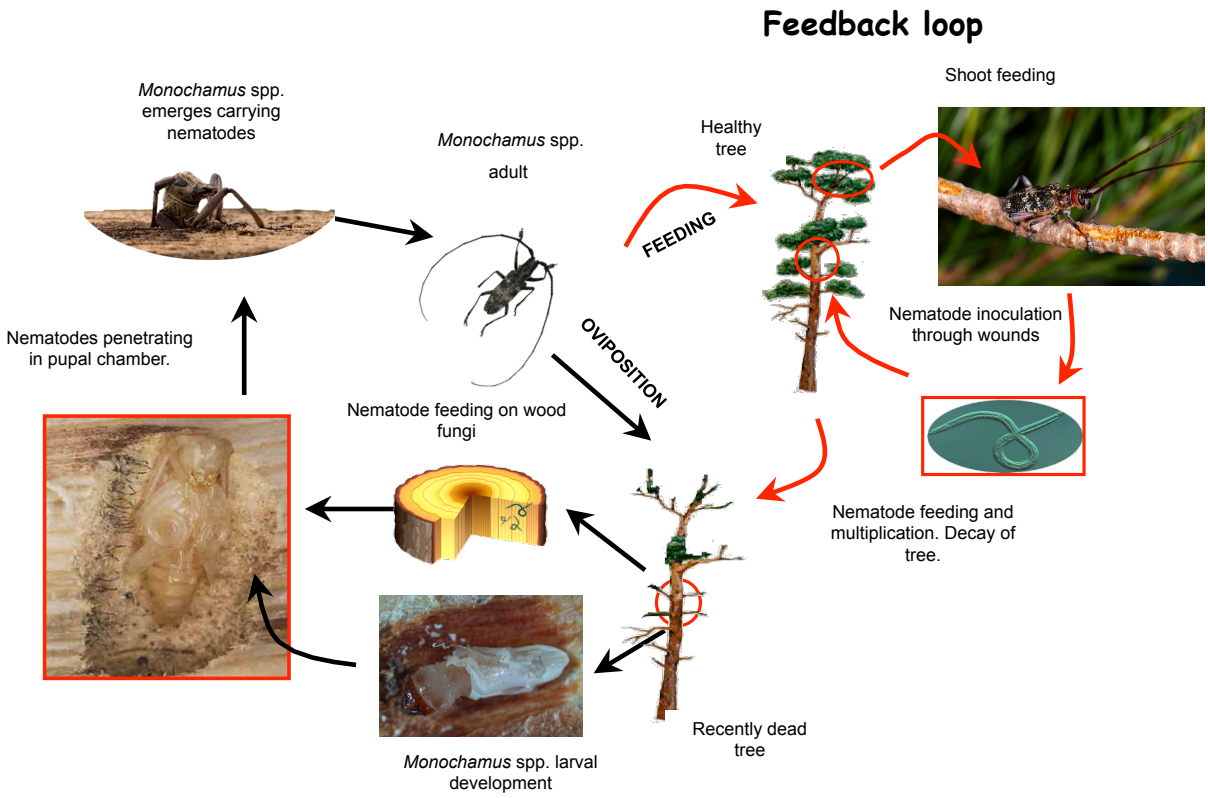


**Figure 1.4.** Pine Wilt Disease infection cycle.

At this point, they moult to 4st dauer larvae, a dispersal-specific stage and enter the adult insect respiratory tract before it emerges. Fresly emerged *Monochamus* adults are sexually immature and need to feed on the fresh phloem of pine shoots of healthy trees during 10-14 days for sexual maturation. Once sexyually mature and after mating, females will look for a suitable host -damaged tree- to oviposit (Togashi, 2008). *B. xylophilus* may be transmitted from male to female beetles and vice-versa during copulation (Arakawa and Togashi, 2002; Lai, 2008). Transported dauer larvae leave the beetles and enter the tree through the feeding wounds (healthy trees) or through the oviposition wounds (dead or dying trees) (Wingfield, 1987). Since shoot feeding for maintenance and oviposition are carried out during all the adult lifespan, nematode transmission may occur during all the time adults are present.

Once nematodes are inside the tree, they rapidly reproduce feeding on the parenchyma cells of resin canals and blue stain fungi leading to sudden decay of the tree (Maehara, 2008). Transmission of *B. xylophilus* during shoot feeding will cause the

development of pine wilt disease in susceptible healthy trees. The trees used by the beetles for oviposition, on the other hand, are already dying or dead from several causes, which may include pine wilt disease (Schröder *et al*, 2009), thus transmission will produce in this case a feedback loop between the nematode and the insect vector leading to the rapid spread of disease.

## Semiochemical management of *Monochamus* beetles

Since PWN was introduced in Asian countries, direct attempts to control the nematode showed unfeasible in large areas. It was then considered that acting on the was on eof the best strategies to deal with PWD. Given the concerns about the convenience of chemical spraying used in those countries, great interest arose in developing semiochemical baited traps as an environmentally friendly tool for monitoring and managing populations of the insect vector.

The new situation in Europe after PWN introduction in Portugal and Spain made surveys for detection of *B. xylophilus* necessary throughout all the continent . An effective trapping system would provide valuable information not only on the insect vector, but also about the nematodes being carried. Since nematodes leave dead insetcs, captured insects must remain alive in the trap if we are to obtain this valuable information. This represents a new target for which traps have not been designed so far. Cross-vane and multiple-funnel traps are used to survey cerambycids (Graham *et al*. 2012) and some studies had reported them also as the most suitable for pine sawyer species (De Groot and Nott 2001, 2003; McIntosh *et al*. 2001; Allison *et al*. 2011; Miller and Crowe 2011). Silhouette of traps had been reported as an important factor when capturing Cerambycids (Morewood *et al*. 2002; Miller *et al*. 2013), so visual cues associated with black traps appeared to be important, but contradictory results have been reported (Pajares *et al*. 2004). In addition, large insects had been reported able to escape even from collectors provided with insecticide (De Groot and Nott 2003; Sweeney *et al*. 2006; Miller and Duerr 2008) and *Monochamus* beetles were also suspected of doing so. Answers to these questions are needed and a evaluation of efficiency and improvement of different trap designs in capturing and keeping alive *Monochamus* adults should be undertaken to provide the efficient tool that PWD management requires.

Early work on effective lures for trapping *M. alternatus* started in Japan. This species was reported to be attracted to volatiles such as α-pinene, β-pinene and ethanol, emmited by damaged trees that were used as kairomonal signals by the insect to locate their hosts (Shimazu, 2008). These early findings led to the development in Japan of the first attractive, terpene based, lure for sampling populations, "Madara-Call" (Sankei Chemical Co. Ltd.), which is still in the market (Shimazu, 2008). Knowledge in this field took a step further when Billings and Cameron (1984) first reported the response of the North American species *M. titillator* to pheromone components emitted by bark beetle species. This observation was later extended to other *Monochamus* species: Allison *et al* (2001) showed that North American *M. clamator, M. notatus*, *M. obtusus*, and *M. scutellatus* were particularly attracted to ipsenol, a pheromone emitted by bark beetles of genus *Ips*. Thus, pine sawyers are able to use pheromones of these insects as kairomones, which was hypothesized to benefit them at finding weakened hosts or even at nourishment improvement by predation of bark beetle larvae (Dodds *et al*., 2001; Allison *et al*., 2003). Pajares *et al*. (2004,) demonstrated that *M. galloprovincialis* was also attracted to several pheromonal compounds of *Ips* spp. Three years later, Ibeas *et al* (2007) proposed a standard kairomonal bait useful in the management of insect populations composed of a combination of ipsenol and 2-methyl-3-buten-2-ol, two bark beetles compounds ,and the pine-host terpene, α-pinene.

After studying the reproductive behavior of *Monochamus alternatus*, Fauziah *et al* (1987) and Kim *et al*. (1992) suggested the existence of a male released sex pheromone used by females to find their partners, but no progress was made in identification. A very remarkable advance in *Monochamus* chemical ecology was produced when this field was made when 2-undecyloxy-1-ethanol was reported as the male produced aggregation pheromone of *M. galloprovincialis* (Pajares *et al*. 2010). This first described *Monochamus* pheromone was later shown to be produced by other species: *M. alternatus* (Teale *et al*., 2011), *Monochamus carolinensis* (Olivier) and *Monochamus titillator* (Fabricius) (Allison *et al*., 2012), *Monochamus scutellatus* (Say) (Fierke *et al*., 2013), and *M. sutor* (Pajares *et al*; 2013). Field experiments found that 2-undecyloxy-1-ethanol was powerfully synergized by the previously described kairomone blend of α-pinene, ipsenol and 2-methyl-3-buten-2-ol allowing high numbers of captures of both sexes of *M. galloprovincialis* (Pajares *et al*., 2010). This achievement led to the development of a commercial lure that soon started to be used in eradication campaigns in Spain.

Previous EAG bioassays had shown that the antenna of this insect was sensitive to these compounds as well as to smoke volatiles from burnt wood, which is consistent with the observation of this species colonizing fire-damaged forest stands. Despite this progress, antennal morphology and physiology at the level of sensillae have not been studied in *M. galloprovincialis*. Such studies are also rare in other cerambycids although Dyer and Seabrook (1975, 1978) described the sensillae of American *M. notatus* and *M. scutellatus*. An approach to *M. galloprovincialis* antenna at this level would provide information about type of sensillae, their distribution along the antenna, their type of response and the degree of specificity to the compounds to which *M. galloprovincialis* is sensitive, as well as possible differences between sexes. Furthermore, this information will be useful for further testing of other more specific attractive compounds. To this respect, α-pinene is a host terpene commonly used by a wide range of forest insects, not only secondary xylophagous looking for available hosts but also their natural predators. Thus, a large number of non-target insects that can play an important role in regulating bark beetle populations (Reeve, 1997; Turchin *et al*., 1991) are also trapped and killed in trapping programs (Schroeder and Weslien, 1994; Erbilgin and Raffa, 2000; Etxebeste *et al*., 2012; Hofstetter *et al*., 2012; Miller *et al*,. 2013, Macias-Samano *et al*., 2014). Further improvement of attractiveness and specificity of the standard lure for *M. galloprovincialis* and development of effective lures for *M. sutor* are thus needed for a sustainable management of PWD in Europe.

As an alternative to the use of chemical insecticides, biological control of the insect vector was approached in the Asian countries. Promising results were obtained by parasitic insects *Dastarcus helophoroides* (Coleoptera: Bothrideridae) and *Scleroderma guani* (Hymenoptera: Bethylidae) and nematodes *Contortylenchus genitalicola* and *Steinernema carpocapsae,* (Shimazu, 2008) but practical application of these agents was not developed. Entomopathogenic fungi have also great potential for biological control of insects. Highly virulent *Beauveria bassiana* strains have been isolated from *M. alternatus* in Asia (Shimazu, 2008; Shin *et al*., 2009) and in Europe Francardi *et al*. (2003) reported good results on *M. galloprovincialis* mortality by commercial *B.bassiana* strain. It has also been reported that entomopathogenic fungi are the most important mortality factors in pine sawyer populations in the area of Portugal affected by PWD (Naves, 2008). These results point to the possibility of finding native strains of entomopathogenic fungi virulent enough to kill *M. galloprovincialis* (Figure 1.5). However, use of these biological agents is greatly

constrained by the need of practical and operational methods of application at the forest scale. In this respect, semiochemically based devices offer a good opportunity for the

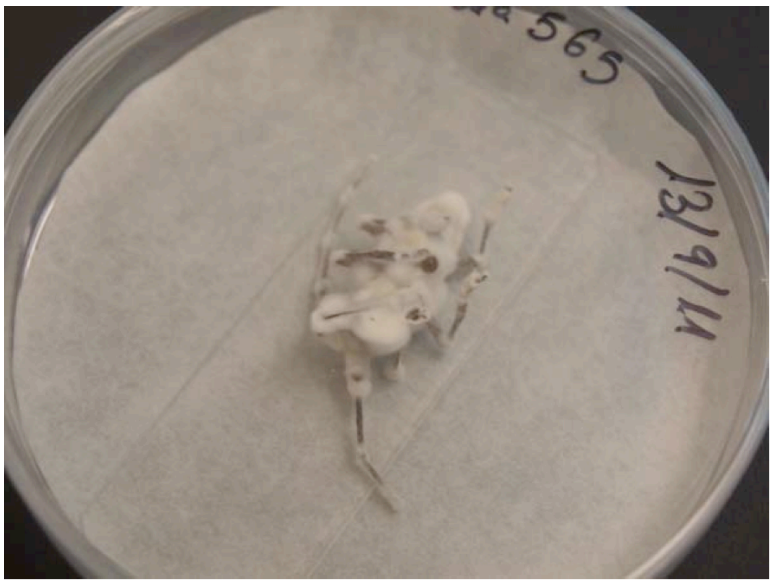

**Figure 1.5.** *M. galloprovincialis* adult infected by an entomopathogenic fungus.

autodissemination of these agents. Thus, improved baits and traps developed for *Monochamus* species may be used also in lure and infect tactics, extending semiochemical management to biological control. It is required though for this to be put in practice that transmission of the pathogen to other adults while mating (horizontal transmission) or to offspring through oviposition (vertical transmission) will occur when contaminated beetles leave the trap.

## Main questions

### *Traps for M. galloprovincialis live trapping (Chapter 2)*

- Can *M. galloprovincialis* escape from traps o avoid being captured?
- Can trap performance be improved at increasing catches and at keeping alive trapped adults?
- Which trap design is more suitable for catching *M. galloprovincialis*?
- What factors determine survival of the captured insects?

### *Olfactory receptor neurons in M. galloprovincialis (Chapter 3)*

- What types of distinguishable sensillae occur in *M. galloprovincialis* antenna?
- What kind of responses chemorreceptors produce? Are they specific to some compounds?
- Is there a correlation between field behaviour of *M. galloprovincialis* and sensillae responses?

### *Effective lures for trapping M. galloprovincialis (Chapter 4)*

- Can pheromone-kaiormone lure power be increased by augmenting pheromone dosage?
- Is α-pinene suitable enough or can it be replaced by other host terpenes?
- Are immature beetles *attracted to M. galloprovincialis* lures attractive to? Can they be attracted by a blend of host compounds?

### *Effective lures for trapping M. sutor (Chapter 5)*

- What host and bark beetle kairomones are suitable to increase pheromone effect in lures?
- Is α-pinene a worth terpene to be used in lures?
- Are smoke volatiles useful for effective attracting this species?

*Entomopathogenic fungi for control of M. galloprovincialis (Chapter 6)*

- Is there native strains of entomopathogenic fungi from *M galloprovincialis* virulent enough to be used in biological control?

- Can virulent strains be horizontally and vertically transmitted?

- What kind of conidial preparation and dosage are more suitable for this purpose?

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# Chapter 2

# Optimization of traps for live trapping of Pine Wood Nematode vector *Monochamus galloprovincialis*

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## Introduction

Increasing movement of people and goods around the globe has facilitated the invasion of insect, plant, and fungal pest species into new areas. Biological invasions into forest ecosystems have become a matter of prime importance due to the severe associated ecological and socioeconomic impacts. Early detection of invasive species is essential for rapid response programs during the establishment phase, prior to growth of infestation (Liebhold *et al*., 1995). Efficacy in capturing may be the difference between detection and non-detection, and the latter may result in wrong treatments applied or in higher associated costs for management of larger infested areas (Miller, 2008). Because resources for surveillance are often limited, maximizing trapping efficiency to increase the likelihood of detection is of major importance (Dodds, 2011). A particularly relevant case currently in Europe is the introduction of the Pine Wood Nematode (PWN) *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle, a pathogen that is vectored by sawyer beetles of the genus *Monochamus* Dejean (Coleoptera: Cerambycidae) (Linit and Akbulut, 2008) and is the causal agent of the Pine Wilt Disease (PWD) (Wingfield *et al*., 1982).

*B. xylophilus,* native to North America where it is regarded as a weak pathogen, causes a rapid wilting and eventually death of susceptible pines (Wingfield *et al*., 1982). Since its introduction into Japan at the beginning of the 20th century, the nematode has

spread over Japan, eastern China, Taiwan and Korea, causing serious economic and environmental losses (Shing, 2008; Zhao *et al*., 2008). Although legislation was issued to prevent the introduction of PWN in Europe (Evans *et al*., 1996), *B. xylophilus* was reported for first time in Portugal in 1999 (Mota *et al*., 1999), causing great concern in Europe. Despite efforts in Portugal to control and eradicate the pest (Rodrigues, 2008), PWD eventually spread over most of the country, devastating large pine stands (Daub, 2008), and crossed into Spain for the first time in 2008 (Zamora *et al*, 2015). Since then, three new infestations of PWD have been detected in Spain to which strong measures are being applied for eradication.

Since PWN was introduced, *Monochamus galloprovincialis* (Olivier) has been reported as its only vector in Europe (Sousa *et al*., 2001). Distributed over this continent and North Africa, *M. galloprovincialis* is a large woodborer colonizing mainly pine, but also fir and spruce trees*,* that are severely stressed, such as by drought or attack by other organisms, especially bark beetles and nematodes (Vives, 2000). Adults feed during all their life on healthy pine twigs for sexual maturation and maintenance, inoculating the trees with *B. xylophilus* (Mamiya and Enda, 1972)*.* Infested trees later become a suitable substrate for *M. galloprovincialis* females to lay eggs, and nematodes also may be inoculated into the dying trees during oviposition. Nematodes aggregate within the pine sawyer pupal chambers and enter into the new adults to be transported to healthy trees after emergence.

Management of the insect vector is a sound strategy to deal with PWD. Soon after PWN detection in Portugal, attempts were made to lure beetles using host and bark beetle kairomones (Pajares *et al*., 2004; Bonifacio *et al*., 2012). Recently, significant progress on understanding *M. galloprovincialis* chemical ecology has been achieved, and very effective pheromone-kairomone lures have been developed for attracting the pine sawyer (Ibeas *et al*., 2007; Pajares *et al*., 2010). These lures in combination with efficient traps may represent the powerful tool that PWD management demands. An efficient trapping system will provide key information not only for vector monitoring but also about the nematodes being carried and may serve also for direct control of the pine sawyer population. Multiple-funnel traps (Lindgren, 1983) and cross-vane traps are often used to capture large cerambycids (Graham *et al*., 2012). Previous studies have shown that these traps are better suited for pine sawyer trapping than other designs, such as panel traps, pan traps,

cylinders or pot traps (Allison *et al*., 2011; De Groot and Nott, 2001; De Groot and Nott, 2003; McIntosh *et al*., 2001; Miller and Crowe, 2011). The multiple-funnel trap, consisting of a series of black plastic funnels arranged vertically over a collection cup is light, easily storable and little affected by strong winds (McIntosh *et al*., 2001), although it offers more surfaces and edges for beetles to alight and cling and thus avoid being captured (Graham, *et al*., 2012). The cross-vane trap, consisting of cross wed plastic vanes suspended vertically over a large funnel and collection cup, is more sensitive to strong winds (McIntosh *et al*., 2001). It can be made of black or clear vanes, the former reported to be more effective (De Groot and Nott, 2001). Several studies have reported cross-vane traps as being more efficient than multiple-funnel traps in catching large insects, such as pine sawyers (Czokajlo *et al*., 2003; De Groot and Nott, 2003; Dodds *et al*., 2010; Graham, *et al*., 2012; McIntosh *et al*., 2001; Morewood *et al*., 2002), though significant differences between them were not found in other cases (Pajares *et al*., 2004).

Optimizing trap efficiency for monitoring both the sawyer and the pathogen vectored may involve three aspects: firstly, to maximize the number of attracted insects falling into the collecting cup. It is known that some large agile wood-borers such as *Monochamus* spp. often do not fall into the collecting cup after landing on the trap, favoured by the multiple edges of the multiple-funnel or by the roughness of the cross-vane traps (De Groot and Nott, 2003; McIntosh *et al*., 2001). In this respect, some attempts have been made to improve trap efficiency using slippery coating with Fluon or Rain-X (Allison *et al*., 2011; Czokajlo *et al*., 2003; De Groot and Nott, 2003; Graham and Poland, 2012; Sweeney *et al*., 2006), though not always successfully (Graham *et al*., 2010). On the other hand, larger collector funnels had negligible effect in preventing insects from falling out (De Groot and Nott, 2003; Morewood *et al*., 2002).

A second objective would be to minimize the number of trapped insects escaping from the collecting cup. It is known that some insects, as pine sawyers, easily escape from collecting cups (e.g. 30% losses estimated for *M. alternatus*; Nakamura, 2008). Up to now, preventing escape of insects has been achieved by killing them using insecticide strips or any kind of preservative liquid, (soapy water, propylene glycol, etc.). However, monitoring nematode loads on *Monochamus* adults requires that the insects stay alive (Schroeder, 2012). Thus, keeping trapped pine sawyers alive and preventing them from escaping at the same time is a key feature for an efficient trapping system. Escape of pine sawyers

may be reduced by extending the collector cup (Nakamura, 2008), but it also could be achieved by coating the inner collector surface with slippery substances as described above for maximizing catches.

Finally, maximizing the number of captured insects that remain alive within the collecting cup would be also important for nematode load checking. In this respect, survival of insects should be inversely related to a) the number of insects caught, which favours aggressive behaviour among confined *M. galloprovincialis* males (pers. obs.), as happens in some other longhorn species (Breidbach, 1990; Wang and Zeng, 2004). b) extreme weather conditions during the season, particularly in warm areas as in southern Europe, and c) the length of time the insects stay within the cup, which will increase the effect of the above factors.

Aiming to provide an efficient tool for PWD management, we present here the results of several experiments conducted in three European countries to evaluate the efficiency of several trap designs in capturing and keeping alive *M. galloprovincialis* adults. In addition, factors influencing survival of captured insects within the trap are analysed.

## Material and Methods

Five trapping experiments evaluating 12 different trap designs (Figure 2.1), baited with the same *M. galloprovincialis* pheromone-kairomone lure Galloprotect 2D® (SEDQ; Barcelona, Spain), were conducted in Spain, France and Portugal from 2010 to 2012 (Table 2.1). All three experiments in Spain were set up in Sierra Espuña (Murcia) Natural Park, South-east Spain (37º 57′ N, 1º 24′ W). The site consisted of a *Pinus halepensis* mature forest planted approximately 110 years ago. The experiment in France was carried out in a *Pinus pinaster* forest plantation located at Marcheprime, France (44° 74’ N, 0° 89’ W). Finally, a field assay was deployed in Herdade da Comporta, Setúbal, Portugal (38° 21’ N, 8° 45’ W) within an open *P. pinaster* pine stand where high mortality by Pine Wilt Disease had occurred during the last ten years.

Experiment 1, conducted from 5 June to 16 September 2010 in Spain, was aimed to evaluate the effect of a slippery coating on trap catches and on retaining live insects within

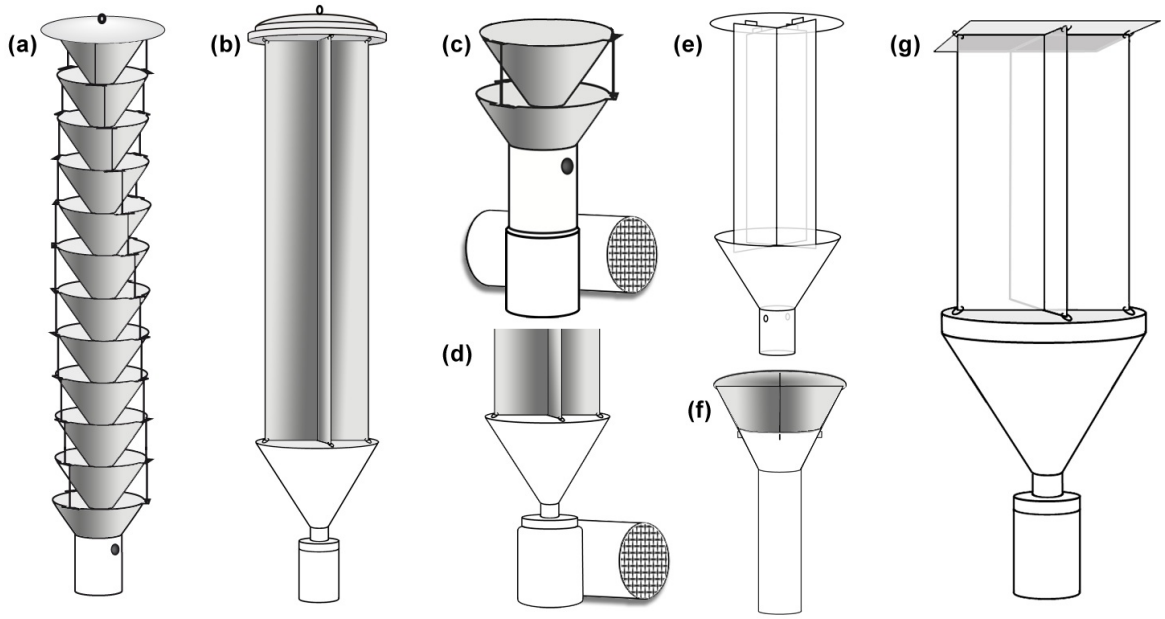


**Figure 2.1.** Trap designs tested in experiments: *Multiple Funnel* (a), *Cross Vane* (b), extended cup in *Multiple Funnel Teflon Trap* (c), extended cup in *Cross Vane Teflon Trap* (d), *Polytrap*® (e), *MasTrap L*® (f), *Cross Vane Transparent* (g).

**Table 2.1.** Name, figure label and manufacturer of trap designs and year/country of the experiments where they were tested

| Name | Label | Manufacturer | Year / Country |
|---|---|---|---|
| Multiple Funnel Fluon Cup Screen Bottom | MFunnel_FCup_SBott | Hand modified on Econex (Murcia, Spain) | 2010 / Spain |
| Multiple Funnel Fluon Trap | MFunnel_FTrap | Hand modified on Econex (Murcia, Spain) | 2010 / Spain |
| Multiple Funnel Fluon Cup | MFunnel_FCup | Hand modified on Econex (Murcia, Spain) | 2010 / Spain |
| Multiple Funnel | MFunnel | Econex (Murcia, Spain) | 2010 / Spain |
| Cross Vane DDVP | CVane_DDVP | Econex (Murcia, Spain) | 2010 / Spain |
| Multiple Funnel DDVP | MFunnel_DDVP | Econex (Murcia, Spain), Contech (Loc,Canada) | 2010 / Spain; 2011 / France, Portugal, Spain |
| Cross Vane Teflon Trap Extendend Cup | CVane_TTrap_ECup | Econex (Murcia, Spain) | 2011 / France, Portugal, Spain; 2012/ Spain |
| Multiple Funnel Teflon Trap Extended Cup | MFunnel_TTrap_ECup | Econex (Murcia, Spain) | 2011, 2012 / Spain |
| Polytrap® DDVP | Polytrap_DDVP | Purpan Engineering School, (Toulouse, France) | 2011 / France, Spain, |
| MasTrap L® DDVP | Mastrap_DDVP | Biotop (Livron sur Drôme, France) | 2011 / France |
| Multiple Funnel Teflon Trap | MFunnel_TTrap | Econex (Murcia, Spain) | 2011 / Portugal |
| Cross Vane Transparent | CVane_Transparent | Hand made | 2011 / Portugal |

the trap. A Teflon coating of trap surfaces was obtained by hand applying a liquid solution of *Fluon*® (DuPont, Wilmington, Delaware, U.S.A). Forty two traps of six types were assayed in seven randomized complete blocks: black *cross-vane* and *multiple-funnel*, both provided with a small piece of 2,2-dichlorovinyl dimethyl phosphate insecticide (DDVP), standard *Multiple Funnel* (no insecticide), *Multiple Funnel Fluon Cup* (only the collector cup Teflon coated), *Multiple Funnel Fluon Cup Screen Bottom* (as before, but with the collector cup extended and also having the whole bottom wire screened) and *Multiple Funnel Fluon Trap* (the whole trap, including the cup, Teflon coated). These modifications were made on standard traps provided by Econex S. L. (Murcia, Spain). Traps were suspended from metal poles at 2 m height, spaced at least 100 m apart with at least 700 m between blocks, and sampled every 7-12 days.

The very promising results obtained by Teflon coating and screened bottom cups in this experiment led us to ask a commercial pheromone company (Econex SL; Murcia, Spain) to produce a trap prototype according to our specifications. Prototype traps so produced were *Multiple Funnel Teflon Trap with Extended Cup* and *Cross Vane Teflon Trap with Extended Cup.* Experiments 2, 3 and 4 were designed to test these traps.

Experiment 2 was conducted from 16 July to 30 August 2011 in Spain to evaluate the effect of Teflon coating and of the screened extended cup bottom in a completely manufactured trap. Twenty eight traps of four different designs were tested in seven randomized complete blocks spaced at least 700 m: *Multiple Funnel DDVP*, *Multiple Funnel Teflon Trap Extended Cup* (the whole trap was Teflon coated, the cup was elongated and its bottom wire screened), *Cross Vane Teflon Trap Extended Cup* (same improvements in a black cross vane trap, commercially branded as *Crosstrap*®) and *Polytrap*® (a transparent cross-vane trap) loaded with DDVP. Traps were placed in the same way as in 2010 and sampled every 7-9 days, with re-randomization after sampling.

Experiment 3 was conducted from 9 to 30 August 2011 in France to test the Teflon manufactured traps in a different environment. Four trap types were tested in twenty traps arranged in five randomized complete blocks: *Polytrap*® with DDVP, *MasTrap L*® (a small grey conical cross vane) with DDVP*, Multiple Funnel DDVP* and *Cross Vane Teflon Trap Extended Cup*. Traps were suspended from wood poles 2 m height. Distance between

traps was at least 100 m and nearest blocks were a minimum of 300 m apart. Traps were sampled twice per week with re-randomization after sampling.

Experiment 4, similarly aimed to test the efficacy of Teflon coating, evaluated sixteen traps of four types deployed in 4 randomized blocks spaced 100 m, from 2 August to 12 September 2011 in Portugal: *Multiple Funnel DDVP*, *Multiple Funnel Teflon Trap*, *Cross Vane Teflon Trap Extended Cup* and a hand-made *Cross Vane Transparent* trap. These were suspended from canopy pine branches at 6 m height and spaced 100 m apart, and sampled and rotated among positions weekly.

Finally, Experiment 5 was conducted from 8 August to 17 September 2012 in Spain to check for the durability of Teflon coating effect after a year of exposure. Three different traps were tested in seven randomized complete blocks spaced 700 m apart: one year *Aged Multiple Funnel Teflon Trap Extended Cup*, one year *Aged Cross Vane Teflon Trap Extended Cup* (*Crosstrap*®) and *New Multiple Funnel Teflon Trap Extended Cup*. Twenty one traps were placed as in Experiments 1 and 2 and sampled every 10 days.

Counts of captured insects were fitted against trap design and block factors to a Poisson or quasi-Poisson (in case of overdispersion) error distribution in a generalized linear model (GLM) (Crawley, 2007). Tukey's honestly significant difference test with Bonferroni adjustment to the value of $\alpha = 0.05$ was used for mean comparisons. To allow for comparisons of the effect of different trap designs on the number of catches across the different experiments, effect sizes on log transformed ratio of means for the number of catches (Hedges ***et al***., 1999), along with their 95% confidence interval (CI), were also calculated and represented in a forest plot, setting the *Multiple Funnel DDVP* trap as the control treatment.

Recording of the number of beetles alive at each sampling occasion in Experiments 1 and 2 in Spain allowed analysis of factors affecting beetle survival within the trap. Only data from non-DDVP Multiple Funnel traps, (standard, Fluon and Teflon coated) were considered, and data from a trapping experiment carried out simultaneously during 2011 in the same area using standard multiple-funnel traps were also included. Living versus dead catches were taken as response variables, because in this way error and variance distributions are not altered, response is not bounded and sample size information can be

used in the model (Crawley, 2007). Variables supposed to be related to survival at each sampling occasion were fitted in a binomial error distribution in a GLM model: number of beetles in the trap, date of capture (week) and elapsed days within the trap. In addition, seven climatic variables were considered: absolute and maximum temperatures, absolute and mean minimum relative humidities, mean precipitation and maximum radiation. Meteorological values for the experimental area were obtained from the Murcian Agrometeorological network (SIAM; http://siam.imida.es), AL51 weather station, located approximately 9 km SE of our site, but 120 m lower in elevation. GLM procedures were performed under the statistical programming environment and language R 2.11.1 (The R Development Core Team, 2010).

## Results

Significant differences in mean *M. galloprovincialis* catches were obtained in Experiment 1 (Spain, 2010) between the Fluon treated trap and the rest of treatments ($F = 8.58$, $P < 0.001$, $df = 5$; Figure 2.2a). Mean catches increased from the standard *Multiple Funnel* (75.6 ± 8.5 insects/trap), to *Multiple Funnel Fluon Cup* (85.6 ± 10.8 insect/trap), *Cross Vane DDVP* (113.4 ± 31.0 insects/trap), *Multiple Funnel DDVP* (123.9 ± 15.8 insects/trap), and *Multiple Funnel Fluon Cup Screen Bottom* (163.6 ± 16.0 insects/trap) traps, though differences were only significant between the least and the most effective of these trap designs. *Multiple Funnel Fluon Trap*, which was coated all over with Fluon, was significantly superior to all others, remarkably catching 280.6 ± 51.1 insects/trap. Insect survival within the trap was similar among all trap designs that did not incorporate insecticide, about a third of trapped beetles. These results clearly showed that the Teflon coating improved trap efficiency, both highly increasing trap catches and preventing live beetles from escaping the collector cup. Traps with Teflon-treated cups that were longer and had the bottoms wire screened (*Multiple Funnel Fluon Cup Screen Bottom)* almost doubled the catches, though not significantly, compared to those that had not (*Multiple Funnel Fluon Cup*), suggesting benefits of this cup modification. The above results led to the production of two commercial trap prototypes incorporating the Teflon coating all over the trap plus the extended cup with screened bottom, which were tested in the following year.

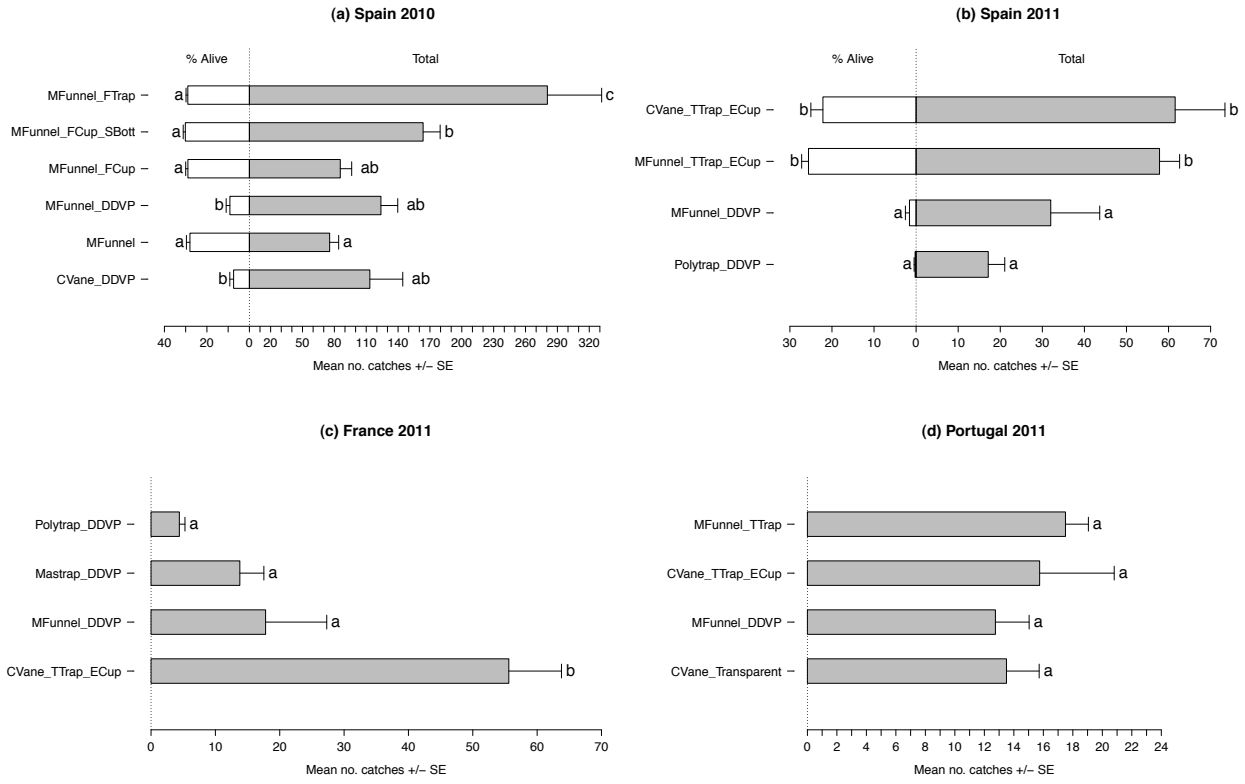


**Figure 2.2.** Mean catches of *M. galloprovincialis* ± SE and percentage of living insects in the experiments carried out in Spain (a, b), France (c) and Portugal (d) for the different trap designs (see Table 2.1 for trap labels). Bars with the same letter are not significantly different (Tukey's honestly significant difference test with Bonferroni adjustment, $\alpha = 0.05$).

Results of Experiment 2 (Spain, 2011) confirmed the higher efficiency of the new prototypes over the other trap designs. Thus, *Multiple Funnel Teflon Trap Extended Cup* and *Cross Vane Teflon Trap Extended Cup* similarly resulted in significantly higher beetle catches (61.6 ± 11.8 and 57.9 ± 4.8 insects/trap respectively) than the standard *Multiple Funnel DDVP* and the *Polytrap*® designs (32.0 ± 11.6 and 17.1 ± 3.9 insects/trap respectively) ($F = 33.35$, $P < 0.001$, $df = 3$; Figure 2.2b). Survival of trapped insects within the extended Teflon-treated cups was similar in both prototypes and close to the values obtained the previous year by the Fluon-treated cups.

Results from Experiment 3 (France, 2011) confirmed the significantly better performance of the cross-vane prototype, the only prototype tested, over the commercial standard trap models ($P < 0.001$, $df = 3$, $F = 14.39$; Figure 2.2c). Catches in the *Cross Vane Teflon Trap Extended Cup* (55.6 ± 8.2 insects/trap) were markedly higher than catches in the *Multiple Funnel DDVP* (17.8 ± 9.5 insects/trap), the *MasTrap L*® (13.8 ± 3.7 insects/trap) or the *Polytrap*® (4.4 ± 0.9 insects/trap) designs.

Results from Experiment 4 (Portugal, 2011) were not so clear cut. Even though both Teflon-treated prototypes obtained higher mean catches than the other two models tested, differences were not significant (F = 0.54, P = 0.67, df = 3; Figure 2.2d). Compared with the other experiments, catches were lower in both the Teflon-treated trap models, *Multiple Funnel Teflon Trap* (17.5 ± 1.6 insects/trap) and *Cross Vane Teflon Trap Extendend Cup* (15.8 ± 5.1 insects/trap), and in the non-Teflon-treated designs, *Multiple Funnel DDVP* (12.8 ± 2.3 insects/trap) and *Cross Vane Transparent* (13.5 ± 2.2 insects/trap).

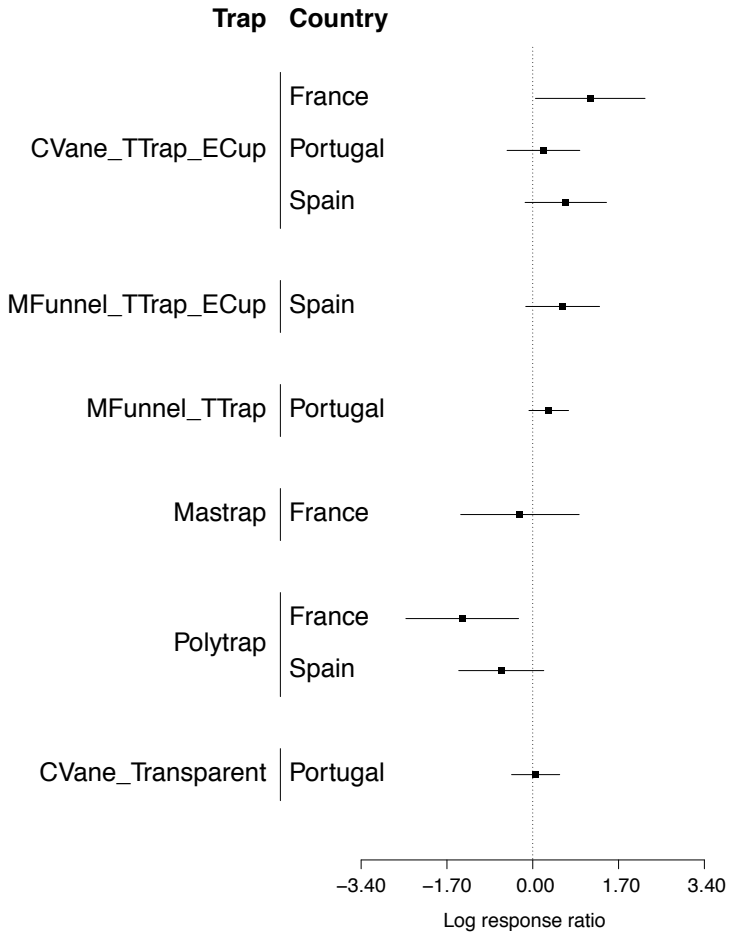


**Figure 2.3.** Forest plot of effect sizes based on log response ratio of means for the number of catches across different experiments and countries along with their 95% confidence interval (CI) widths. Multiple-Funnel DDVP trap has been set as the reference treatment. Confidence interval widths overlapping ratio = 0 do not represent a significant improvement in number of catches relative to the reference treatment.

Experiment 5 (Spain, 2012) showed that the Teflon coating was effective for at least two consecutive years. No differences were found between catches obtained in the one year *Aged Multiple Funnel Teflon Trap Extended Cup* (7.0 ± 1.3 insects/trap) and *Aged Cross Vane Teflon Trap Extended Cup* (6.3 ± 1.9 insects/trap) compared to *New Multiple Funnel Teflon Trap Extended Cup* (5.7 ± 1.8 insects/trap) traps (F = 0.44, P = 0.65, df = 2).

Overall results of seven trap designs that were tested in 2011 are summarised in the forest plot in Figure 2.3. Teflon-treated models with extended and wire screened cups scored large effect sizes (>0.8), with their lower tails of estimated 95% CIs away or slightly overlapping the zero value. Besides detected improvement effect of *Cross Vane Teflon Trap Extended Cup* was consistent across experimental sites. Even if these designs have their 95% CI of *log transformed ratio of means* overlapping several of the other tested traps, they could be regarded as the most suitable for live trapping *M. galloprovincialis* among tested models.

A model for insect survival within the trap was fitted. Six significant univariate GLM were fitted for survival of captured beetles: number of beetles in the trap (P = 0.0351), date of capture (week in the year) (P = 0.0037), elapsed time between samplings (P = 3.97e-13), absolute minimum relative humidity (P = 0.02), mean relative humidity (P = 4.03e-5) and maximum radiation (P = 3.25e-8). The best multivariate GLM obtained using uncorrelated predictors (<0.7 Pearson correlation index) reduced the deviance of the null model 12.1% and included just the three most significant variables, resulting in the form:

$$Survival\_rate = \frac{e^a}{1+e^a}$$

Where a = -5.88 -0.15·D + 0.044· MRH + 0.003·MR; D: number of elapsed days between samplings (P = 3.06e-11); MRH: mean relative humidity (P = 2.24e-9) and MR: maximum radiation during the period that insects remained in the trap (P = 3.81e-11).

## Discussion

Results obtained in 2010 suggested that a sizable proportion of insects captured in the standard *Multiple Funnel* traps escaped from collector cups, in accordance with previous reports on trapping large and relatively agile insects (De Groot and Nott, 2003; Graham and Poland, 2012; Miller, 2008; Sweeney *et al*., 2006). It has been shown that some of these insects are even able to escape from collectors with insecticide (De Groot and Nott, 2003; Miller, 2008; Sweeney *et al*., 2006). Although providing insecticide to collectors in these traps resulted in a 50% increase in *M. galloprovincialis* catches, this improvement was not significant. Applying Fluon to collector cups only produced a minor increase in catches, indicating that some individuals were able to climb the hand-applied Teflon-treated collector walls and leave the trap. It has been reported that hand application of Teflon may be less effective than expected, because detachment of Teflon flakes can occur after manipulation or insect attempts to escape (Graham and Poland, 2012). Nakamura (2008) reported that escape and mortality of pine sawyers were reduced in Japanese traps that had longer collector cups. Our *Multiple Funnel Fluon Cup Screen Bottom* was designed to prevent escape and increase survival of captured adults by improving thermal conditions in elongated collector cups. A completely wire screened bottom allows the air to circulate through it, cooling the collector cup, and captured insects seemed to remain quiet on the screen bottom most of the time, greatly reducing their activity and attempts to escape. A chimney effect for eluting semiochemicals in baited mutiple-funnel traps (Lindgren, 1983) might also be favoured by the screened bottom. Thus, either by reducing escape, by increasing the number of caught beetles, or by both, *Multiple Funnel Fluon Cup Screen Bottom* resulted in significantly higher pine sawyer catches (110%) than the standard model. Although not different in proportion, the number of captured insects sampled alive was higher than in the other cup treatments. It is known that landing adults easily grip the trap edges and may not fall into the cup (Allison *et al*., 2011). Applying Teflon to the whole multiple-funnel trap was thought to increase catches by minimizing this detrimental effect. Our results showed that Teflon coating significantly improved trap performance (275% increase over standard model), confirming that slippery surfaces are key not only to minimize escape of captured beetles but to maximize the number of those attracted that are eventually caught. Allison *et al*., (2011) had previously recorded significantly more *Monochamus* spp. beetles captured in both non-sticky silicon and in Teflon-treated traps, and De Groot and Nott, (2003) captured more *M. s. scutellatus*

in traps treated with Rain-X than in untreated controls. However, dry collectors were less effective than wet collectors, pointing to retention of captured insects as a problem.

Implementing these findings in completely manufactured traps and comparing these with other trap models in different forest environments was intended in 2011. The two manufactured prototypes, *Multiple Funnel Teflon Trap Extended Cup* and *Cross Vane Teflon Trap Extended Cup*, demonstrated a remarkable and significantly higher performance than the standard *Multiple Funnel DDVP* model (from 180% to 312% higher) when tested in Spain and France, confirming the results obtained in 2010. Results from Portugal showed also a higher, but not significant, performance by the Teflon-treated prototypes. In this case, lower catches (i.e., 0.32 insects/trap/day in the standard *Multiple Funnel DDVP* compared to 0.46 insects/trap/day and 0.85 insects/trap/day in Spain and France respectively) may have influenced results. Release of natural attractants from abundant dying pine trees due to PWN and bark beetle attacks in the experimental area may have competed with the synthetic lures. Even if there are contradictory reports that black cross-vane traps are more suitable than multiple-funnel traps for large woodborers due to a more prominent silhouette (Miller *et al*., 2013; Morewood *et al*., 2002), our results showed that both types of Teflon-treated traps with modified collectors caught a similar number of pine sawyers and held a similar proportion of them (28.9 – 30.2%) alive when sampled. Both types of traps retained their improved performance after one year of being exposed to real outdoor conditions, confirming that Teflon coating is not rapidly degraded by weather conditions (Graham *et al*., 2010) and it is effective at least one year after treatment. However, catches in this experiment were quite low, likely related to unusual weather conditions, so results should be confirmed. Long term durability of the slippery effect in Teflon-treated traps is so far unknown and should be determined because this is particularly important to prevent the beetles from escaping the collector cup.

Microscopic analysis of nematode load carried on vectors requires the insects to be alive, so obtaining live beetles when sampling the traps is key for nematode monitoring in PWD management programs. Even if Teflon-treated traps are able to hold a number of beetles suitable for such analysis, there are other factors that strongly influence survival of insects within the trap. Our model revealed that number of beetles in the trap, date of sampling, elapsed time between consecutive samplings, mean relative humidity and maximum radiation were the five most influential, especially the last three. High insolation

and dry weather, particularly during southern European summers, may represent insurmountable conditions for survival of captured insects. Lethal temperatures of 45-49 ºC have been recorded inside collecting cups on sunny summer days in Japan (Nakamura *et al*., 1999). The longer the insects are retained in the trap, the higher the number of insects that may accumulate and the longer the time that they are subjected to unsuitable weather conditions, so a rapid decrease in the number of surviving insects is to be expected. Thus, if managers wish to maximize the likelihood of having pine sawyers alive for nematode monitoring, trap exposure to the sun should be minimized and they should be sampled as frequently as possible.

In summary, our results show that Teflon coating of trap surfaces plus elongation and ventilation of collector cups strongly improves performance of multiple-funnel and cross-vane traps for catching pine sawyers by both maximizing trapping of attracted beetles and by reducing escape of living trapped beetles. Both trap designs are equally effective in capturing *M. galloprovincialis* and can provide a suitable sample of live insects for nematode monitoring if excessive sun exposure of the trap is avoided and a reasonable sampling schedule is implemented. These findings are currently incorporated into newly developed commercial traps (*Econex Multifunnel-12*® and *Crosstrap*®*;* Econex, Murcia, Spain*)* that proved highly effective in capturing the large and agile insect *M. galloprovincialis* when used in combination with the attractants developed for this species (Pajares *et al*., 2010). The development of this trapping system is rendering PWD vector population monitoring and even control through mass trapping feasible, representing a valuable new tool for control of this threatening disease.

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# Chapter 3

# Smoke, pheromone and kairomone olfactory receptor neurons in males and females of the pine sawyer *Monochamus galloprovincialis* (Olivier) (Coleoptera: Cerambycidae)

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## Introduction

Most longhorned beetle species are considered secondary forest pests, but those in the genus *Monochamus* Dejean (Coleoptera: Cerambycidae) have become economically important worldwide as they vector the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer) (Linit and Akbulut, 2008), which causes pine wilt disease (PWD) in vulnerable pine species (Wingfield, 1982). *B. xylophilus* is endemic to North America but, beginning early last century, PWD was accidentally introduced in several countries with devastating environmental and economic consequences (Shing, 2008; Zhao *et al*., 2008). In Europe, PWD was reported for the first time in Portugal (Mota *et al*., 1999). It spread over most of the country, despite efforts to control it (Rodrigues, 2008), and it was detected in Spain for the first time in 2008 (Zamora *et al*, 2015). Four infection foci have been declared in Spain since then, and measures for eradication (Evans *et al*., 1996) are currently being implemented.

One of the most promising strategies in PWD management in Europe is to act on *Monochamus galloprovincialis* (Olivier), the only known vector described for this disease in the continent (Sousa *et al*., 2001). In recent years, significant progress on the chemical

ecology of *M. galloprovincialis* has been made with the aim of developing more effective monitoring and management tools (Pajares *et al*., 2004; Ibeas *et al*., 2007; Pajares *et al*., 2010; Álvarez *et al*., 2014). The genus *Monochamus* responds to host tree volatiles such as α-pinene or ethanol, as well as to pheromone components of pine-scolytids, such as ipsenol, ipsdienol, *cis*-verbenol and 2-methyl-3-buten-2-ol (Billings and Cameron, 1984; Billings, 1985; Allison *et al*., 2001, 2003; De Groot and Nott, 2004; Miller and Asaro, 2005). Some of these compounds have been described as attractants of *M. galloprovincialis* in Europe (Pajares *et al*., 2004; Ibeas *et al*., 2007; 2008). In addition, male *M. galloprovincialis* release an aggregation pheromone (2-undecyloxy-1-ethanol) which attracts both males and females (Pajares *et al*., 2010). The potency of this pheromone increases with the addition of host and bark beetle kairomones (Pajares *et al*., 2010). The antenna of *M. galloprovincialis* responds to the aggregation pheromone and to bark beetle and host kairomones (Pajares *et al*., 2010). Furthermore, several volatiles of the smoke produced by burning wood elicited EAG responses in *M. galloprovincialis* male and female antenna and increased attraction to pheromone and kairomones when they were released together in field tests (in preparation). Up to now, morphological and physiological studies at the sensillum and olfactory receptor neuron (ORN) levels have not been carried out on *M. galloprovincialis*, and are rare in cerambycids (Dyer and Seabrook, 1978; Barata *et al*., 2002; Mitchell *et al*., 2012), although functional specificity in olfactory receptor neurons has been found in other beetles (Bengtsson *et al*, 2009; Andersson *et al*, 2009).

The present paper reports studies of the different types of sensillae and their distribution along the antenna of *M. galloprovincialis*, and records the response of the olfactory receptor neurons (ORNs) housed within to smoke and host-plant volatiles, pheromone components of pine scolytids and the aggregation pheromone of *M. galloprovincialis*, in order to gain a better understanding of the function and specificity of these receptors.

## Material and Methods

*Insects*

Logs containing *M. galloprovincialis* larvae were collected from fire-damaged trees in Valencia, Spain in the winter-spring of 2012 and were left in an outdoor cage until adult

emergence during the following summer. Adults were stored individually in 1-l glass jars under a 15L:9D photoperiod and 15:22ºC temperature regime, and were provided with fresh pine twigs for at least 2 weeks before being tested to ensure enough time to reach sexual maturity (Naves *et al*., 2006).

*Scanning electron microscopy*

Four antennae from each sex were cut and mounted on microscope holders with conductive double-side adhesive black tape. Preparations were air dried at 60ºC for 2 days, fixed with osmium tetroxide, dehydrated with acetone and coated using a sputter coater (Balzers SCD 050, Leica Microsystems, Madrid, Spain), with 50 nm gold particles for 3 minutes from a distance of 50 mm, with a current of 45 mA and argon as cooling gas. Samples were scanned using a Zeiss DSM940A microscope with 10 kV at 200X to 500X magnifications. A rough estimate of the relative distributions of different types of sensillae was obtained by counting the number of sensillae inside a 200 µm-side square placed over the proximal, medial and distal regions and in the lateral side of flagella 1, 3, 6 and distal. Length and basal widths of all types of sensillae contained in the sample area (*N* = 10 when feasible) were measured.

*Electrophysiological recordings*

Whole insects were immobilized and fixed on a microscope slide with parafilm and double-sided adhesive tape (Figure 3.1). Antennae were attached to the microscope slide using double-sided adhesive tape and immobilized with a strip of dental wax over the first flagellum and with several small pieces of tape throughout its length, but leaving proximal, medial and distal areas of each segment exposed. The slide was attached to a metal antivibration table (63–511, TMC Ametek, USA) with a magnet (4x2x1 cm). The electrodes consisted of electrolytically (20% $KNO_2$) sharpened tungsten microelectrodes (0.125 mm diameter, 99.98% purity, Advent Research Materials Ltd, England). The reference electrode was inserted into the first flagellum while the recording electrode was placed in the base of randomly chosen sensillae on the opposite antenna, with the help of a manual micromanipulator (NMN-25, Narishige, Japan) under a stereo-microscope (objective 2 x, oculars 25 x, zoom range 0.8-12.5, Leica Microsystems, Madrid, Spain). The signal was preamplified (PR-05, Syntech, Germany), filtered and digitized (low pass = 200 Hz, high pass = 3 KHz, sampling rate = 10.666 $s^{-1}$) (IDAC-4-USB, Syntech, Germany) and analyzed

in the computer (Autospike v.3.9, Syntech, Germany). The setup was shielded by a Faraday cage.

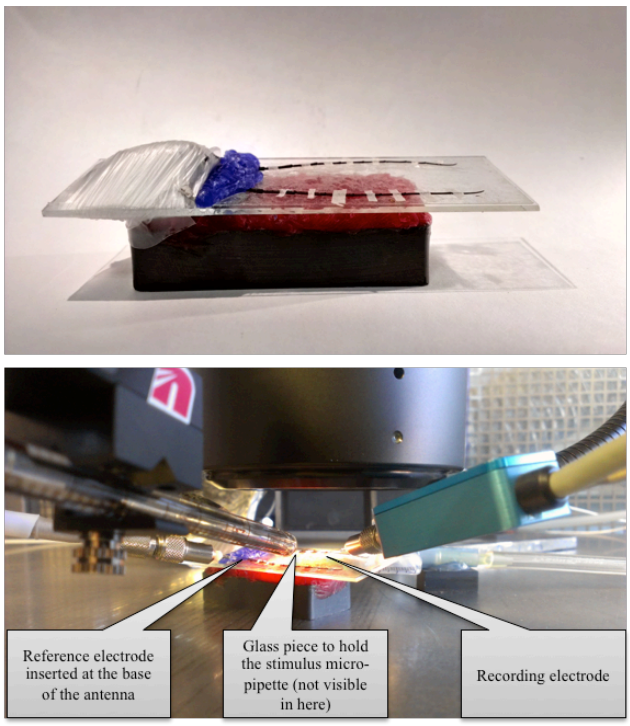


**Figure 3.1.** Insect preparation for SSR recordings. The body was immobilized on a microscope slide using paraffin plastic, the head was immobilized with dental wax and the antennae lay on double-sided sticky tape and is held with several strips of painter´s tape. The slide is fixed on a magnet using dental wax. After recordings, insects were returned to their rearing containers and resumed their normal behavior apparently unharmed.

*Stimuli and stimulation*

A total of 18 compounds (Table 3.1) were used in single sensillum recordings. These include kairomonal compounds (host-plant volatiles and scolytid beetle pheromone components) that attract *M. galloprovincialis* (Pajares *et al*., 2004) and the male-produced aggregation pheromone of *M. galloprovincialis* (Pajares *et al*., 2010). Volatile components of smoke released by burning wood (Schütz *et al*., 1999; Hall *et al*., 2006), were also tested since there is circumstantial evidence that *M. galloprovincialis* is attracted to burnt trees and that the addition of synthetic smoke volatiles to pheromone-kairomone lures increased attraction of this species (in preparation).

For ORN characterization compounds were tested at a 10 µg dose, and for dose-response curves they were tested at 1 ng to 10 µg doses, in decadic steps. Dilutions were maintained at -20 ºC until used. Stimulus cartridges were prepared by applying 1 µl of solution onto a 20 x 1 mm piece of filter paper (#1, Whatman International Ltd, England)

which was introduced into a 100 µl glass micropipette (1.2 mm internal diameter, Blaubrand® Intramark, Germany).

**Table 3.1:** Synthetic compounds used in the bioassays.

| Compound | Abbr. | BP (ºC) | Solvent | Purity | Source |
|---|---|---|---|---|---|
| *Aggregation pheromone* | | | | | |
| 2-undecyloxy-1-ethanol | PHER | 390 | n-hexane | 98% | NRI |
| *Host-plant volatiles* | | | | | |
| (+)-Limonene | LIMO | 176 | n-hexane | ≥93% | SEDQ |
| (+)-Camphene | CAM | 159 | n-hexane | ≥90% | SEDQ |
| p-Cymene | CYM | 177 | n-hexane | ≥97% | SEDQ |
| α−Pinene | APIN | 155 | n-hexane | ≥97% | SEDQ |
| β−Myrcene | MYR | 167 | n-hexane | ≥90% | SEDQ |
| β−Pinene | BPIN | 155 | n-hexane | ≥97% | SEDQ |
| 3-Carene | 3CAR | 168.5 | n-hexane | ≥90% | SEDQ |
| *Bark beetle pheromones* | | | | | |
| 2-Methyl-3-buten-2-ol | 2M3B | 98.5 | n-hexane | ≥98% | SEDQ |
| Cis-verbenol | CISV | 214.9 | $CH_2Cl_2$ | 97% | SEDQ |
| Ipsdienol | IPSD | 233.59 | n-hexane | 93% | SEDQ |
| Ipsenol | IPSE | 222.2 | n-hexane | 93% | SEDQ |
| *Smoke volatiles* | | | | | |
| 2-Methoxyphenol | 2M | 205 | $CH_2Cl_2$ | ≥98% | SA |
| 4-Methyl-2-methoxyphenol | 4M2M | 221.5 | $CH_2Cl_2$ | ≥98% | SA |
| 4-Vinyl-2-methoxyphenol | 4V2M | 224 | $CH_2Cl_2$ | ≥98% | SA |
| Eugenol (2-methoxy-4-allylphenol) | EUG | 254 | $CH_2Cl_2$ | ≥98% | SA |
| Iso eugenol (2-methoxy-4-propenylphenol) | IEUG | 266 | $CH_2Cl_2$ | 99% | SA |
| Vanillin (4-hydroxy-3-methoxybenzaldehyde) | VAN | 285 | $CH_2Cl_2$ | ≥97% | SA |

BP, boiling point. NRI, Natural Resources Institute, Chatham Maritime, Kent, UK. SEDQ, Sociedad Española de Desarrollos Químicos, Barcelona, Spain. SA, Sigma-Aldrich, Gillingham, Dorset, UK.

Both filter paper and micropipette were precleaned with *n*-hexane. Blank stimuli were prepared with 1 µl of the solvents used to make the dilutions, *n*-hexane and dichloromethane. Stimulus pipettes were prepared daily and kept in individual odor-clean glass tubes with teflon-lined screw caps. To avoid stimulus loss, the base and the tip of micropipettes were sealed with parafilm until puffed.

A stimulus controller unit (CS-55, Syntech, Germany) produced a constant charcoal-filtered and humidified room-air flow of 0.5 l/min at 10 mm from the antenna (velocity at exit = 0.4 m/s). The stimulus pipette was placed at 5 mm from the contact point of sensillum and electrode, and a puff of charcoal-filtered room air sent stimulus-loaded air from the pipette to the preparation for 0.2 s (velocity at exit = 2.9 m/s). During the puff the

continuous flow was stopped. The air around the preparation was constantly renewed with an exhaust to minimize contamination.

Every session started and finished with blank stimuli (*n*-hexane and $CH_2Cl_2$) and the other compounds were tested in a randomized sequence, preventing adaptation of receptors by leaving 60 s between puffs. In all, 31 and 28 sensillae from 5 males and 7 females respectively were stimulated. For dose-response curves a new set of 21 and 19 sensillae from 2 males and 3 females was used. First we identified neurons with high sensitivity to sex pheromone, *cis*-verbenol or one of two blends, each containing half of the remaining compounds. When a sensitive cell was identified, it was stimulated with the 5 doses of the blend compounds.

*Spike and statistical analyses*

When two spike amplitudes were detected in the same sensillum, they were considered as different ORNs. To calculate relative spike frequency, the number of action potentials (spikes) during 1 s immediately prior to stimulation was subtracted from the number of spikes during 1 s following stimulation. Hierarchical cluster analysis with Ward's minimum variance method was used to group cells according to their response pattern. We observed considerable difference in latency (i.e., the time elapsed between stimulation and the ORN response) among compounds, so we analysed this parameter for those compounds that produced clearly distinguishable excitations on a minimum of 7 ORNs. A general linear model (GLM) (Crawley, 2007) was fitted to compare latencies among compounds, and differences between means were tested for significance by Tukey's honestly significant difference test. Statistical analyses were performed under the statistical programming environment and language R, version 2.11.1 (R Core Team, 2012).

## Results

*Morphology*

*M. galloprovincialis* shows the typical antennal sexual dimorphism of the *Monochamus* genus, in which male antennae are almost twice as long, in relation to the body, than those of females. Antennae have two basal segments, scape and pedicel, and nine flagellomers which get progressively smaller towards the apical segment, which is

**Table 3.2.** Number of different sensillae types (mean ± SEM) sampled in 200 x 200 µm sections of segments 1, 3, 6 and distal, and in the scape and pedicel of *M. galloprovincialis* males and females

| | | Antennal segment | | | | | |
|---|---|---|---|---|---|---|---|
| | Sensillum type | Scape | Pedicel | 1 | 3 | 6 | Distal |
| Males | Stout chaetica | 10.00 ± NA | 13.75 ± 1.80 | 3.42 ± 1.15 | 9.92 ± 5.35 | 17.92 ± 6.22 | 24.44 ± 8.68 |
| | Male peg | 0.00 ± NA | 0.00 ± 0.00 | 9.75 ± 2.08 | 10.83 ± 2.21 | 8.67 ± 2.14 | 18.67 ± 3.13 |
| | Trichoidea | 0.00 ± NA | 0.00 ± 0.00 | 0.41 ± 0.19 | 0.83 ± 0.27 | 1.50 ± 0.31 | 3.11 ± 0.89 |
| | Basiconica | 0.00 ± NA | 0.00 ± 0.00 | 5.25 ± 4.57 | 14.50 ± 9.28 | 24.33 ± 10.19 | 0.00 ± 0.00 |
| Females | Stout chaetica | 9.50 ±1.71 | 25.50 ±2.53 | 27.58 ± 1.32 | 43.25 ± 3.62 | 59.92 ± 6.65 | 75.67 ± 11.01 |
| | Trichoidea | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.33 ± 0.14 | 1.08 ± 0.43 | 2.58 ± 0.82 | 2.92 ± 1.07 |
| | Basiconica | 0.00 ± 0.00 | 0.00 ± 0.00 | 2.17 ± 2.17 | 16.67 ± 9.83 | 35.25 ± 12.20 | 26.17 ± 8.34 |

longer than the preceding one. All the sensillae types described here have already been characterized by SEM and TEM in two other *Monochamus* species (Dyer and Seabrook, 1975), providing valuable comparative information about their possible function, which will be used in here. In both sexes, the most conspicuous sensillae on the antennae were the "stout sensillae chaetica" (*sensu* Dyer and Seabrook, 1975) (Figure 3.2A). As their name suggests, these are large and solid sensillae, 58.95 ± 1.5 µm long, and 6.93 ± 0.53 µm wide at the base (mean ± SEM, *N* = 10), and they increased in numbers towards the distal end of the antenna (Table 3.2). In males they became gradually thicker and laid flatter on the surface towards the dorsal side of the antennae (Figure 3.2A), receiving the name of "male peg sensillae chaetica" (Dyer and Seabrook, 1975). These were 40.98 ± 1.46 µm long and 15.15 ± 1.71 µm wide at the base (*N* = 5, only males).

On the latero-ventral surface of segments 2 to 8 there were two sensory fields, one in the proximal half consisting of two grooves in the cuticle (Figure 3.2B), and another located in the distal half and shaped as a plate area (Figure 3.2C and D). In the first segment only the distal sensory field was present. In both sexes these areas were carpeted with hundreds of small sensillae which, following Dyer and Seabrook (1975), are described as sensillae basiconica. Two subtypes were distinguished within these sensillae basiconica, one being more cylindrical (15.47 ± 1.16 µm in length and 2.87 ± 0.15 µm in diameter, *N* = 5), and the other one more flattened (12.04 ± 0.75 µm in length and 3.93 ± 0.24 µm in width, *N* = 10), with the first being relatively more abundant than the second (Figure 3.2E). A third type of sensillae, probably sensillae trichoidea according to the similarity of those described by Dyer and Seabrook (1975), was found in small numbers, increasing in abundance towards the distal end of the antenna (Table 3.1, Figure 3.2F). They presented longitudinal fluting and were 45.36 ± 1.7 µm in length and 4.23 ± 0.16 µm

in diameter (*N* = 10). Distribution and abundance of these sensillae types were not different between sexes (Table 3.2).

The distal end of each flagellomere was surrounded by a ring of large sensillae chaetica that overlapped with the following segment (Figure 3.2G). They were 189.87 ± 20.55 µm in length and 10.51 ± 0.40 µm in diameter near the base. Sensory cells in these sensillae fired action potentials in response to movement, so they probably act as proprioceptors that indicate antennal segment position. Long sensillae chaetica (Figure 3.2H) were found in so small numbers that they almost never fell in our sampling area, so their abundance is not reported. They were 158.35 ± 5.79 *µ*m in length and 5.02 ± 0.21 *µ*m in diameter (*N* = 4). Two dome-shaped organs were observed in our entire sensillar sampling, both of them inside the sensory fields, and they consisted of a small vault of about 2.5 µm in diameter (Figure 3.2I). Gland pores clustered around stout sensillae chaetica (Figure 3.2J) as described by Dyer and Seabrook (1975).

*Single sensillum recordings*

No action potentials were obtained from the abundant stout/male peg sensillae chaetica. However, electrophysiological contact with neurons within sensillae basiconica were relatively easy to achieve, so we focused our sampling on these sensillae. Fifty of the 60 sensillae (83%) contained a single ORN and the remaining 17% contained two ORNs that could be separated by their differences in spike amplitude (Figure 3.3a). A diversity of electrophysiological responses was obtained, including both excitations and inhibitions with maximum values of +97 and –20 spikes $s^{-1}$, respectively (Figure 3.4). Some cells could respond with excitation to one compound and with inhibition to another (e.g., females 2S and 25S, Figure 3.4). When more than one ORN was housed in the same sensillum they rarely showed similar responses to the odour panel (Figure 3.4).

Groups of distinct functional types of cells were obtained by hierarchical cluster analysis. Because the groups were similar in males and females (data not shown), we combined ORNs from both sexes in a single and larger dataset to gain more resolution. Six roughly uniform ORN types were obtained this way (Figures 3.4 and 3.5), and

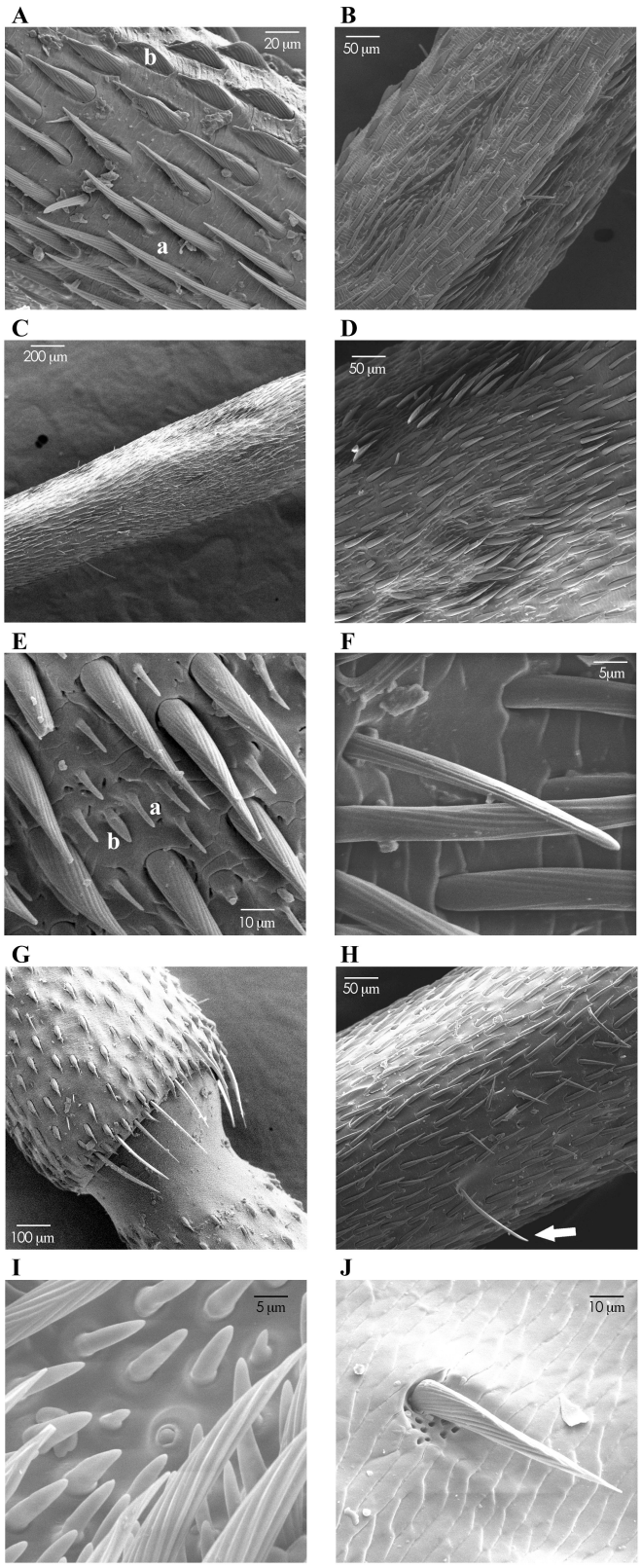


**Figure 3.2.** Scanning electron micrographs of *M. galloprovincialis* antennae. **(A)** Stout sensillae chaetica (a) and male peg sensillae chaetica (b) (male). **(B)** Groove-shaped sensory field located at the proximal end of the flagellum (male). **(C)** Plate area sensory field located at the distal end of the flagellum (female). **(D)** Detail of plate area sensory field showing the small sensillae basiconica (male). **(E)** Cylindrical (a) and flattened (b) sensillae basiconica. **(F)** Probably sensillae trichoidea (female). **(G)** Distal end sensillae chaetica; **(H)** Long sensillae chaetica (female); **(I)** Dome shaped organ (male); **(J)** Cluster of pores behind stout sensillae chaetica (male).

averaged responses of each type to all the test compounds are shown in Figure 3.6. The largest group of cells (13 male and 10 female, 33 % of the total) did not respond to any stimuli and so were labelled as "Unresponsive". The next large group (20 % of the ORNs, 3 male and 11 female) contained "Generalist" cells which showed heterogeneous, but

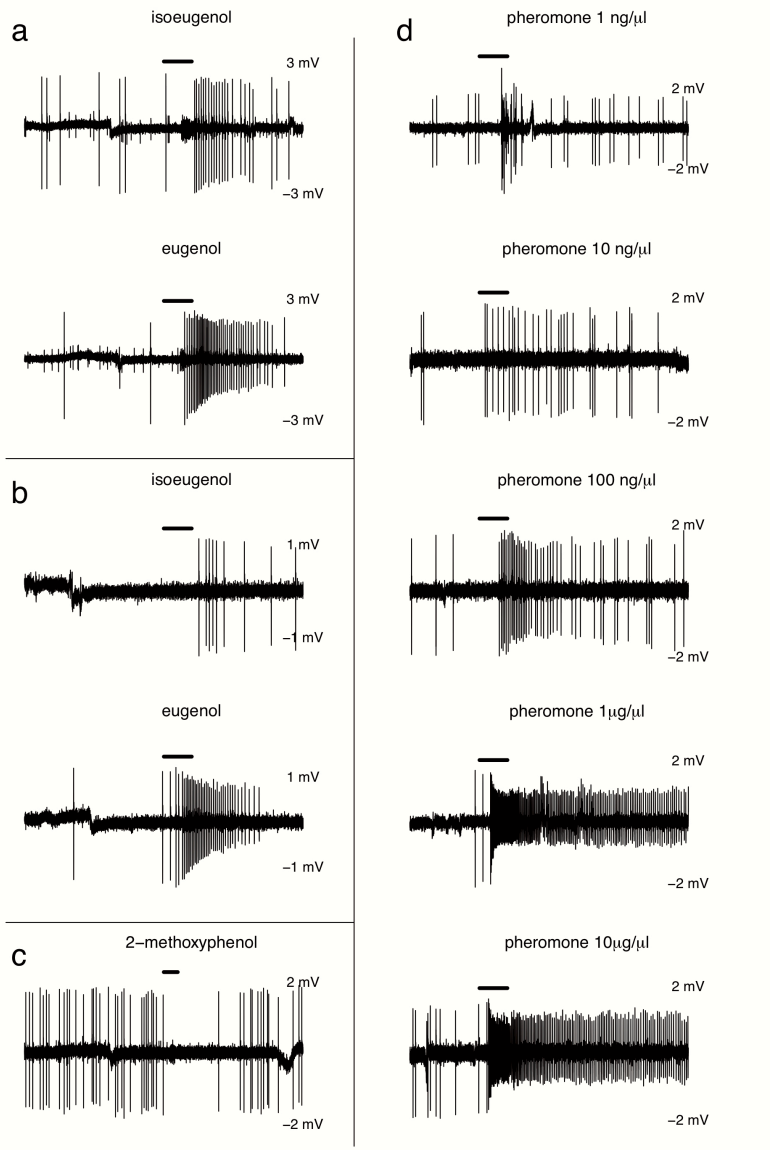


**Figure 3.3.** Single sensillum recordings from sensillae basiconica in the plate areas. (a) Two cells in the same sensillum of a female, distinguishable by their action potential amplitude (the large one is about 6 mV, and the small one is about 1 mV). The large action potential cell is excited by isoeugenol (top) and by eugenol (bottom), and shows different latency to each one of them. The small action potential cell appears to be excited by isoeugenol. (b) A recording of a female sensillum with an isolated large neuron with very different response intensity and latency to isoeugenol (top) and eugenol (bottom). (c) Complete inhibition of response to 2-methoxyphenol in a female ORN. The right half of the figure (d) shows a sequence of responses of a single male ORN to several doses of the sex pheromone, which illustrates both the increased spike frequency and decreased latency as the concentration increases. Horizontal bar represents the 200 ms stimulation.

relatively strong, responses to one or more compounds, but with a different pattern in each cell. A group of 10 ORNs labelled "Smoke inhibited" (14% of the total, 4 male and 6 female) showed moderate but consistent inhibition by smoke compounds. A group of 8 cells (11% of the total, 3 male and 5 female) was strongly excited by the pine volatile α-pinene and, depending on the cell, also responded to other host compounds (mainly (+)-

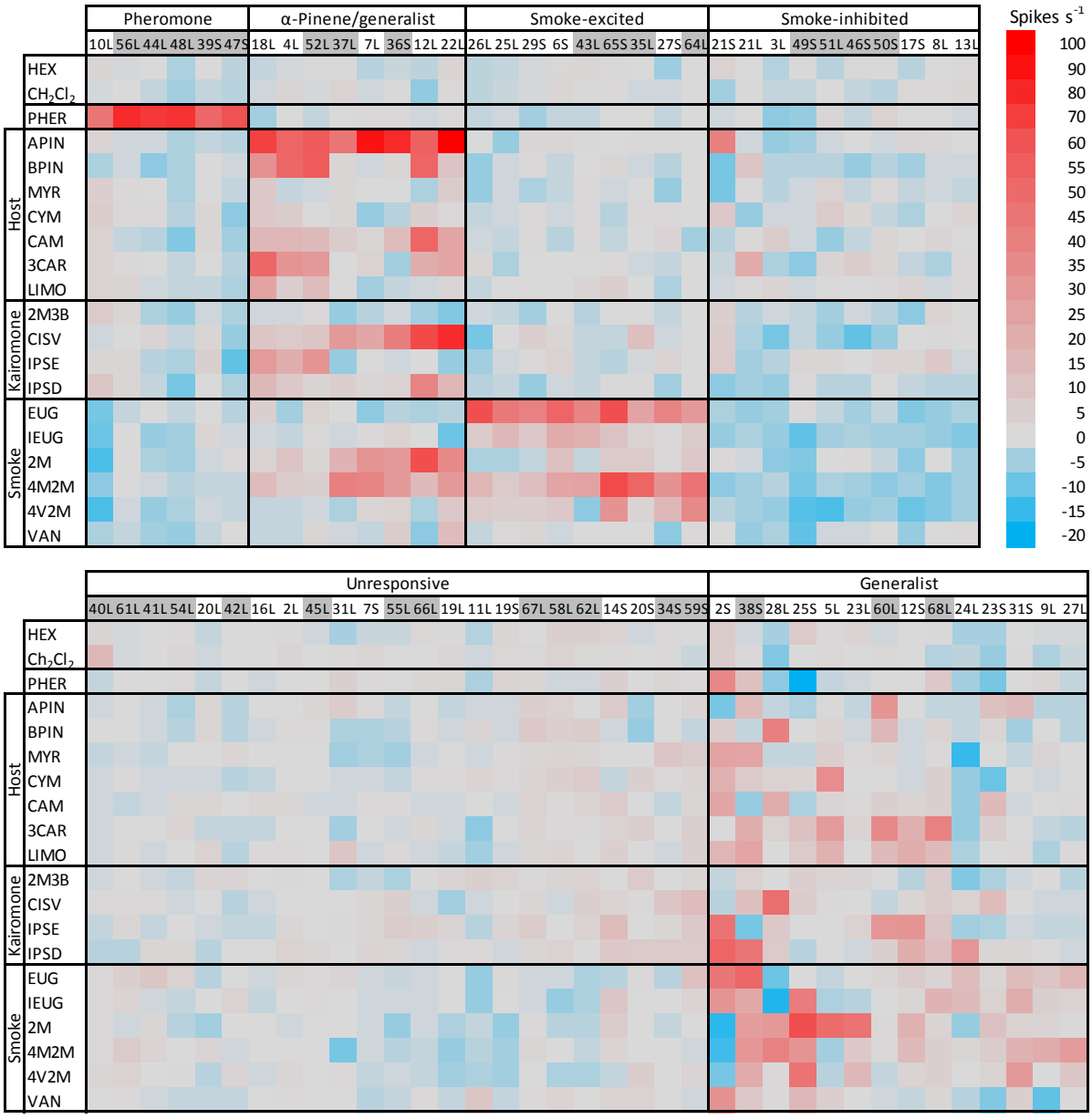


**Figure 3.4.** Response espectra of ORNs of *Monochamus galloprovincialis* to 10 μg of pheromone, kairomone and smoke odorants. Bar shows color code for inhibition (blue), no or minor response (gray range), and moderate to large excitation (red). Sensillae could house one or more (small [S] or large [L] action potential amplitude) cells. Female and male cells colored white and gray, respectively. Responses to the solvents (n-hexane or CH2Cl2) are also shown.

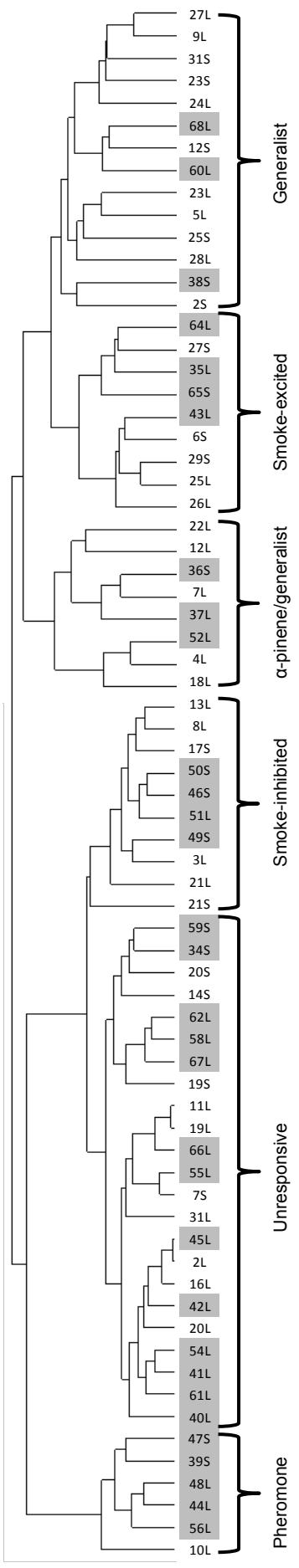


**Figure 3.5** Dendrogram showing ORNs classes in *Monochamus galloprovincialis* males (empty cells) and females (gray cells). The dendrogram is based in a hierarchical cluster analysis using Ward's minimum variance method.

camphene) and to bark beetle kairomone (mainly *cis*-verbenol) and smoke compounds (mainly 2-methoxyphenol and 4-methyl-2-methoxyphenol), so it was labelled "α-pinene/generalist". A group of 9 cells (13 % of the total, 4 male and 5 female) responded strongly to the smoke compounds eugenol and 4-methyl-2-methoxyphenol, and were labelled as "Smoke-excited". Finally, a group of 6 cells (9 % of the total, 5 male and 1 female) responded very strongly and very specifically to the male-produced aggregation pheromone, so they were considered as "Pheromone" specialist cells.

Dose-response curves were obtained for some compounds (Figure 3.7). Pheromone cells showed the typical sigmoidal-shape curve in the log(conc) scale (Byers, 2013) at the doses tested (0.1 to 10,000 ng), with no apparent levelling-off that would suggest saturation at the maximum concentration. Male and female pheromone ORNs showed no significant differences in their intensity of response to pheromone ($F$ = 2.99, $P$ = 0.085, df = 1). Although only 9% of all the ORNs were pheromone-specific while screening odorants (Figure 3.5), in the dose-response curves these cells were readily found, so they may be more abundant than what the initial screening indicates. α-Pinene/generalist cells were also relatively easy to contact and each of the 6 cells tested showed a clear dose-response pattern. Five smoke (eugenol) cells were recorded and showed a slightly different pattern from the other cells in that they responded very little to the 1 to 1,000 ng doses but peaked at the 10 µg dose (Figure 3.7). Only one cell was tested with ipsenol. Response peaked at 100 ng, then sharply decreased at 1 µg and stabilized at the highest dosage (Figure 3.7).

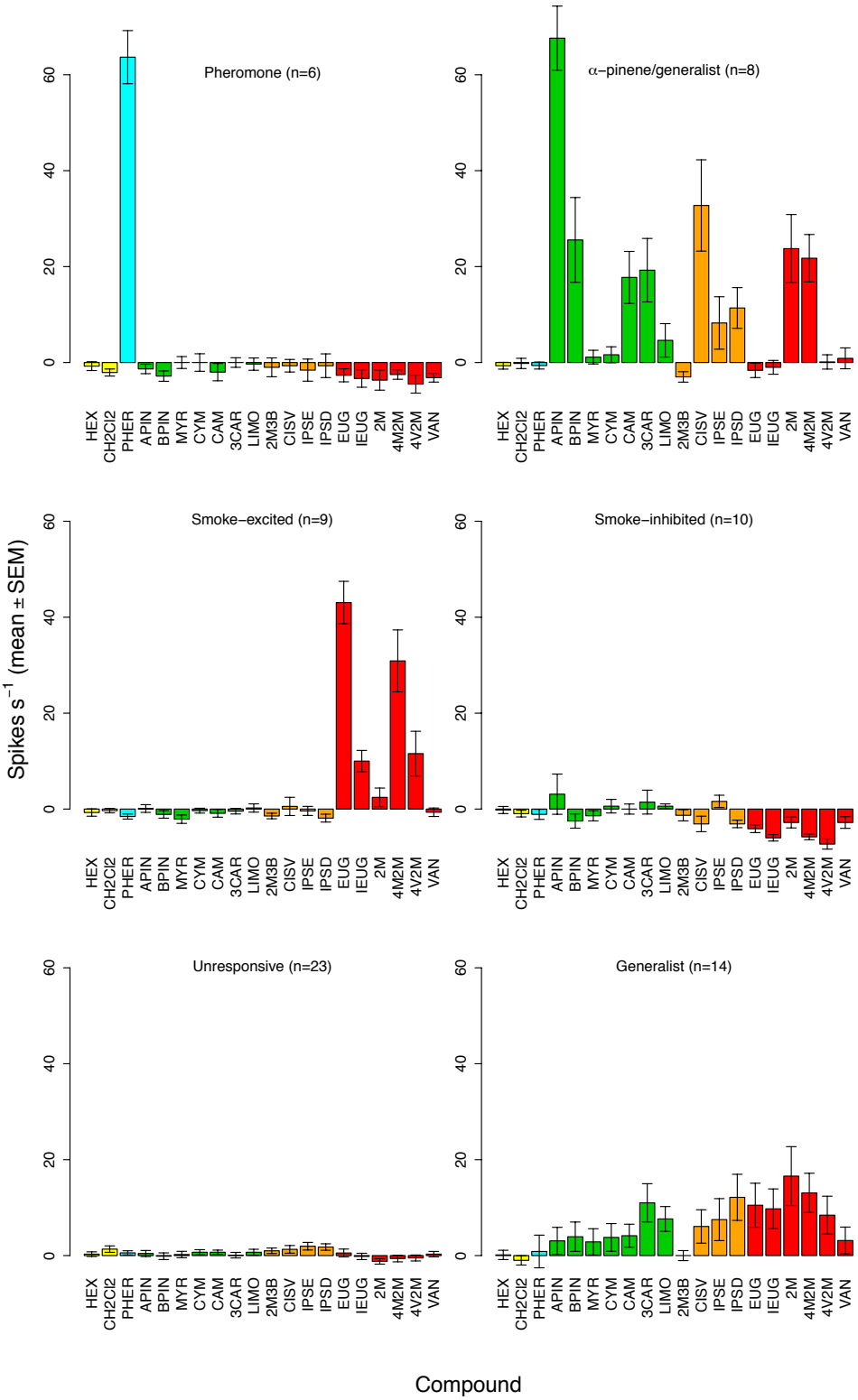


**Figure 3.6**. Average response of ORN classes obtained from hierarchical cluster analysis (Figure 3.5) to stimulation with 10 *μ*g of test compounds. Colors indicate different categories of volatiles (yellow: solvents, blue: *M. galloprovincialis* aggregation pheromone, green: host monoterpenes, orange: bark beetle kairomones, and red: smoke volatiles; abbreviations as in Table 3.1).

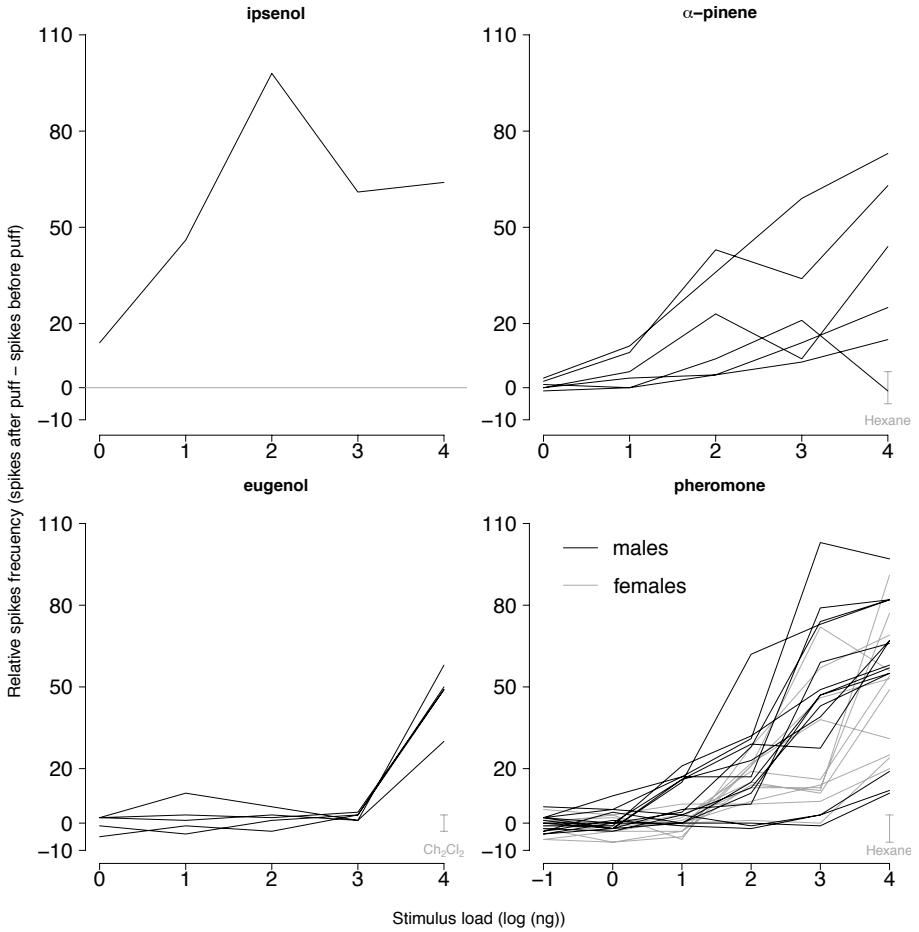


**Figure 3.7.** SSR doses-responses curves of *Monochamus galloprovincialis* ORNs to ipsenol, α-pinene, eugenol and aggregation pheromone. Each line represents a different ORN from a different sensillum. For ipsenol only one ORN was recorded. Ipsenol and eugenol were tested on males, α-pinene was tested on females and pheromone was tested on both sexes. Solvent response of individual ORNs is indicated as a horizontal gray line, or as a vertical line showing the range when there are many cells in the same plot.

Latency of response varied among compounds ($F$ = 2.47, $P$ = 0.005, df = 13; Figure 3.8 and illustrated in Figure 3.3a, b), but differences were significant only between isoeugenol and pheromone, two kairomonal compounds (α-pinene, 3 carene), and 3 smoke compounds (2-methoxyphenol, 4 methyl-2 methoxyphenol and eugenol). Although in the calculation of latency the same dose was used for all compounds, each of them has a different boiling point, and so it is likely that the number of molecules hitting the antenna varied among them. In moths, higher stimulus concentration result in shorter latencies (Jarriault *et al*., 2010), and this was the same in *M. galloprovincialis* when we compared the latency of response with the concentration of pheromone ($F$ = 20.31, $P$ < 0.001, df = 77). Therefore, it is possible that differences in latency reflected differences in compound quantity, in addition to, or instead of, the specific interaction of each compound with the olfactory receptor machinery (olfactory receptor protein, odor degrading enzymes, etc.). To

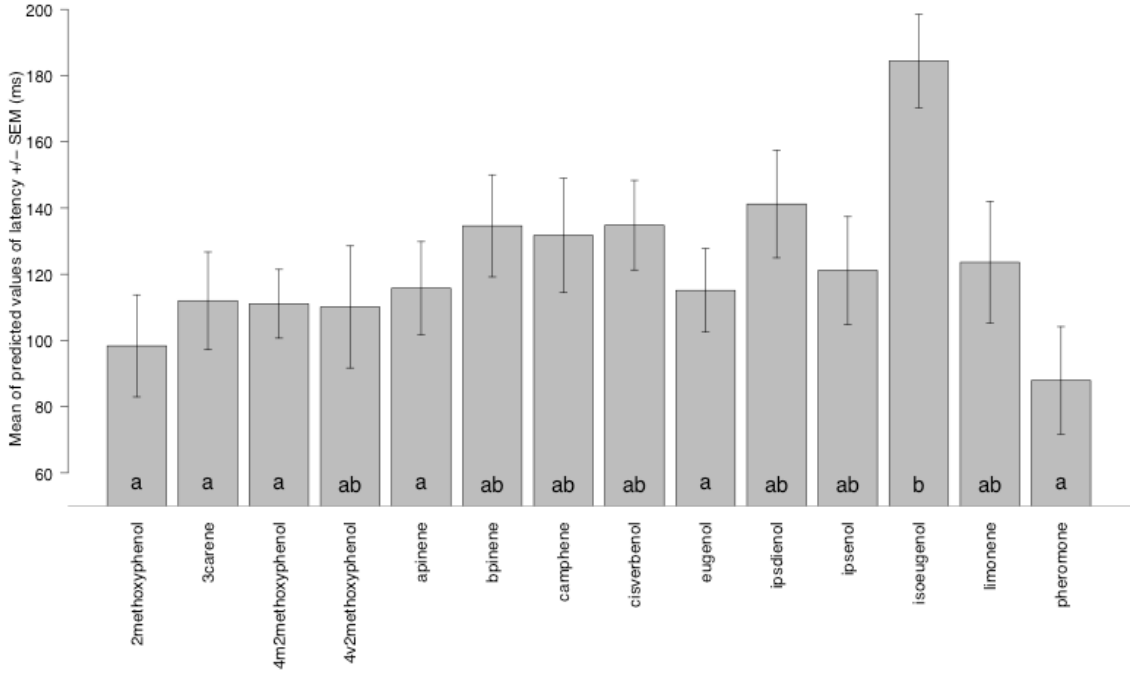


**Figure 3.8.** Mean (± SEM) latency of response of *Monochamus galloprovincialis* antennal ORNs to host, bark beetle, pheromone and smoke odorants. Different letters indicate significantly different latencies (Tukey's honestly significant difference test, α = 0.05 after GLM). Only compounds with 7 or more replicates were used for comparison. The data shown in here are the predicted means and SEMs after GLM model analysis.

address this possibility, we compared the intensity of the response with the boiling point of the compounds (pooling several ORNs) and found no relationship between these two variables ($F$ = 0.90, $P$ = 0.345, df = 167), which suggests that the volatility of the compounds does not fully explain differences in latency.

## Discussion

The sensillae assortment of *M. galloprovincialis* closely resembles that of its sister species *M. notatus* (Drury) and *M. scutellatus* (Say) described by Dyer and Seabrook (1975). The function of the conspicuous and abundant stout and male peg sensillae chaetica is unknown. We did not obtain action potentials from them, but Dyer and Seabrook (1975) reported in the other two *Monochamus* species that some of these sensillae are innervated, and suggested they probably serve as mechanoreceptors. Sensillae trichodea of *M. notatus* and *M. scutellatus* have several characteristics of contact chemoreceptors, such as a pore at the tip, which could well be used to sense the cuticular hydrocarbons which are so relevant in chemical communication of cerambycids (Allison *et al*., 2004) including *M. galloprovincialis* (Ibeas *et al*., 2008; Ibeas *et al*., 2009). When

manually bent, the distal sensillae chaetica of *M. galloprovincialis* responded with spike trains (data not shown), which demonstrated that these are mechanoreceptors. Other structures, such as the dome shaped sensillae and the glands associated with stout sensillae chaetica are similar to those described for *M. notatus* and *M. scutellatus* (Dyer and Seabrook, 1975) and they probably have a similar, but yet unknown, role in these three species. These pores, and their associated glands, probably secrete cuticular hydrocarbons and related substances which normally cover the antennal surface (Böröczky *et al*, 2013).

Clearly, from an olfactory perspective, the most interesting sensillae type in *M. galloprovincialis* are the small sensillae basiconica located in the sensory fields. The two types of sensillae basiconica reported in here are similar in morphology to those described for *M. notatus* and *M. scutellatus* (Dyer and Seabrook, 1975) and other cerambycids, such as *Psacothea hilaris* (Pascoe) (Dai and Honda, 1990) and *Phoracanta semipunctata* Fab. (Lopes *et al*., 2002). In *M. galloprovincialis* they are confined to two sensory fields in each flagellum, similar to *M. notatus* and *M. scutellatus* (Dyer and Seabrook, 1975). As in these two species, in *M. galloprovincialis* there was no remarkable sexual dimorphism with respect to the distribution of sensillae basiconica and their abundance along the antenna, suggesting that these sensillae have a similar olfactory role in both sexes. This hypothesis is supported by the finding that both males and females are similarly attracted to host plant odour, bark beetle pheromones and *M. galloprovincialis* aggregation pheromone (Pajares *et al*., 2004; Ibeas *et al*., 2007; Pajares *et al*., 2010). In *M. notatus* sensillae basiconica also host ORN's tunned to host volatile and a variety of responses, including excitation and inhibition, have also been noted (Dyer and Seabrook, 1978).

As with the North American *Monochamus* species (Allison *et al*., 2001; 2003; De Groot and Nott, 2004; Miller and Asaro, 2005), *M. galloprovincialis* adults of both sexes locate suitable host trees for breeding by following pine volatiles and sex pheromones of sympatric bark beetle species (Pajares *et al*., 2004; Ibeas *et al*., 2007), which correlates with the presence of ORNs tuned to these compounds. Host monoterpenes are defensive compounds located in trunk and leaves of woody plants. Some of these compounds are released in large quantities by stressed or recently damaged tissues, which signal optimal host conditions to potential predators. Among the monoterpenes, $\alpha$-pinene is a major component in local pine species (Santos *et al*., 2006) and seems to be a key component

for *M. galloprovincialis* to locate potential hosts (Ibeas *et al*., 2007; Pajares *et al*. 2010), which is consistent with ORNs responses found in our work. We also found ORN responses to β-pinene and 3-carene, which are also abundant monoterpenes in typical pine species of the Iberian Peninsula (Santos *et al*., 2006). Field bioassays have shown that α-pinene, 3-carene and β-pinene, in that order, were the best pheromone synergists for *M. galloprovincialis*, out of the seven pine terpenes tested (Álvarez *et al*, unpublished). On the contrary, Santos *et al*., (2006) reported myrcene as a relatively abundant monoterpene, but most tested cells did not respond to it.

All four bark beetle pheromone components tested by SSR are emitted by the European *Ips* species infesting pines: *Ips sexdentatus* (Boerner), *Ips acuminatus* (Gyllenhall), *Ips mannsfeldii* (Watchl) and *Ips (Orthotomicus) erosus* (Wollaston) (Kohnle *et al*. 1988, 1993). Although these are generally secondary species breeding in stressed, fallen or dying trees, they can kill healthy trees under favourable conditions. Since the colonization flights of these species overlap widely with that of *M. galloprovincialis* during the summer, it has been suggested that it is advantageous for the pine sawyers to respond to the pheromonal signals released by these secondary bark beetle species (Pajares *et al*., 2004). In North America, *cis*-verbenol does not attract *Monochamus* species (Allison *et al*. 2001, 2003; De Groot and Nott 2004), while the effect of ipsdienol varies according to the *Monochamus* species, being attractive to *M. titillator* (Miller and Asaro 2005), *M. clamator* and *M. scutellatus* (Allison *et al*. 2003), and unattractive to *M. scutellatus* and *M. mutator* (De Groot and Nott 2004). Field tests with *M. galloprovincialis* have shown that ipsdienol and *cis*-verbenol are behaviourally active, although less active than ipsenol and 2-methyl-3-buten-2-ol, and these two compounds constitute the kairomonal basis of commercial lures developed for *M. galloprovincialis* (Ibeas *et al*. 2007). Despite the behavioural importance of the bark beetle kairomones for *M. galloprovincialis*, we have not identified an ORN "type" that preferentially responds to them, except for the "α-pinene/ generalist" cells that in addition of responding to α-pinene and smoke compounds, also responded to *cis*-verbenol. However, the isolated dose-response curve to ipsenol showed that at least some ORNs have high sensitivity to some of these compounds.

Forest fires debilitate or kill trees, and some xylophagous insects, mainly bark beetles, benefit from attacking weakened hosts, so it is to their advantage to locate burnt trees (Allison *et al*., 2004; Schwilk *et al*., 2006). Beetles detect recently burnt trees in two

ways, by using infrared sensors located on their cuticle (Schmitz *et al*., 1997), and by smelling smoke compounds (Schütz *et al*., 1999). These authors used the antenna of *Melanophila acuminata* Eschscholtz beetles to identify, by means of GC-EAD, the smoke compounds that may attract them to burnt trees, and these were mainly phenolic compounds similar to the ones we have tested, with 2-methoxyphenol (guaiacol) producing the strongest responses. Although not proved experimentally, there is some evidence that *Monochamus* beetles are attracted to burnt trees (Parmelee, 1941; Ross, 1960). They have been reported on burnt hosts (Markalas, 1997), and larvae are relatively easy to find in burnt pine stands (personal observation). Furthermore, in GC-EAG studies we observed that *M. galloprovincialis* male and female antennae responded to the smoke volatiles 2-methoxyphenol, 4-methyl-2-methoxyphenol, 2,6-dimethoxyphenol, phenol, 4-methylphenol and eugenol, the two first compounds eliciting the strongest responses. When some of these volatiles were field tested, individually or in blends, they synergized the pheromone and bark beetle kairomones increasing attraction of *M. galloprovincialis* to traps (in preparation). In *M. galloprovincialis* three groups of neurons responded to smoke compounds as determined by hierarchical cluster analysis. The "smoke-excited" group was fairly specific in its response to smoke odorants, especially to eugenol and to 4-methyl-2-methoxyphenol. Besides being a smoke-related compound, eugenol is also a widespread floral and plant volatile that attracts a number of insects, including several coleoptera (El-Sayed, 2014), and at least one cerambycid betle (Iwabuchi *et al*., 1985). Therefore it might be argued that these “smoke-excited” ORNs are indeed plant ORNs. However all these ORNs also responded to a smoke-only compound such as 4-methyl-2-methoxyphenol (Figure 3.4). Besides, floral volatiles have never been implicated in the chemical ecology of *M. galloprovincialis*, and indeed eugenol is not a main ingredient of fresh pine effluvia (Mumm *et al*., 2004; Santos *et al*., 2006). Furthermore, physiological and behavioural responses of *M. galloprovincialis* to smoke volatiles (personal observation) and the repeated observation of colonization of fire-damaged trees suggest that the role of eugenol ORNs is to detect smoke volatiles from burnt trees. A second group of cells, the "α-pinene/generalists", was not so smoke-specific, as it responded to α-pinene *cis*-verbenol and other kairomones, in addition to the smoke compounds 2-methoxyphenol and 4-methyl-2-methoxyphenol. A third group of cells was slightly inhibited by smoke compounds. Therefore, *M. galloprovincialis* seems to be equipped with a refined sensory system dedicated to the detection of smoke-related volatiles.

In *M. galloprovincialis* the aggregation pheromone, 2-undecyloxy-1-ethanol, is released by males, elicits EAG responses in both sexes, and attracts both males and females in the field (Pajares *et al*, 2010). The same molecule is also the aggregation pheromone of *M. alternatus* (Hope) (Teale *et al*., 2011), *M. carolinensis* (Olivier) and *M. titillator* (Fabricius) (Allison *et al*., 2012), *M. scutellatus* (Fierke *et al*. 2012), and *M. sutor* (L.) (Pajares *et al*., 2013). In addition, it has also been shown to be a likely pheromone component for *M. clamator* (LeConte) and *M. obtusus* Casey (Macias-Samano *et al*. 2012), and *M. notatus* (Fierke *et al*. 2012). The presence of 2-undecyloxy-1-ethanol-specific ORNs in both sexes confirms the ecological importance of this attractant for *M. galloprovincialis*. Although only one pheromone-specific ORN was found in females, as compared with 5 in males, these cells were relatively easy to find to make the dose-response curves, so we believe that these cells are more common in both sexes than what our initial ORN screening shows. Given the importance of this compound for other *Monochamus* species, it is very likely that they also have specific receptors for it, and that they are most likely located in the basiconic sensillae of the plate areas.

We conclude that the antennal sensillae of *M. galloprovincialis* are similar morphologically and electrophysiologically to those described for other species in the same genus. Sensillae basiconica are chemoreceptors that respond to odours from host plants, bark beetles, smoke volatiles and aggregation pheromone. A few of the ORNs could be considered specific to pheromone or smoke compounds, and a large proportion are generalist or unresponsive, which suggest that many semiochemicals relevant to this species remain still unknown. There are no marked differences between sexes in relation to their receptors, so it is likely that behavioural activity depends on central integration of different stimulus. The presence of smoke-detector ORNs in *M. galloprovincialis* supports the hypothesis that they use smoke odour to locate suitable mating and oviposition sites.

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# Chapter 4

# Combining pheromone and kairomones for effective trapping of the Pine Sawyer Beetle *Monochamus galloprovincialis*

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## Introduction

Most forest cerambycids are considered secondary tree pests. These longhorn beetles usually colonize woody plants that are severely stressed or near death due to the action of fire, drought or pathogens. However, pine sawyer beetles of the *Monochamus* Dejean (Coleoptera: Cerambycidae) genus have become a phytosanitary issue since they were reported as vectors of the pine wood nematode (PWN) *Bursaphelenchus xylophilus* (Steiner and Buhrer), (Linit and Akbulut 2008). This native North American nematode species causes pine wilt disease (PWD), a pathology characterized by rapid wilting and death of pines and other susceptible conifers (Wingfield 1982). It was accidentally introduced into Southeast Asia in the early 20th century and spread across Japan, eastern China, Taiwan and Korea in the following decades, causing dramatic economic and environmental losses (Shing 2008; Zhao *et al*. 2008). In 1999, PWD was first reported on the European continent, in Portugal (Mota *et al*. 1999). Despite containment efforts, PWD spread throughout this country, devastating large pine areas (Daub 2008; Rodrigues 2008). It eventually crossed into Spain four times since 2008 (Zamora *et al*. 2015), where strong eradication measures (Evans *et al*. 1996) are currently being implemented.

Control of PWD has proven difficult worldwide (Kamata 2008). Direct attempts to control the pathogen, such as trunk injection, were convenient for ornamental trees but impractical in large areas (Shin 2008). Management of the insect vectors seems a better

strategy for dealing with the disease. Accordingly, chemical insecticides have been widely used in Asian countries (Shin 2008; Zhao *et al*. 2008), but are inappropriate due to their questionable effectiveness and potential hazard to environmental and human health (Takatsuka 2007). As an alternative to insecticides, traps baited with specific lures have proven useful and environmentally friendly for both monitoring and even reducing the populations of several species. Coniferous woodborers are known to be attracted by host odours (Morewood *et al*. 2002; Miller 2006). Conventional lures for these species use compounds that simulate the bunch of volatiles released by suitable hosts, such as stressed or dying trees. Several studies have documented the attraction of North American *Monochamus* species to kairomones from host trees such as α-pinene or ethanol, which are synergized by bark beetle (Coleoptera: Scolytinae) pheromone components such as ipsenol, ipsdienol and *cis*-verbenol (Billings and Cameron 1984; Allison *et al*. 2001, 2003; De Groot and Nott 2004; Miller and Asaro 2005). *M. galloprovincialis* (Olivier), the only known PWN vector in Europe (Sousa *et al*. 2001) is reportedly attracted by the four main pheromone compounds emitted by European Ipini bark beetles (Pajares *et al*. 2004). Accordingly, a blend of α-pinene, ipsenol and methyl-butenol was proposed as the best kairomonal lure for trapping this species (Ibeas *et al*. 2007). A further advance in unravelling *Monochamus* chemical ecology occurred when the *M. galloprovincialis* 2-undecyloxy-1-ethanol male aggregation pheromone was identified and described. This pheromone attracts both males and females and is powerfully synergized by a blend of α-pinene, ipsenol and methyl-butenol. The combination creates an effective attractant for trapping and potentially managing this species (Pajares *et al*. 2010). However, α-pinene is perceived by a wide range of forest insects, including secondary xylophagous beetles and bark beetle natural enemies as *Temnochila coerulea* (Olivier) or *Thanasimus formicarius* (L.) which may be also trapped and killed. Thus, this terpene should be replaced to minimize the trapping of non-target organisms. Other host terpenes show potential as effective pheromone synergists. The present work reports the results of several experiments that tested *M. galloprovincialis* pheromone and its blending with bark beetle kairomones and pine terpenes, aimed at developing an improved and more specific lure for *M. galloprovincialis* that can be used to monitor and possibly control this PWN vector.

## Material and Methods

*GC-EAG Analyses*

*Insects*

Insects that emerged from infested *P. pinaster* logs were collected in the field and brought to the laboratory in Spain, then sexed and kept individually. Some of these were fed on *P. halepensis* pine shoots for two weeks in order to induce sexual maturation while others were kept unfed and immature. Both insect types were then placed in individual tubes and sent by courier to the UK, where they arrived within two days. Mature beetles were maintained on *P. halepensis* shoots in an insectary with a 12L:12D cycle at room temperatures of 25°C (0700-1900 h) or 20°C (1900-0700 h) and ambient humidity. Immature insects were kept unfed under the same conditions.

*Collection of Volatiles*

For collection of volatiles, pine shoots of Scots pine, *Pinus sylvestris* L., or maritime pine, *Pinus pinaster* Aiton, were held in a silanized flange flask (1 l). Air was drawn in through a charcoal filter (10 cm x 2 cm; 10-18 mesh, Fisher Scientific) at 2 l/min and volatiles were trapped on Porapak Q (50-80 mesh; 200 mg; Waters Corp., MA, USA) packed between glass wool plugs in a Pasteur pipette (i.d. 4 mm). The Porapak Q was purified previously by Soxhlet extraction with chloroform for 8 h and filters were washed well with dichloromethane before use. Trapped volatiles were eluted from the filter with dichloromethane (3 x 0.5 ml) and used directly for analyses.

*Synthetic Compounds*

(±)-α-pinene (98%), (-)-β-pinene (99%), (S)-(-)-limonene (96%), p-cymene (99%), (+)-3-carene (90%), (+)-camphene (> 80%), myrcene (> 90%), β-caryophyllene (90%) and terpinolene (> 90%) were obtained from SigmaAldrich (Gillingham, Dorset, UK). 2-undecyloxy-1-ethanol was synthesized at NRI and was >98% pure.

*Gas Chromatography (GC) Coupled to Electroantennographic (EAG) Recording*

For GC-EAG analyses an HP6890 GC (Agilent Technologies, Stockport, Cheshire, UK) was used, fitted with fused silica capillary columns (30 m x 0.32 mm i.d. x 0.25 µm film thickness) coated with both polar DB Wax (Agilent) and non-polar SPB-1 (Supelco, Gillingham, Dorset, UK) phases. The ends of the two columns were connected to a short

piece of deactivated fused silica tubing with a glass, push-fit Y-piece. The outlet from this was then split by means of a similar Y-piece with half going to the flame ionization detector and half to a silanized, glass T-piece (arms 5 cm, i.d. 4 mm), using similar lengths of deactivated fused silica tubing. One arm of the T-piece was connected to a device delivering compressed air (300 ml/min) continuously or in a 3-sec pulse at 17-sec intervals. The third arm of the T-piece passed through the GC oven wall to the insect EAG preparation (Cork *et al*. 1990). All analyses were carried out on the polar GC column with helium carrier gas (2.4 ml/min), splitless injection (220°C), flame ionization detection (250°C) and oven temperature programmed from 50°C for 2 min then at 10°C/min to 240°C. In some analyses the pheromone, 2-undecyloxyethanol (10 ng) was added as a positive control. EAG recording was carried out with a portable device (INR-02; Syntech, Hilversum, The Netherlands) consisting of integrated electrode holders, micromanipulators and amplifier. Electrodes were silver wires fitted with glass electrodes pulled to a fine point with an electrode puller and containing saline solution (0.1 M potassium chloride with 1% polyvinylpyrrolidine to reduce evaporation). An antenna was excised at the base and suspended between the glass electrodes which were cut so that they just accommodated the ends of the antenna. The signal was amplified x 10 and the amplifier was connected to the GC as a detector device. Data were processed with EZChrom Elite v3.0 (Agilent).

*GC Coupled to Mass Spectrometry (GC-MS)*

Volatile collections were analysed GC-MS in electron impact (EI) ionization mode with a Saturn 2200 MS (Varian, now Agilent) interfaced with a CP3800 GC fitted with a fused silica capillary columns (30 m × 0.25 mm i.d. × 0.25 µm film thickness) coated with polar DB-Wax (Agilent). Injections were made in splitless mode (220 °C), using helium carrier gas (1 ml per min) and the oven temperature was held at 40°C for 2 min, then increased by 10°C per min to 250 °C.

**Table 4.1.** Synthetic compounds used in experiments

| Semiochemicals | Description | Release rate (mg/day) | Supplier | Experiments |
|---|---|---|---|---|
| P | *M. galloprovincialis* pheromone | 0.8[1] | SEDQ | 1, 2, 3, 4 (2009); 4, 5 (2010) |
| K | Single dispenser releasing ipsenol and 2-methyl-3-buten-2-ol | 2.4[2]<br>11[2] | SEDQ | 1, 4 (2009); 5, 6 (2010) |
| Kp | Two dispensers, one releasing ipsenol and the other 2-methyl-3-buten-2-ol | 0.4[3]<br>11[3] | Contech | 1 (2009) |
| α | α-pinene, host terpene | 500[2] | SEDQ | 1, 4 (2009); 5, 6 (2010); 7, 8 (2011); 9, 10 (2013) |
| G2D | Two dispensers, one releasing ipsenol and 2-methyl-3-buten-2-ol (K) the other the *M. galloprovincialis* pheromone (P) | 2.4[2]<br>11[2]<br>2[1] | SEDQ | 7, 8 (2011); 9 (2013) |
| β-pinene | host terpene | 500[2] | SEDQ | 7, 8 (2011); 10 (2013) |
| 3-carene | host terpene | 500[2] | SEDQ | 7, 8 (2011); 10 (2013) |
| (+)-limonene | host terpene | 500[2] | SEDQ | 7, 8 (2011); |
| camphene | host terpene | 500[2] | SEDQ | 7, 8 (2011); |
| myrcene | host terpene | 500[2] | SEDQ | 7, 8 (2011); |
| p-cymene | host terpene | 500[2] | SEDQ | 7, 8 (2011); |
| terpinolene | host terpene | 500[2] | SEDQ | 9, 10 (2013) |

[1] Release rates under lab conditions, 27ºC and 2 m/sec windspeeed SEDQ, Sociedad Española de Desarrollos Químicos, Barcelona, Spain.
[2] Release rates under field conditions, SEDQ, Sociedad Española de Desarrollos Químicos, Barcelona, Spain.
[3] Release rates under lab conditions at 25ºC, Contech Enterprises, Victoria, Canada.

### *Field Experiments*

Nine experiments (Table 4.1) were conducted in Spain in 2009, 2010, 2011 and 2013, and one in France in 2011, to test several combinations of attractants. The first eight experiments in Spain were set up in the Sierra Espuña Natural Park (Murcia), (37° 57' N, 1° 24' W) in a mature *Pinus halepensis* forest planted 110 years ago. The traps used from 2009-2011 were 12-unit, multiple-funnel traps (Econex SL, Murcia, Spain) suspended from ropes between trees, with the top funnel 2 m above ground. The collecting cups were fitted with a small piece of DDVP (dimethyl 2, 2-dichlorovinyl phosphate) insecticide strip (Econex S. L., Murcia, Spain) in order to kill trapped beetles. In 2013, Teflon-coated multiple-funnel traps with extended collector cups for live trapping (Econex Multifunnel-12®) were used. All the experiments were deployed in seven randomized complete blocks. Traps were at least 100 m apart and blocks were arranged 700 m apart. Captured *M. galloprovincialis* were collected every 7-12 days, identified and sexed (Vives 2000). To assess lure specificity, captured *T. coerulea* (Coleoptera: Trogossitidae), a relatively abundant natural bark beetle predator, were also counted and recorded.

A ninth experiment, specifically designed to test pine volatiles as attractants for immature adults, was set up in Calzadilla de la Cueza (Palencia, Spain) (42º 19' N, 4º 45' W) in 2013. To guarantee the absence of competitive sources of attraction (i.e. volatiles from pine trees), the bioassay was carried out in a non-forested area free of any pine tree. Four pairs of multiple-funnel traps (Econex Multifunnel-12®) were regularly spaced within each of three concentric circles of 100m, 250m and 500m radii. Traps in each pair were 30 m apart; one of them was baited with pine volatiles, while the other was left un-baited as a control. One-hundred ninety fresh immature insects that had emerged in outdoor cages from infested P. *sylvestris* logs collected in the field were marked with bee tags and released within 5 days from the center of the circles. Traps were sampled every 3-4 days from 25 July to 9 August.

The experiment in France was conducted in 2011 in a mature *Pinus pinaster* forest plantation located at Marcheprime (44° 74' N, 0° 89' W). Multiple-funnel traps (Econex SL, Murcia, Spain) were suspended from wood poles 2 m height. Distance between traps was at least 100 m and nearest blocks were a minimum of 300 m apart. Traps were assessed twice a week during four weeks, with re-randomization after sampling.

*Experiments in 2009*

Experiment 1 was conducted from 4 July to 7 August 2009 in order to: 1) confirm the synergistic effect of bark beetle kairomones (ipsenol and methyl-butenol) with the *M. galloprovincialis* aggregation pheromone, as reported by Pajares *et al*. (2010); 2) test the effect of adding $\alpha$-pinene to the blend of bark beetle kairomones and *M. galloprovincialis* aggregation pheromone and 3) compare the joint release of the bark beetle kairomones ipsenol and methyl-butenol from an integrated dispenser with the release of both compounds separately. Seven combinations of attractants were tested: *M. galloprovincialis* pheromone (P); ipsenol and methyl-butenol released from a single dispenser (SEDQ, Barcelona, Spain) plus $\alpha$-pinene (K+α); ipsenol and methyl-butenol released form separate dispensers (Contech Enterprises, Victoria, Canada) plus $\alpha$-pinene (Kc+α); *M. galloprovincialis* pheromone plus ipsenol and methyl-butenol from a single dispenser (P +K); the same blend plus α-pinene (P+K+α); *M.galloprovincialis* pheromone plus ipsenol and methyl-butenol from separate dispensers (P+Kc); and the same blend plus α-pinene (P+Kc+α).

Experiments 2 and 3 were conducted from 4 July to 7 August and 7 August to 14 September 2009, respectively. They were designed to test the dose-response effect of *M. galloprovincialis* pheromone 2-undecyloxy-1-ethanol when released at 500, 1000 and 1500 mg/day (Experiment 2) or 500, 1500 and 2500 mg/day (Experiment 3).

Experiment 4 ran from 7 August to 14 September 2009 and was intended to: 1) corroborate the synergistic effect of *M. galloprovincialis* pheromones with bark beetle kairomones when released from a single dispenser; 2) evaluate if the dose-response effect of the *M.galloprovincialis* pheromone also occurred when it was synergized by the bark beetle kairomones; and 3) examine again the synergistic effect of $\alpha$-pinene with the pheromone-bark beetle kairomones blend. Four treatments were tested: *M. galloprovincialis* pheromone (P); pheromone plus ipsenol and methyl-butenol from a single dispenser (P+K); a triple dose of *M. galloprovincialis* pheromone plus ipsenol and methyl-butenol from a single dispenser (3P+K) and *M. galloprovincialis* pheromone plus bark beetle kairomones and $\alpha$-pinene (P+K+$\alpha$).

*Experiments in 2010*

Experiments 5 and 6 were carried out from 5 June to 12 August and from 12 August to 16 September 2010, respectively, with the same objectives as Experiment 4 (2009). Experiment 5 tested the same treatments as in Experiment 4, except for the *M. galloprovincialis* pheromone released alone, and Experiment 6 evaluated the same three treatments as Experiment 5 plus a fourth consisting of *M. galloprovincialis* pheromone and $\alpha$-pinene (P+$\alpha$) blend, in order to compare the synergistic effect of $\alpha$-pinene to that of bark beetle kairomones.

*Experiments in 2011*

Two similar field experiments were conducted in Spain and France from 1 June to 16 July 2011 (Experiment 7) and July 18 to August 12 (Experiment 8), respectively. Both were designed to assess the performance of six selected pine terpenes, after results from the GC-EAG bioassays (Figure 4.1), as synergists for the commercial lure Galloprotect 2D (G2D) which consists of two dispensers: one releasing the *M. galloprovincialis* pheromone and the other releasing ipsenol and methyl-butenol. A seventh terpene, limonene (+) was also tested as potential repellent. (+)-Limonene was found the major compound of the resin of Stone pine (*Pinus pinea*. L.), which was reported to be unsuitable to *M.*

*galloprovincialis* feeding and reproduction (Naves *et al*. 2008). Because Galloprotect 2D was known to be unable to attract *M. galloprovincialis* adults before they were sexually mature (i.e. after at least 10 days of feeding following emergence, unpubl. data), another objective of experiment 7 was to check whether any of the tested terpenes would also attract immature beetles. Eight treatments were assayed: G2D alone and G2D plus α-pinene or β-pinene, 3-carene, p-cymene, myrcene, camphene or (+)-limonene. In Experiment 7 a sub-sample of trapped females was collected each time the trap was emptied. They were taken to the laboratory and dissected to check for sexual maturation (e.g. the presence of eggs).

*Experiment in 2013*

Further EAG bioassays have shown that terpinolene, not previously tested, elicited a notable antennal response in *M. galloprovincialis* males and females. Thus, one field bioassay (Experiment 9) was conducted from 12 August to 23 September 2013 in order to evaluate the role of terpinolene as synergist for G2D standard lure. Treatments were: G2D; G2D plus α-pinene and G2D plus terpinolene.

The pine volatiles tested in the 2011 experiment did not attract immature beetles. However, EAG recording had shown that immature beetles responded to some of the tested terpenes (α -pinene, 3-carene, p-cymene; Figure 4.2). Because competition with pines releasing terpenes in the wild could not be ruled out as a reason for this fact, a subsequent bioassay (Experiment 10) was carried out to test antennally active pine terpenes in an area deprived of any pines. Traps were baited with a blend of terpinolene, 3-carene and α-pinene. The blend also incorporated β-pinene as it was proved to be the best pheromone synergist for mature beetles in Experiments 8. Paired un-baited traps with a tree-like silhouette were set as controls. Immature beetles were released from the centre of the trapping set up.

Statistics

The number of caught insects were fitted to a Poisson or quasi-Poisson (in case of over-dispersion) error distribution in a generalized linear model (GLM), using attractant and block as fixed factors (Crawley 2007). Ten Tukey's HSD tests were performed for mean comparisons. To allow for comparisons of the effect of α-pinene in combination with the blend of *Monochamus* pheromone plus ipsenol and methyl-butenol on trap capture

across the different experiments, effect sizes were calculated. We used the log ratio of capture obtained with α-pinene divided by capture with no α-pinene (Hedges ***et al***. 1999), and estimated its 95% confidence interval (CI).

## Results

*GC-EAG Analyses*

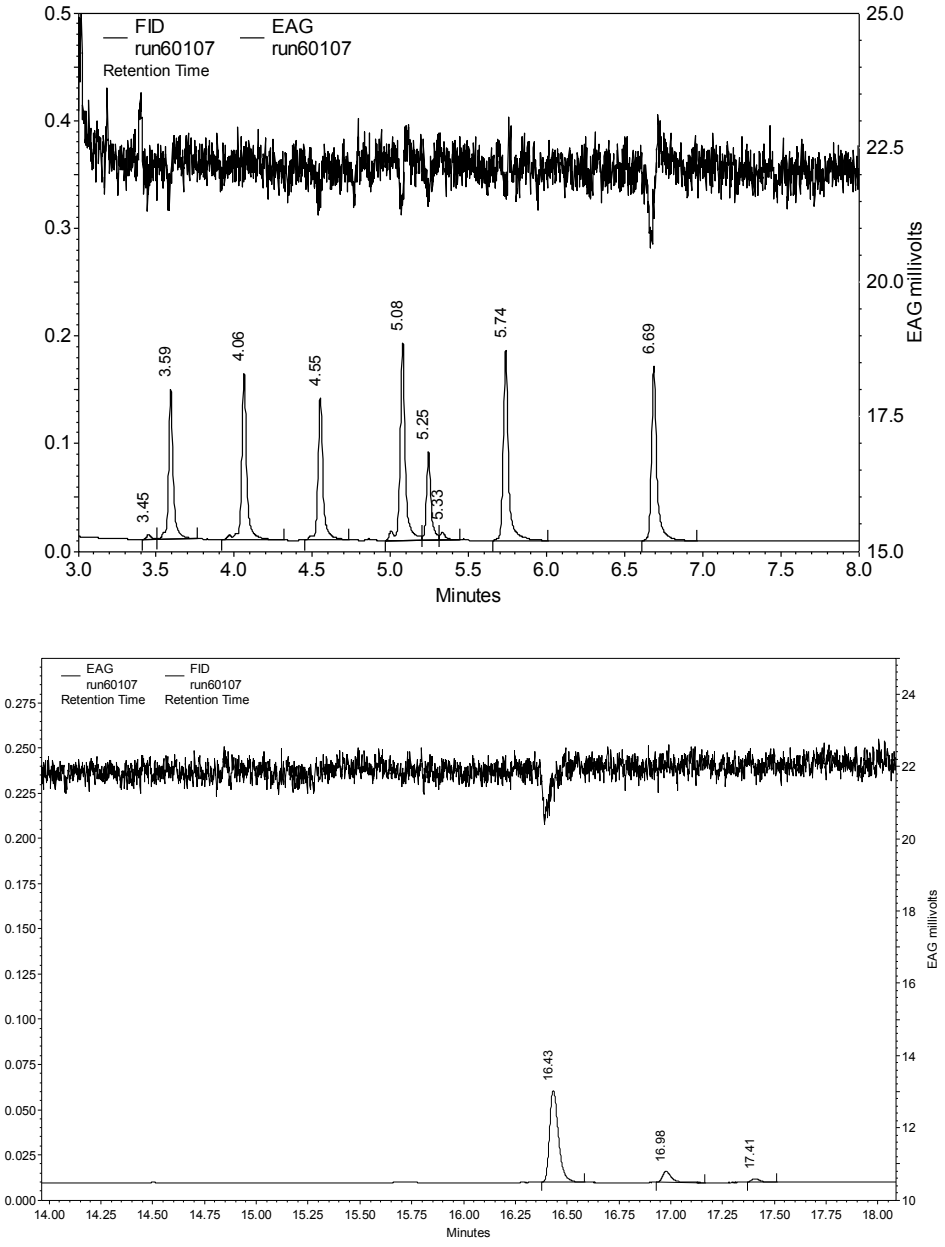


**Figure 3.1**. GC-EAG Analyses of synthetic monoterpenes (100 ng injected, 50 ng to insect) and pheromone with antenna of mature female *M. galloprovincialis* (upper trace is EAG, lower GC; 3.59 min α-pinene, 4.06 min camphene, 4.55 min β-pinene, 5.08 min 3-carene, 5.23 min myrcene, 5.74 min limonene, 6.69 min *p*-cymene, 16.43 min pheromone).

The GC-EAG system was used to test responses of mature *M. galloprovincialis* to synthetic monoterpenes using continuous delivery. EAG Responses were observed to most of the compounds tested and particularly to α-pinene, 3-carene and p-cymene (Fig. 3.1). For analyses with immature *M. galloprovincialis* it was necessary to use the intermittent, puffed delivery of the GC effluent which increases sensitivity (Cork *et al*.

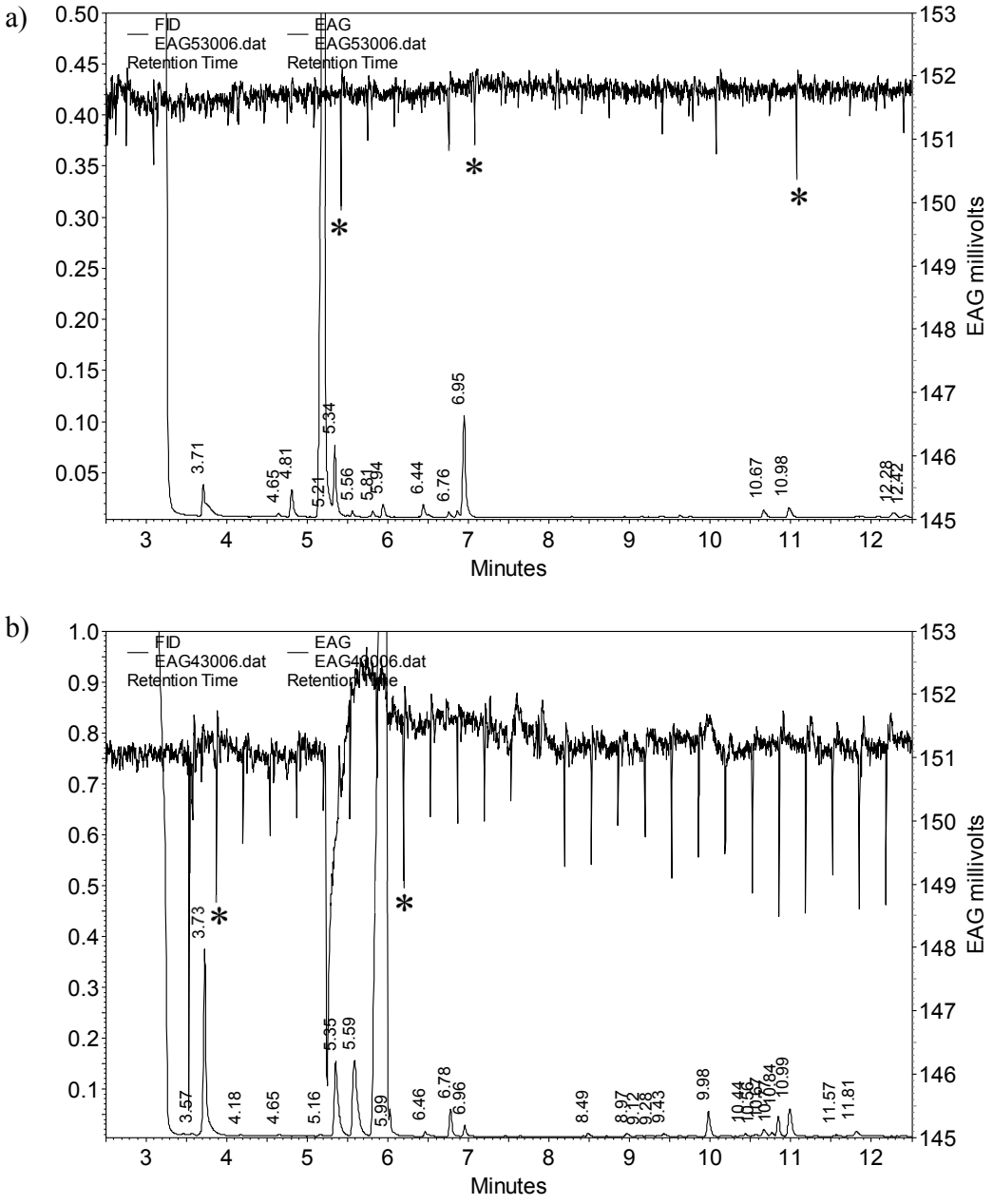


**Figure 3.2.** GC-EAG Analysis of volatiles with antenna of immature male from *M. galloprovincialis* from Scots pine (a) (upper trace is EAG, lower GC; 5.21 min 3-carene; 6.76 min *p*-cymene; 6.95 min terpinolene; 10.98 min β-caryophyllene), and from maritime pine (b) (upper trace is EAG, lower GC; 3.73 min α-pinene; 5.99 3-carene)

1990). Analyses of volatiles from Scots pine showed EAG responses to the major component, 3-carene, and to minor components p-cymene, terpinolene and β-caryophyllene (Fig. 3.2a). Analyses of volatiles from maritime pine showed EAG responses to the major component α-pinene (e.g. Fig. 3.2b).

*Field experiments*

*Experiment 1*

Significant differences in *M. galloprovincialis* captures between trapping treatments were found ($F = 10.55$, $P<0.001$, d.f. = 6; fig. 3.3). All tested semiochemical combinations proved significantly more attractive than the *M. galloprovincialis* pheromone alone. Results also showed the synergistic effect between the *M. galloprovincialis* pheromone and bark beetle or host kairomones.

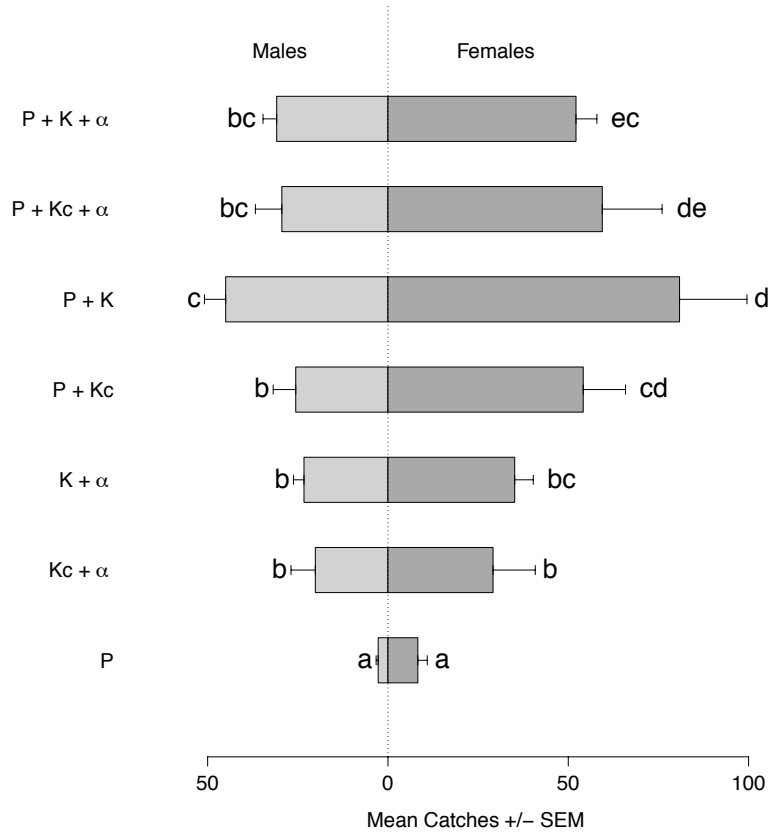


**Figure 3.3**. Mean (± SE) catches of *Monochamus galloprovincialis* males and females in experiment 1 (2009). Bars with the same letter are not significantly different (Tukey's honestly significant difference test, α = 0.05).

Thus, higher catches were obtained when the pheromone and kairomones were released together (83.00 ± 8.6 insects/trap for P+K+α−pinene and 88.86 ± 23.51 insects/ trap for P+Kc+α−pinene) rather than separately (11.00 ± 2.79 insects/trap for P, 58.43 ± 7.26 insects/trap for K+α−pinene and 49.29 ± 18.34 insects/trap for Kc+α−pinene). No differences were observed between captures obtained with ipsenol and methyl-butenol released separately (Kc; Contech) or from a single dispenser (K; SEDQ). However, when α-pinene was removed from the all-compounds blend, results differed somewhat. With separate kairomone dispensers, removal of α-pinene from the P+ Kc+α−pinene blend resulted in a non-significant decrease in captures (79.71 ± 17.92 insects/trap with P+Kc), whereas removal of α-pinene from the P+K+α−pinene blend led to a sizable increase in catches, but only significant in females (125.86 ± 23.00 insects/trap with P+K). No significant differences were found between mean capture with K vs. Kc lures in any of the paired comparisons, except for males being more attracted to P+K than P+Kc.

*Experiments 2 and 3*

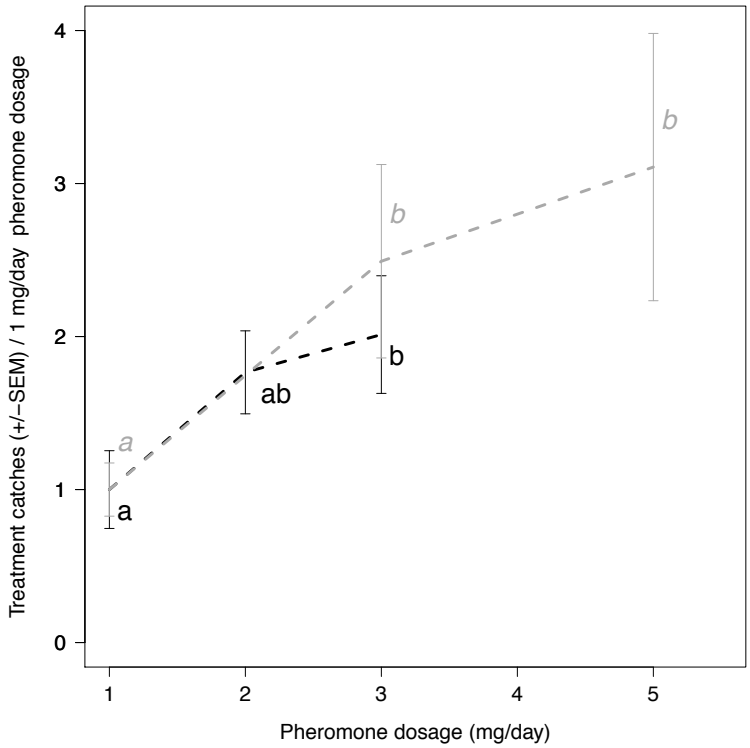


**Figure 3.4.** Mean (± SE) catches of *M. galloprovincialis* referred to single dose (1P=1 mg/day) of 2-undecyloxy-1-ethanol in experiments 2 (black) and 3 (gray) (2009). For each experiment, dosages with the same letter were not significantly different (Tukey's honestly significant difference test with Bonferroni adjustment, α = 0.05).

Results from these experiments showed a typical dose-response in the pheromone trapping of *M. galloprovincialis* (F =2.86 , P<0.097 d.f. = 2 [exp 2]; F =4.86 , P<0.028 d.f. = 2 [exp 3]; fig. 3.4). Catches increased significantly –in fact doubled– when the pheromone release was tripled and further increased when the pheromone dose was raised to five times the initial dose. These results suggested the possibility of improving pheromone-kairomone lure performance by increasing pheromone release rate. This was tested in the following three experiments.

*Experiments 4, 5 and 6.*

A significant increase in attraction was observed again when bark beetle kairomones were released along with the *M. galloprovincialis* pheromone in Experiment 4 (72.00 ± 17.57 insects/trap for P+K compared to 9.29 ± 1.61 insects/trap with P) (F =6.01, P<0.005 d.f. = 3; fig. 3.5a). However, tripling the pheromone release rate in this blend did not result in increased catches (3P+K vs. P+K). Addition of α-pinene to the simple pheromone-kairomones blend only slightly increased catches (89.86 ± 33.25 insects/trap P+K+α). Similar results were obtained in Experiment 5., as tripling the pheromone dose or adding the pine terpene did not resulted in significantly higher catches than the simple pheromone-kairomone lure (109.57 ± 17.31 and 113.71 ±14.12 insects/trap, compared to 72.14 ± 9.14 insects/trap) (F= 2.193, P = 0.154, df = 2; Figure 4.5b). By contrast, significant differences were observed in Experiment 6 when the pheromone release rate was increased in the blend (F = 3.77, P<0.029, d.f. = 3): catches increased by 100% when a triple dose of pheromone was used (105.29 ± 26.05 3P+K, compared with 51.71 ± 9.19 insects/trap P+K; fig. 3.5c). Non-significant increase was obtained with the addition of α-pinene in P+K (72.00 ± 4.53 insects/trap).

*Experiments 7, 8, 9 and 10*

The results of Experiment 7 to test the effect of several pine terpenes as synergists for the standard pheromone-kairomone lure showed significant differences in both sexes (F = 3.503, P=0.0048, d.f. = 7; and F=3.47, P=0.005, df=7; Figure 4.6a). Four terpenes resulted in increased catches when released together with G2D: $\alpha$-pinene (29.5% increase), 3-carene (+24.8%), camphene (+14.3%) and β-pinene (+2.7%), whereas catches were lower than with the standard lure (G2D) when p-cymene (-33,5%), myrcene (-33,5%) and (+)-limonene (-46%) were added. Differences were only significant between

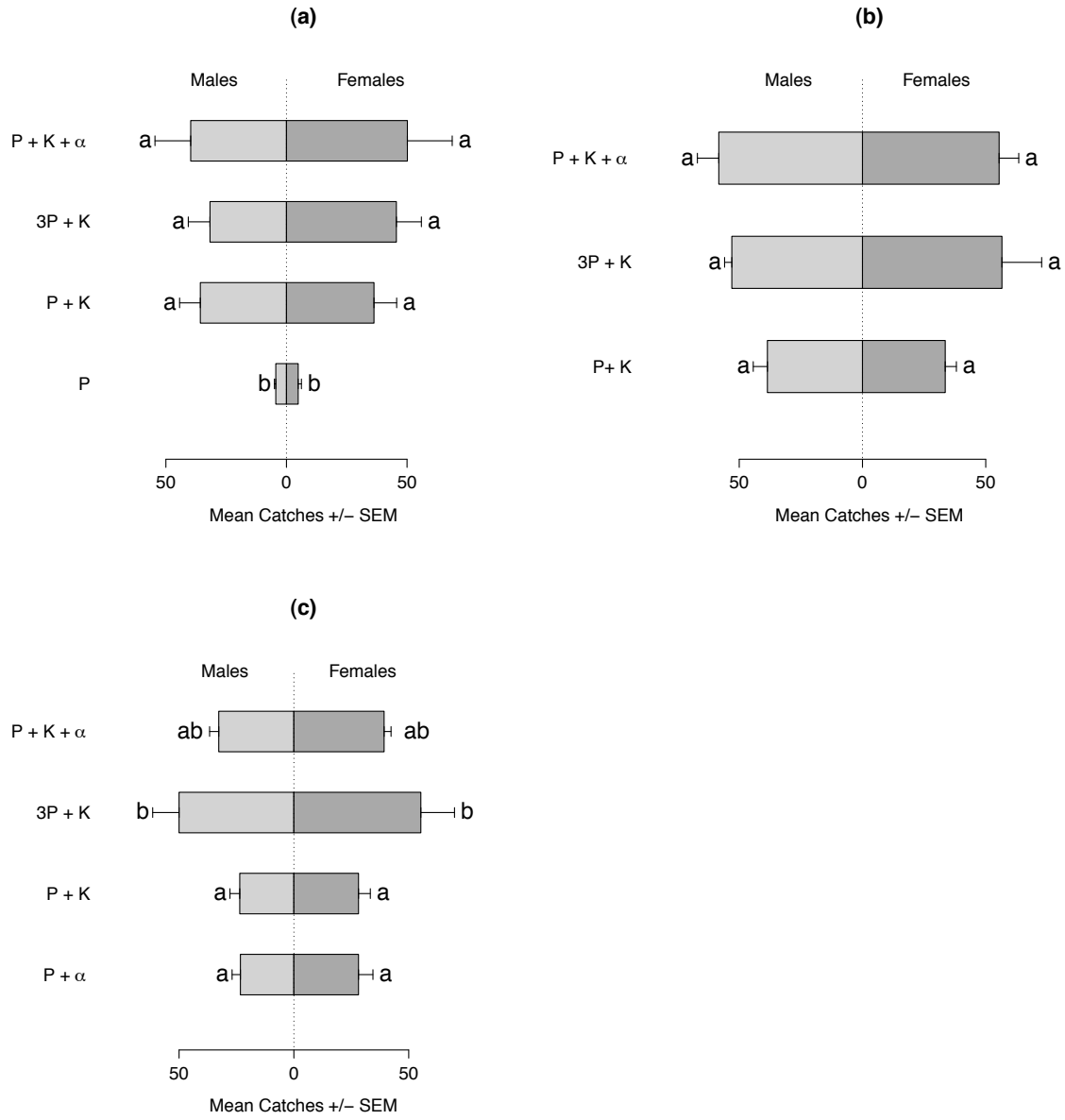


**Figure 3.5.** Mean (± SE) catches of *M. galloprovincialis* in experiments 4 (2009) (a), 5 (2010) (b) and 6 (2010) (c). Bars with the same letter are not significantly different (Tukey's honestly significant difference test, α = 0.05).

the blends incorporating α-pinene or 3-carene and (+)-limonene. All analyzed females trapped by any of the lures were sexually mature (i.e had eggs in their ovaries).

Comparable results were obtained from Experiment 8 in France. Though significant differences were not found (F = 1.49, P = 0.231, d.f. = 7; Figure 4.6b), the addition of β-pinene (+113.3%), α-pinene (+62.2%) and 3-carene (+33.33%) resulted in much higher catches, whereas p-cymene (-2.22%) and (+)-limonene (-24.4%) seemed to reduce catches.

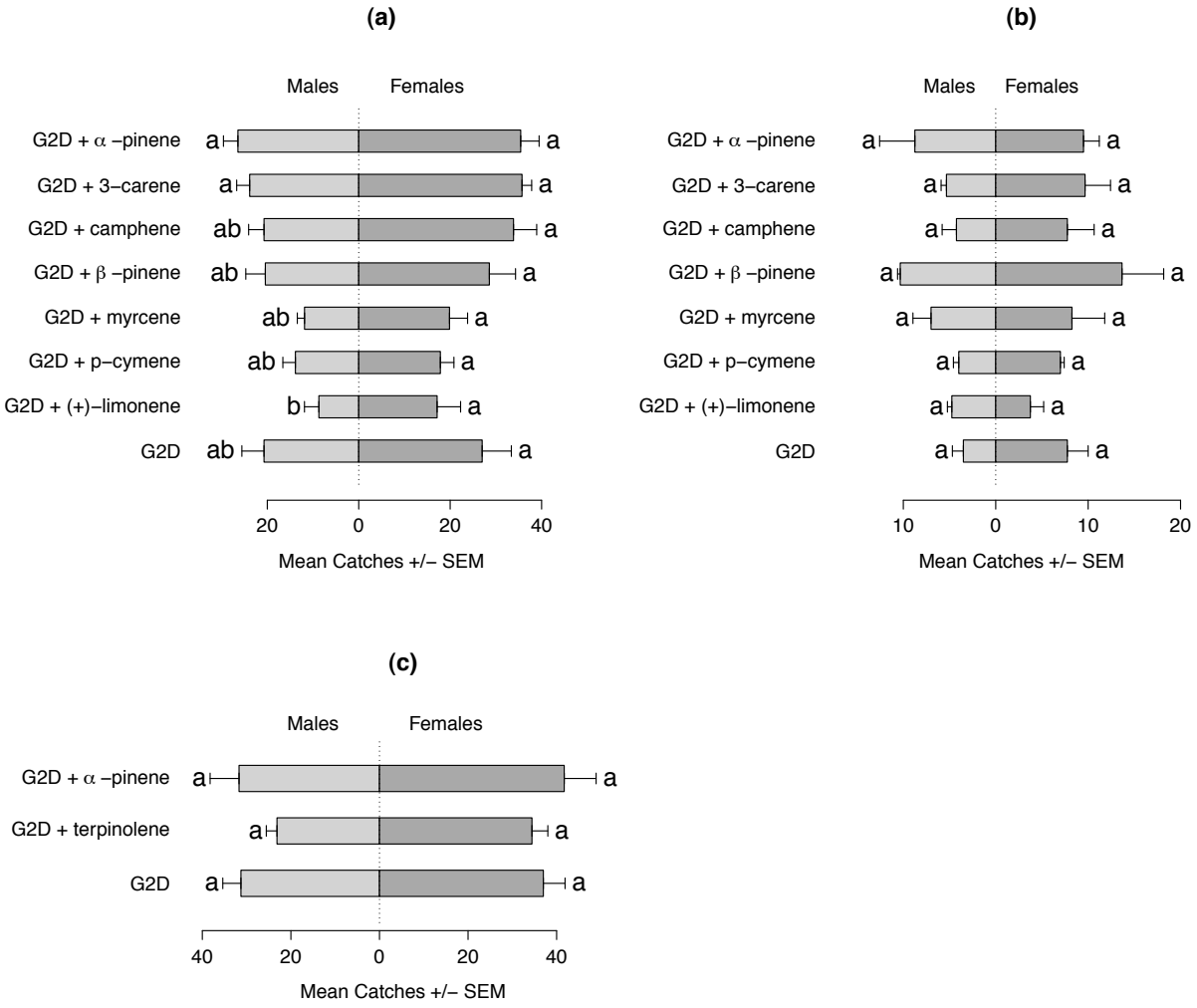


**Figure 3.6.** Mean ± SE catches of *M. galloprovincialis* in experiments 7 (2011) (a), 8 (2011) (b) and 9 (2013) (c). Bars with the same letter are not significantly different (Tukey's honestly significant difference test, α = 0.05).

Similarly, in the experiment where the effect of terpinolene was tested, no differences among treatments were observed (F = 1.04, P = 0.3841, d.f. = 2; Figure 4.6c). When added to the standard G2D lure, neither terpinolene nor α-pinene significantly increased catches (57.57 ± 5.58 insects/trap with G2D, 73.43 ± 13.67 with terpinolene added and 68.29 ± 7.75 with α-pinene added).

Finally, none of the 190 *M. galloprovincialis* immature adults that were released in Experiment 10 were re-captured in the terpene-baited or in the unbaited traps, suggesting that the terpene blend tested (α-pinene, β-pinene, 3-carene and terpinolene) was not attractive to these beetles, even in the absence of other likely competitive sources of attraction.

The overall combined results from all experiments where the effect of α-pinene was tested as synergist for standard pheromone-bark beetle kairomones blend (P+K or G2D) is shown in figure 3.7. Though all experiments except Experiment 1 showed positive effects ranging from 0.07 to 0.48, only in Experiment 5 it could be considered significant. The grand mean effect size (weighted mean of the seven individual effect sizes) resulted in +19.5% with confidence interval ranging from -8.5% to +44.6%, showing no significant effect of α-pinene.

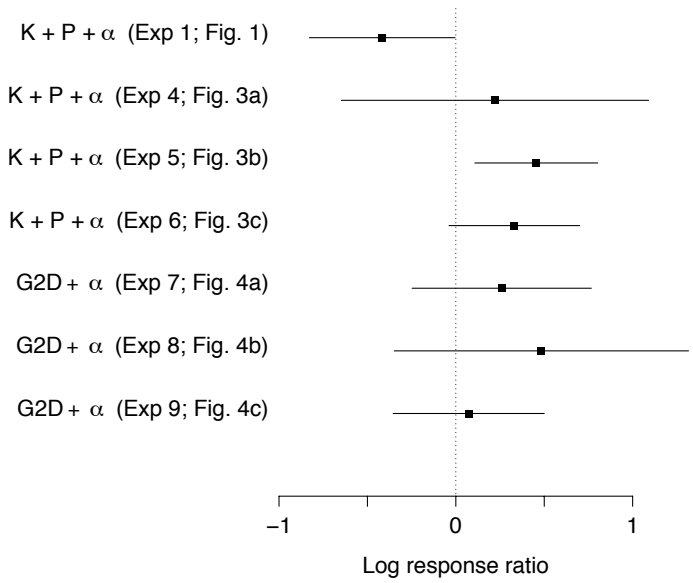


**Figure 3.7.** Forest plot of effect sizes based on log response ratio of means for the number of catches across different experiments, along with their 95% confidence interval (CI). Blend of *M. galloprovincialis* aggregation pheromone plus the bark beetle kairomones ipsenol and 2-methyl-3-buten-2-ol (notated P+K or G2D) has been set as the control treatment. Confidence intervals overlapping zero indicate the lack of any significant difference in number of catches with the control treatment.

Neither *T. coerulea* nor other non-target insects were collected from traps loaded only with *M. galloprovincialis* pheromone. However, when ipsenol and methyl-butenol were added to the pheromone lure (P+K or G2D), many individuals of this predator species were trapped. *T. coerulea* catches further increased (from 0.7-5.2 to 4.1-18 insects/trap) when the α-pinene was added (Table 4.2). This effect was also found with β-pinene.

**Table 4.2.** Mean *Temnochila coerulea* captured per trap in experiments. Mean values followed by the same letter are not significantly different (Tukey's honestly significant difference test, α = 0.05).

| Experiment | Attractants | Mean± SE | |
|---|---|---|---|
| 1 | P+ K | 5.29 ± 1.58 | a |
| | P+K+α | 18 ± 4.84 | b |
| | P | 0 ± 0.00 | c |
| 4 | P+ K | 0.43 ± 0.3 | a |
| | P+K+α | 6.71 ± 2.78 | b |
| | P | 0 ± 0.00 | c |
| 5 | P+ K | 5 ± 2.07 | a |
| | P+K+α | 17.43 ± 3.4 | b |
| 6 | P+ K | 0.71 ± 0.36 | a |
| | P+K+α | 4.14 ±1.3 | b |
| 7 | G2D | 5 ± 1.68 | a |
| | G2D+α | 14.14 ± 2.65 | b |
| | G2D+β | 9.86 ± 2.66 | b |
| | G2D+3c | 5.57 ± 1.21 | a |
| | G2D+cam | 7.86 ± 1.86 | ab |
| 9 | G2D | 0.86 ± 0.4 | a |
| | G2D+α | 4.29 ± 1.19 | b |

## Discussion

*Combination of pheromone and bark beetle kairomones*

*M. galloprovincialis* has been observed to locate suitable host trees for breeding by using kairomone-like compounds, i.e. host pine volatiles or bark beetle pheromones (Pajares *et al*. 2004; Ibeas *et al*. 2007), much like what has been reported for other North American *Monochamus* species (Allison *et al*. 2001, 2003; de Groot and Nott 2004; Miller and Asaro 2005). More precise identification of the *M. galloprovincalis* pheromone led to the discovery of synergistic effects with some of these kairomones, in particular α-pinene, ipsenol and 2-methyl-3-buten-2-ol (Pajares *et al*. 2010). If response to host terpenes such as α-pinene can orientate beetles toward suitable, i.e. recently damaged host, attraction by bark beetle pheromones may point to a higher degree of suitability for *Monochamus* oviposition in hosts where defences (i.e resin pressure) have been reduced by bark beetle attacks (Dyer and Seabrook 1978; Pajares *et al*. 2004). Pine sawyers might also benefit from breeding in material occupied by bark beetles, as *Monochamus* larvae have been found to prey on larvae of bark beetles and associated subcortical species (Dodds *et al*. 2001). The kairomonal volatiles ipsenol and 2-methyl-3-buten-2-ol are essential compounds of many aggregation pheromones in European Ipini species (Kohnle *et al*.

1988, 1993) and have been identified as two essential components of an effective lure for *M. galloprovincialis* (Ibeas *et al*. 2007, Pajares *et al*. 2010). Our results corroborate these previous findings (Pajares *et al*. 2010; Rassati *et al*. 2012) showing the synergistic effect of these volatiles in association with the *M. galloprovincialis* pheromone. In Experiment 1, for example, the triple combination of pheromones, bark beetle and host kairomones (P+K+$\alpha$-pinene and P+Kc+$\alpha$-pinene) caught 19.6% and 47.4% more insects, respectively than if the catches of the pheromone alone plus the kairomone blends alone (K+$\alpha$-pinene and Kc +$\alpha$-pinene) were added. This effect, though, was not as high as the 80% to 140% synergism reported by Pajares *et al*. (2010), probably due to differences in insect population levels.

Previous studies noticed that blends composed of the male-produced pheromone and the bark beetle kairomones (Pajares *et al*. 2010), or the kairomones alone (Ibeas *et al*. 2007) were usually more attractive for female *M. galloprovincialis*. In Experiments 1, 7, 8 and 9 slightly more females than males were captured, while in Experiment 5 the opposite occurred and in Experiments 4 and 6 no differences were apparent between sexes. From these results and the findings of Rassati *et al*. (2012), it cannot be concluded that females were more attracted than males by the pheromone-kairomone blends. However, our findings corroborated the bias of the pheromone alone toward capturing females. We observed a male to female ratio of 1:3 that was similar to the 1:3.5 ratio reported by Pajares *et al*. (2010).

*Quantitative effect of pheromone dose*

A clear dose-response was observed with *M. galloprovincialis* pheromone. Catches doubled when the pheromone dose was tripled, and tripled when five times the initial amount of pheromone was released. This confirms a dose-response reported by Pajares *et al*. (2010), with a similar magnitude as three times more females were captured when pheromone release was 6-fold increased. This finding led us to test whether increasing pheromone dose would also result in higher catches with the pheromone-bark beetle kairomones blend. Though no significant improvement was observed in the first two trials (Experiments 4 and 5), a significant increase was clearly observed in Experiment 6, where the number of catches with the 3P+K lure doubled that of P+K. This result was communicated to the lure manufacturer (SEDQ, Spain), which in 2011 began producing an

standard Galloprotect 2D lure that released a much higher rate of the *M. galloprovincialis* pheromone (Table 4.1).

*Combination of pheromone, bark beetle and host tree kairomones*

Alpha-pinene had previously been proposed as a host pine kairomone worth including in any effective kairomone or pheromone-kairomone blend for capturing *M. galloprovincialis* (Pajares *et al*. 2004; Ibeas *et al*. 2007; Francardi *et al*. 2009; Pajares *et al*. 2010; Rassati *et al*. 2012). However, this unspecific monoterpene was also found to attract a wide range of non-targeted xylophagous beetles in monitoring and mass trapping programs (Schroeder and Weslien 1994; Erbilgin and Raffa 2000; Etxebeste *et al*. 2012; Hofstetter *et al*. 2012; Miller *et al*. 2013, Macias-Samano *et al*. 2014). We observed the same side effects since significantly higher numbers of the predator *T. coerulea* (Coleoptera: Trogossitidae) were caught in pheromone traps if $\alpha$-pinene was added to the lures (Table 4.2). These natural enemies that can play an important role in regulating bark beetle populations (Reeve 1997; Turchin *et al*. 1991) are known to respond to bark beetle pheromones but also to pine semiochemicals such as $\alpha$-pinene (Schroeder 2003; Pajares *et al*. 2004; Gallego *et al*. 2008; Panzavolta *et al*. 2014). A few studies have investigated how changing trap designs might help to reduce trapping non-target species (Ross and Daterman 1998; Martin *et al*. 2013) but with no success. Pajares *et al*. (2010) also tried unsuccessfully to replace $\alpha$-pinene by verbenone, an oxidized derivative. In addition our experiments demonstrated that mean catches of pine sawyers obtained when $\alpha$-pinene was combined with standard pheromone-bark beetle kairomones blends did not increase significantly in most cases (Figure 4.5). Thus, the advantages of incorporating this terpene into the standard attractant lure are clearly outweighed by the detrimental effects of luring predators and other non-target organisms.

Further experiments looking at alternative host kairomones showed that none of the other tested terpenes performed significantly better, though several proved equally detrimental in attracting predators (Table 4.2). Interestingly, some of them resulted in lower catches than the standard lure alone; (+)-limonene in particular reduced catches by 46% and 25% in experiments in Spain and France, respectively. This result may align with hypotheses about the deterrent effect of (+)-limonene explaining the absence of infection

of PWN on *P. pinea* in Portugal (Naves *et al*. 2008; Sanchez Husillos *et al*. 2013). The potentially repellent effect of this terpene therefore merits further research.

*Trapping immature beetles*

Previous experiments using the mark-release-recapture technique to study pine sawyer dispersal found that immature *M. galloprovincialis* adults were not caught in traps baited with the Galloprotect 2D standard lure (Sánchez-Husillos *et al*. 2015). Only mature insects (10-14 days of shoot feeding after emergence) were lured into the traps. This fact is biologically coherent, since both the *M. galloprovincialis* agreggation pheromone and bark beetle kairomones are likely to signal the beetle a suitable host for breeding, something that would not be of interest to immature beetles. In contrast, we reasoned that host terpenes would signal suitable hosts for shoot feeding and therefore be meaningful to immature beetles. Previous EAG recordings had shown antennal response by immature beetles to several host terpenes. However, our trials within a pine forest stand found that no immature adults were caught in traps baited with a combination of Galloprotect 2D and some of the antennally bioactive terpenes. It was suggested that the high levels of terpenes emitted by nearby pine trees may have outcompeted the lures. This led us to ask whether immature beetles could be attracted by host volatiles in areas deprived of hosts, as might occur in cases where long distance human-transported beetles arrive in ports, warehouses, wood mills, etc., a situation where the monitoring of *M. galloprovincialis* is highly relevant to PWD management. Unfortunately, our results indicated that immature beetles remained unresponsive, even to a blend of pine volatiles that elicited EAG response released at the high dose of 2 g/day.

## Conclusions

Our experiments have corroborated the strong synergistic effect of ipsenol and 2-methy-buthen-3-ol with the *M. galloprovincialis* aggregation pheromone in attracting mature individuals of this species. The attractiveness of this combination was further improved by increasing the pheromone release rate, leading to the development of a new efficient commercial lure.

The addition of α-pinene or other pine terpenes is not advisable since it did not significantly improve the pheromone trapping effectiveness of the target species while inducing high catches of non-target species, including useful natural enemies. Only when maximizing the removal of vectors is considered a priority (i.e. PWD foci under eradication) the use of pine terpenes would be acceptable.

Future studies are needed to determine whether lure attractiveness and specificity can be further improved using other volatiles that are known to be detected by *M. galloprovincialis* antenna (e.g. smoke volatiles, Álvarez ***et al***. 2015) while being non attractive to non-target organisms. Another important research objective should be the identification of active compounds for trapping immature pine sawyers.

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# Chapter 5

# Attractants for management of the pine sawyer beetle *Monochamus sutor*

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## Introduction

Most longhorned beetles are considered secondary forest pests as they usually colonize woody plants previously weakened by other factors. However, those belonging to the *Monochamus* (Dejean) genus have become a primary concern because several of its species have been described as vectors of the Pine Wood Nematode (PWN) *Bursaphelenchus xylophilus* (Steiner and Buhrer), the causal agent of Pine Wilt Disease (PWD) (Linit and Akbulut, 2008). This North American nematode was introduced during the last century into several East Asian countries, with dramatic consequences (Mamiya & Enda, 1972; Shing, 2008; Zhao, 2008). It reached Portugal in 1999 (Mota *et al*. 1999), where despite containment efforts spread throughout the country and devastated large pine stands (Daub, 2008; Rodrigues, 2008). PWD crossed into Spain for the first time in 2008; four infection foci have been declared to date and eradication attempts are underway (Zamora *et al*., 2015). The obvious risk of contagion to other European countries has created an urgent need for tools that can detect and monitor both the nematode and the insect species that vectors it. Currently, only two European *Monochamus* species, *M. saltuarius* and *M. galloprovincialis*, have been confirmed as vectors of *B. xylophilus* (Schröder *et al*, 2009). However, *M. sutor*, which is widely distributed throughout Europe and Asia (CABI, 2012), has been hypothesized as capable of transmitting *B. xylophilus* if the nematode became associated to it (Schroeder & Magnusson, 1992; Evans *et al*., 1996).

Since the introduction of PWN into Asian countries, management of PWD through its insect vectors has been shown as a promising strategy to reduce spread of the disease.

Traps baited with specific lures have been shown to be preferable to insecticides which are hazard to non-target organisms and human health. In recent years, significant progress has been made in understanding the chemical ecology of European species such as *M. galloprovincialis* (Ibeas, 2007; Pajares 2004; 2010), resulting in highly attractive lures with practical applications in PWD management (Álvarez-Baz *et al*; 2015a). Improved traps (Álvarez *et al*; 2014) baited with a blend of pine sawyer pheromone, bark beetle and host kairomones are currently being used to eradicate pine wood nematode in the three active foci where pine wilt disease has been detected in Spain.

Recent identification of 2-undecyloxy-1-ethanol as the male-produced aggregation pheromone for *M. sutor* by Pajares *et al* (2013) has opened doors to the possibility of developing an efficient lure for this species. The same authors also reported that blends of the European *Ips* bark beetle pheromone components ipsenol, ipsdienol, cis-verbenol and methyl-butenol alone or supplemented by host volatiles α-pinene and 3-carene, attracted this species. Furthermore, pheromone attraction increased when combined with these supplementary blends. Though some of the host compounds tested (i.e α-pinene, 3-carene and ethanol) neither attracted on their own nor increased catches when added to the pheromone, other host volatiles might be. Some smoke compounds from burning wood have been shown to elicit EAG responses in *M. galloprovincialis*, due to smoke-specialized olfactory receptor neurons detected in the antennae of this species (Álvarez-Baz *et al*; 2015b). Apart from the ease with which they can be found in burnt pine stands, research has provided evidence that *Monochamus* beetles are attracted to burnt trees and colonize them (Parmelee, 1941; Ross, 1960; Forsslund, 1934; Ehnström *et al*., 1995). Thus, smoke volatiles might play a role in *M. sutor* host location.

In this work, we present the results of three years of field trapping assays in three countries, aimed at developing an effective lure based on the aggregation pheromone and kairomones that could be used operationally for management of *M. sutor*. The work included studies of EAG and field responses of M. sutor to four European *Ips* kairomones, the *Pityogenes chalcographus* pheromone, chalcogran, as well as field responses to host volatiles $\alpha$-pinene and a to blend of smoke volatiles as pheromone synergists.

## Materials and methods

*Insects*

During 2012 and 2013, adult *M. sutor* were collected in multifunnel traps baited with 2-undecyloxy-1-ethanol, ipsenol, ipsdienol, and α-pinene (cf. Pajares *et al*., 2010) in Torla, Huesca, Spain, 42º37'40"N, 0º5'24"W. They were sexed, placed individually in glass tubes, and shipped by courier to the Natural Resources Institute, Greenwich (UK). They arrived within 2 days and were maintained on Scots pine shoots, *Pinus sylvestris* L. (Pinaceae) in an insectary on an L12(25 °C):D12(20 °C) photo-thermo cycle at ambient humidity. Adult beetles collected in the same way were allowed to oviposit on Scots pine logs kept in the laboratory at the University of Valladolid in Palencia (Spain) under ambient conditions of temperature, humidity, and lighting. Emerging adults were collected daily, kept separate by sex under the same conditions and fed on Scots pine shoots for 2 weeks before shipping to the UK as above.

*Gas Chromatography (GC) Coupled to Electroantennographic (EAG) Recording.*

For the GC-EAG analyses an HP6890 GC (Agilent Technologies, Stockport, Cheshire, UK) was used, fitted with fused silica capillary columns (30 m x 0.32 mm i.d. x 0.25 µ film thickness) coated with both polar DB Wax (Agilent) and non-polar SPB-1 (Supelco, Gillingham, Dorset, UK) phases. The ends of the two columns were connected to a short piece of deactivated fused silica tubing with a glass, push-fit Y-piece. The outlet was then split by means of a similar Y-piece, with half going to the flame ionization detector and half to a silanized glass T-piece (arms 5 cm, i.d. 4 mm), using similar lengths of deactivated fused silica tubing. One arm of the T-piece was connected to a device delivering compressed air (300 ml/min) either continuously or in a 3-sec pulse at 17-sec intervals. The third arm of the T-piece passed through the GC oven wall to the insect EAG preparation (Cork *et al*. 1990). All analyses were carried out on the polar GC column with helium carrier gas (2.4 ml/min), splitless injection (220°C), flame ionization detection (250°C) and oven temperature programmed at 50°C for 2 min then increasing by 10°C/min to 240°C. In some analyses the 2-undecyloxy-1-ethanol (10 ng) pheromone was added as a positive control.

EAG recording was carried out with a portable device (INR-02; Syntech, Hilversum, The Netherlands) consisting of integrated electrode holders, micromanipulators and an amplifier. Electrodes consisted of silver wires fitted with glass electrodes pulled to a fine point with an electrode puller and containing saline solution (0.1 M potassium chloride with 1% polyvinylpyrrolidine to reduce evaporation). An antenna was excised at the base and suspended between the glass electrodes which were cut so that they just accommodated the ends of the antenna. The signal was amplified 10x and the amplifier was connected to the GC as a detector device. Data were processed with EZChrom Elite v3.0 software (Agilent).

Bark beetle kairomones ipsenol, ipsdienol, *Cis*-verbenol and verbenone, and *M. sutor* pheromone were tested on the antenna of both sexes. Besides, 3-carene, terpinolene, 2-methyl-3-buten-2-ol and chalcogram were tested on *M. sutor* females. All bark beetle kairomones were obtained from the Sociedad Española de Desarrollos Químicos (SEDQ, Barcelona, Spain), except for *Cis*-verbenol (95%) and verbenone (94%), that were obtained, together with the two host terpenes, from SigmaAldrich (Gillingham, Dorset, UK). Chalcogran was obtained from Witasek (Feldkirchen in Kärnten, Austria).

*Field tests in Spain*

Two field trials were carried out, in 2012 and 2013, in the Pyrenees mountains near Torla (Huesca, Aragon, Spain), between 42º37’40”N, 0º5’24”W and 42º38’44”N, 0º4’07”W, and from 1609 to 2066 m altitude. The area was forested by *M. sutor* hosts, mainly natural stands of *Pinus uncinata* Mill. ex Mirb mixed with *P. sylvestris* at lower altitudes. To assay the increase in attractiveness of bark beetle and host kairomones when released together with the *M. sutor* pheromone, six treatments were tested each year in 12-unit multiple funnel Teflon-coated traps fitted with 2-l wire-screened bottom collection containers designed for live trapping (ECONEX MULTIFUNNEL-12®, Econex SL, Murcia, Spain). Treatments tested in 2012 (Experiment 1) were as follows: The *M. sutor* pheromone 2-undecyloxy-1-ethanol (P) released at ca. 2 mg per day; three combinations of the pheromone plus two bark beetle pheromone components: racemic ipsenol (ca. 2.5 mg per day) and (S)-cis-verbenol (ca. 2.5 mg per day) (P+I+cV), racemic ipsenol and racemic ipsdienol (ca. 2.5 mg per day) (P+I+Id), and racemic ipsenol and racemic 2-methyl-3-buten-2-ol (ca. 10 mg per day) (P+I+M), the prior blend plus the host volatile (±)-α-pinene

(ca. 500 mg per day) (P+I+M+α), and the last combination plus a blend of 6 smoke volatiles (2-methoxyphenol, 4-methyl-2-methoxyphenol, phenol, 4-methylphenol, 2,6-dimethoxyphenol and eugenol (ca. 80 mg per day) (P+I+M+α+S).

In 2013 (Experiment 2), the following treatments were tested: *M. sutor* pheromone (P), the pheromone released together with several bark beetle volatiles: chalcogran (c.a 50 mg per day) (P+C), racemic 2-methyl-3-buten-2-ol (P+M), racemic ipsenol (P+I), racemic ipsenol plus racemic 2-methyl-3-buten-2-ol (P+I+M), and racemic ipsenol plus chalcogran (P+I+C).

All volatiles except the smoke blend were released individually. Pheromone, host and bark beetle dispensers were provided by SEDQ (Barcelona, Spain), and chalcogran dispenseres were provided by Witasek (Feldkirchen in Kärnten, Austria). The smoke blend was prepared by NRI (U. of Greenwich). Traps were hung ca. 2 m above ground from ropes tied between trees. A replicate of each treatment was randomly deployed within each of six experimental blocks in a randomized complete block design. Traps were at least 90 m apart and nearest blocks were 500 m apart. Traps were deployed from 27 June to 8 August 2012 and from 4 July to 4 September 2013. Trapped beetles were collected weekly, identified and sexed (Vives, 2000).

*Field tests in Sweden*

Field experiments were carried out in the Uppland province of central Sweden in 2012 (one experiment) and 2013 (four experiments) within a 12 x 36-km area centred at 60º07'N, 18º07'W. *Pinus sylvestris* and Norway spruce, *Picea abies* (L.) Karst., are the dominant tree species in the region. More than 95% of the forest land consisted of even-aged stands, managed by thinnings from below and harvested by clear-cutting at an age of about 80 years. All experiments were conducted on spruce clear-cut areas that had been harvested during the previous winter. Beetles were caught in 12-unit multiple funnel traps provided with collector cups containing water (ECONEX MULTIFUNNEL-12®, Econex SL, Murcia, Spain) and hung from 1.8 m wooden poles. The traps were arranged 50 m apart in blocks and at a distance of 50 m from stand edges facing the clear-cuts. When more than one block was placed on the same clear-cut, the distance between blocks was at least 50 m. In all experiments, traps were set up in randomized block design

with 10 blocks. Trapping periods are listed in the legend of Fig. 3. In the 2012 experiment, the blocks were distributed on six clear-cuts; in 2013 the blocks were distributed on three clear-cuts in Experiments 2 and 3 and 10 clear-cuts in Experiments 4 and 5. Intervals between trap emptying varied from 4 to 16 days.

One field trial (Experiment 1) was conducted in 2012, with the following six treatments: *M. sutor* pheromone 2-undecyloxy-1-ethanol (P); three combinations of the pheromone plus two bark beetle pheromone components: racemic ipsenol and (S)-cis-verbenol (P+I+cV), racemic ipsenol and racemic ipsdienol (P+I+Id), the pheromone plus racemic ipsenol and racemic 2-methyl-3-buten-2-ol (P+I+M), the pheromone and a blend of 6 smoke volatiles: 2-methoxyphenol, 4-methyl-2-methoxyphenol, phenol, 4-methylphenol, 2,6-dimethoxyphenol and eugenol (P+ S) and unbaited control traps. The sources and release rates for the compounds were as described for the experiments in Spain. In 2013 four experiments (2 to 5) were conducted with the following treatments: (Exp. 2) one unbaited control and one chalcogran bark beetle volatile treatment (C); (Exp. 3) one *M. sutor* pheromone (P) treatment and a combination treatment of the pheromone plus chalcogran bark beetle volatile (P+C); (Exp. 4) six treatments consisting of the *M. sutor* pheromone (P), the four bark beetle volatiles racemic ipsenol (I), racemic ipsdienol (Id), (S)-cis-verbenol (cV) , racemic 2-methyl-3-buten-2-ol (M) and an unbaited control, and (Exp. 5) six treatments consisting of the *M. sutor* pheromone (P), racemic ipsenol (I), and four combinations of the pheromone plus one of the bark beetle volatiles: racemic ipsenol (P+I), racemic ipsdienol (P+Id), (S)-cis-verbenol (P+cV) and racemic 2-methyl-3-buten-2-ol (P+M). Experiment 2, which is not included in Fig. 5, was conducted from 11 to 30 July. For the other experiments trapping periods are given in legend of Fig. 5. The sources and release rates of the compounds were as described for the experiments in Spain.

*Field tests in Austria*

Four field trials were carried out in Austria in 2012, 2013 and 2014 using traps and volatile compounds with the same sources and release rates as those described for the experiments in Spain and Sweden. Traps were hung from 2 m high wooden poles and were set up in open areas of mixed coniferous forests in mountainous zones with substantial amounts of dead wood after windthrow and bark beetle attack. The minimum distance between individual traps was 40 m.

Experiments were set up in the Dürrenstein wilderness area in 2012 and 2013 (47°46'N, 15°02'E; 900-970 m elevation). Forest stands consist of mixed forests dominated by Norway spruce and beech. The mature spruce stands have incurred significant mortality since 2000 due to bark beetle (*Ips typographus*) attack and an avalanche in 2009. Three treatments were tested in the 2012 experiment (Experiment 1) involving the *M. sutor* pheromone combined with: racemic ipsenol and racemic 2-methyl-3-buten-2-ol (P +I+M) bark beetle volatiles, the prior combination plus the (±)-α-pinene host volatile (P+I +M+α), and the previous combination plus the 6 smoke volatile blend (P+I+M+α+S). Traps were set up in a randomized block design in four blocks (= replicates). Trapping lasted for 40 days starting July 10; assignment of treatments to positions was re-randomized every 10 days. Traps were checked and emptied every 3 or 4 days. Three treatments were tested on the same site in 2013 (Experiment 2): *M. sutor* pheromone (P) and a combination of the pheromone plus racemic ipsenol and racemic 2-methyl-3-buten-2-ol (P +I+M) or plus racemic ipsenol and chalcogran (P+I+C). Traps were set up in five blocks. Trapping lasted for 42 days starting July 8; assignment of treatments to positions was re-randomized every 14 days. Traps were checked and emptied every 3 or 4 days.

One experiment in 2013 (Experiment 3) was set up in the Oiswald natural forest reserve (47°46'N, 15°12'E; 1150-1260 m elevation). Windthrow in 2007 and a subsequent extended outbreak of *Ips typographus* (beginning in 2009) had caused significant tree mortality in a mature spruce-dominated stand. Two treatments were tested: *M. sutor* pheromone combined with racemic ipsenol and racemic 2-methyl-3-buten-2-ol (P+I+M), and the bark beetle kairomones alone (I+M). Traps were installed in five blocks on July 2 for a 42 days trapping period. They were checked and emptied weekly, and the assignment of treatments to positions was re-randomized half-way through the trapping period. Finally, one experiment in 2014 (Experiment 4) was set up in a managed, spruce dominated forest at Nasswald (47°46N, 15°39'E; 1040-1220 m elevation). Wind and snow break had created breeding material for beetles. Three treatments were tested in the experiment: the *M. sutor* pheromone (P) alone, a blend of racemic ipsenol and racemic 2-methyl-3-buten-2-ol (I+M), and a combination of the three (P+I+M). Traps were set up in five blocks on July 3 for a 42 day trapping period. They were checked and emptied weekly, and the assignment of treatments to positions was re-randomized every two weeks.

*Statistical analysis*

The total number of beetles caught during each of the experimental periods was used as the response variable. The response was fitted against treatment and block factors and to a Poisson error distribution in a generalized linear model (GLM) (Crawley, 2007). If significant treatment effects (P<0.05) were detected, Tukey's honestly significant difference test with Bonferroni adjustment to the value of α = 0.05 was used for mean comparisons (Reeve & Strom, 2004). All statistical computing was carried out using the R language and environment (The R Development Core Team, 2011). To allow for comparisons of the synergistic effect of ipsenol (I) and 2-methyl-3-buten-2-ol (M) on the *M. sutor* pheromone across the different experiments, effect sizes on log transformed ratio of means for the number of catches (Hedges *et al*., 1999), along with their 95% confidence interval (CI), were also calculated and represented in a forest plot, setting the *M. sutor* pheromone (P) as the control treatment.

## Results

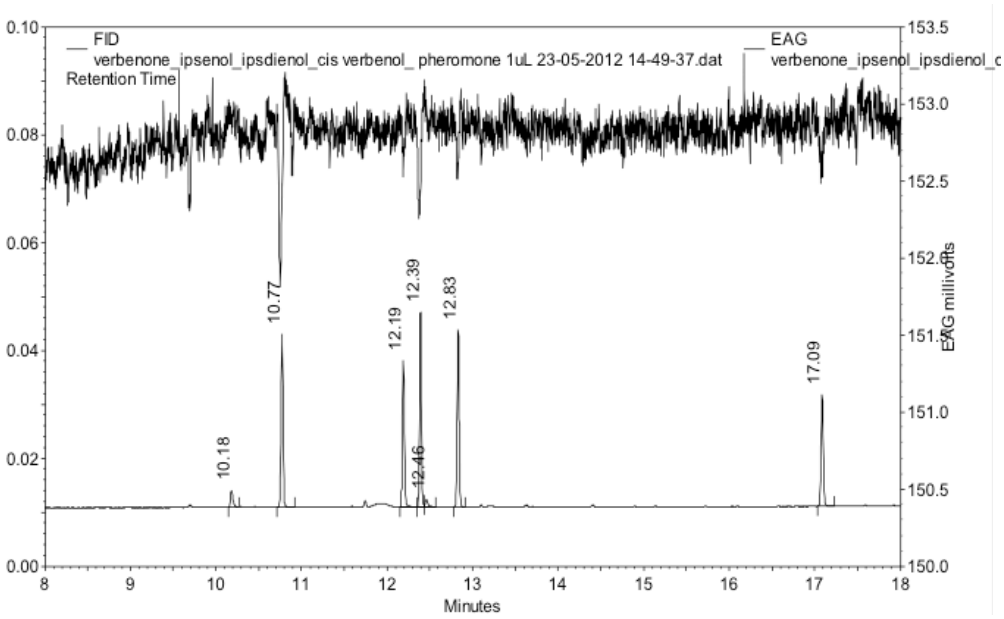


**Figure 5.1** - GC-EAG Analyses of kairomones with antenna of *Monochamus sutor* male (10 ng injected, 5 ng to EAG; ipsenol 10.77 min; *cis*-verbenol 12.19 min; ipsdienol 12.39 min; verbenone 12.83 min; pheromone 17.08 min)

*Electroantennogram recording*

*M. sutor* males and females registered consistent EAG responses to ipsenol and ipsdienol (Figures 5.1 and 5.2). Females sometimes gave a strong response to 2-methyl-3-buten-2-ol (Fig. 5.2) and both sexes responded weakly to verbenone (Figs. 5.1 and 5.2) but not to cis-verbenol. Females responded to chalcogran (Fig. 5.3), but it was not tested on males.

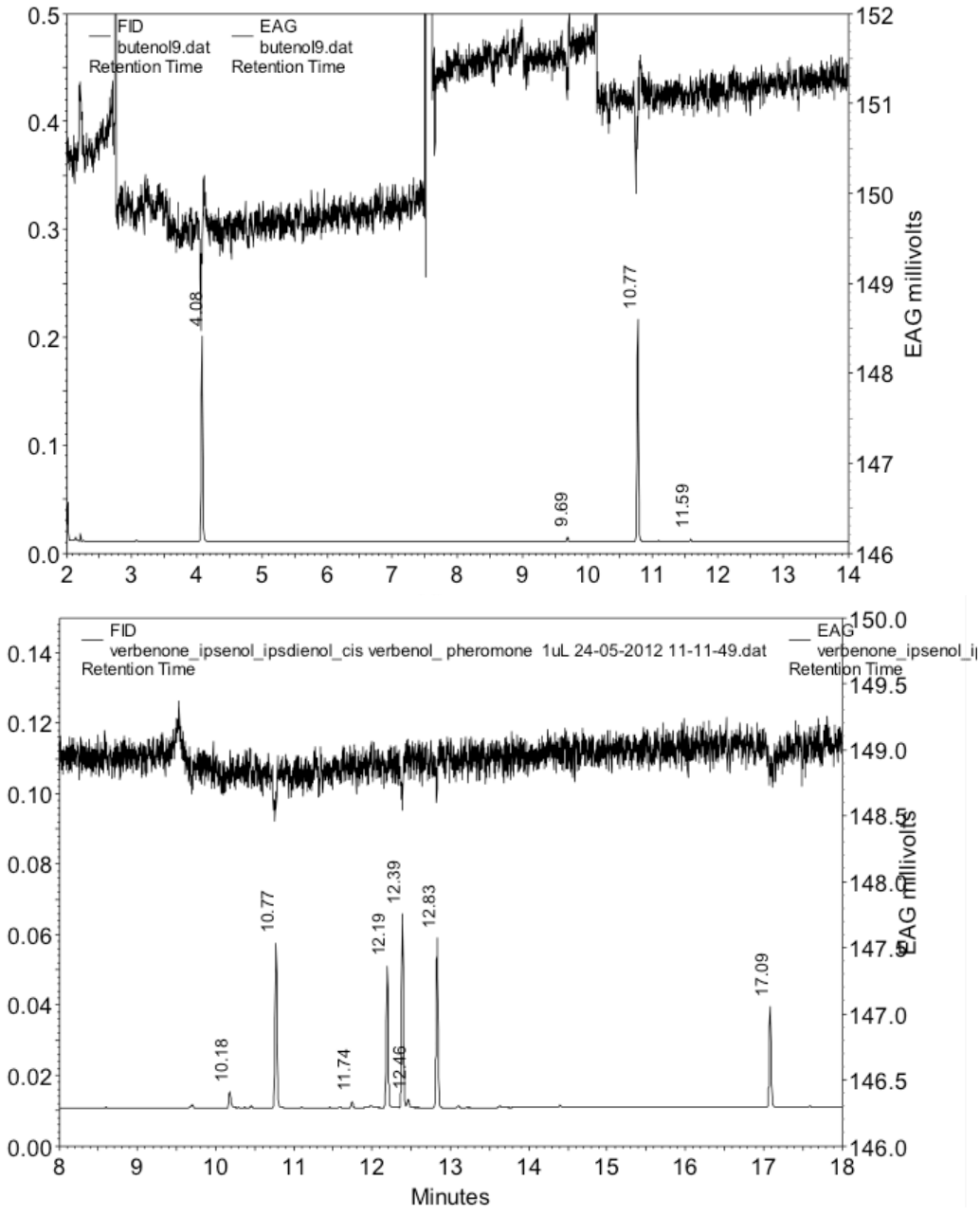


**Figure 5.2.** GC-EAG Analyses of kairomones with antenna of *Monochamus sutor* female (2-methyl-3-buten-2-ol 4.08 min; ipsenol 10.77 min; *cis*-verbenol 12.19 min; ipsdienol 12.39 min; verbenone 12.83 min; pheromone 17.09 min)

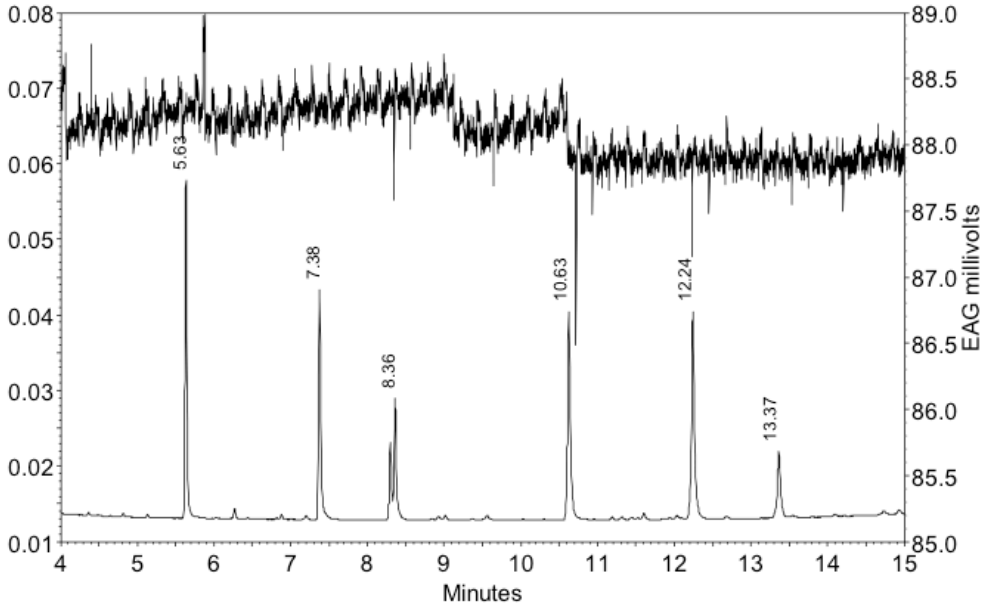


**Figure 5.3.** GC-EAG Analysis on polar GC column with *M. sutor* female EAG preparation (10 ng injected, 5 ng to EAG; 3-carene 5.63 min, terpinolene 7.38 min, chalcogran 8.36 min (2 isomers), ipsenol 10.63 min, ipsdienol 12.24 min, methyl salicylate 13.37 min)

*Field test in Spain*

No significant differences between captured male (F =1.97, P=0.11, d.f. = 5) or female *M. sutor* (F=2.24, P=0.08, d.f.=5) were found in 2012 Experiment 1 in Spain (Fig 5.4a). Results showed that even if the joint release of the pheromone and any of the kairomone blends resulted in a noticeable increase in catches, the differences were only significant for females caught by the pheromone plus ipsenol and 2-methyl-3-buten-2-ol treatment (P+I+M) compared to the pheromone alone (P). This combination obtained the highest male and female catches of all treatments. When the bark beetle pheromone components were tested individually or in two compound blends with the pheromone in Experiment 2 (2013), significant differences were found for males (F=3.84, P=0.01, d.f.=5) but not for females (F=2.33, P=0.07, d.f.=5) (Fig 5.4b). Release of the pheromone with either 2-methyl-3-buten-2-ol (P+M) or chalcogran (P+C) did not cause any increase in catches of of *M. sutor* compared with pheromone alone (P), but more sawyers were caught when ipsenol was used (P+I) (5.83 ± 2.12 and 8.5 ± 2.67 insects/trap for males and females, respectively) compared to the pheromone alone (P) (2.33 ± 0.61 and 3.67 ± 1.31 insects/trap for males and females, respectively). None of the two-compound bark beetle blends releasing ipsenol resulted in further significant increases in catches compared to

ipsenol alone. However, as in the previous experiment, the pheromone, ipsenol and 2-methyl-3-buten-2-ol combination (P+I+M) obtained the highest mean catches for males (6.83 ± 1.27 insects/trap) and females (8.83 ± 1.72 insects/trap).

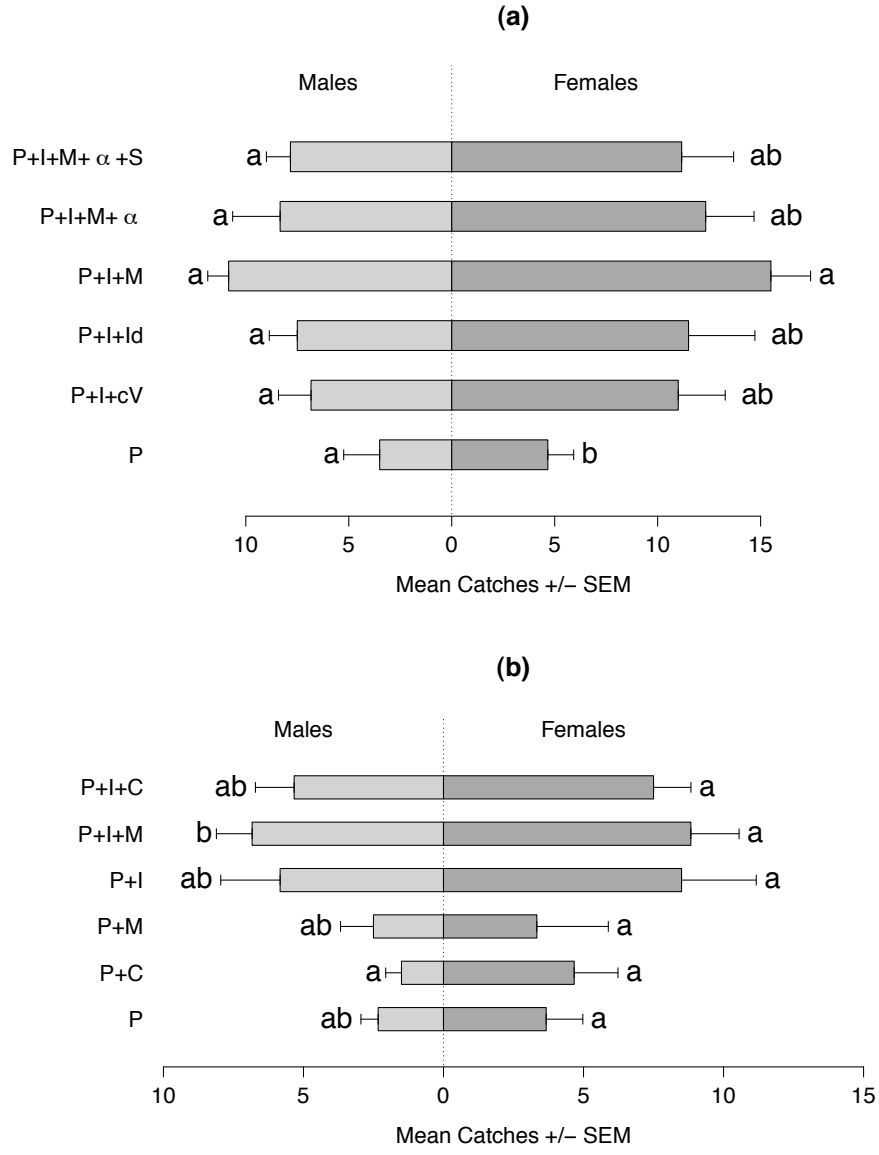


**Figure 5.4.** Mean catches (+ SE) of *Monochamus sutor* between 27 June and 8 August 2012 2012 (a) and between 4 July and 4 September in 2013 (b) in Huesca, Aragon, Spain, using multi-funnel traps. Treatments were (a) *M. sutor* pheromone (P), pheromone plus ipsenol and *cis*-verbenol (P+I+cV), pheromone plus ipsenol and ipsdienol (P+I+Id), pheromone plus ipsenol and 2-methyl-3-buten-2-ol (P+I+M), the previous blend plus (±)-α-pinene (P+I+M+α), the previous blend plus a blend of 6 smoke volatiles (P+I+M+α+S) (n = 6 replicates); and (b) pheromone (P), pheromone plus chaocogran (P+C), pheromome 2-methyl-3-buten-2-ol (P+M), pheromone plus ipsenol (P+I), pheromome plus ipsenol plus 2-methyl-3-buten-2-ol (P+I+M) and pheromome plus ipsenol plus chalcogran (P+I+C) (n = 6 replicates). Means with different letters are significantly different (Tukey's HSD test after Bonferroni correction: $P<0.05$).

*Field tests in Sweden*

Significant differences among treatments were found for males (F=8.25, P<0.001, d.f.=5) and females (F=11.44, P<0.001, d.f.=5) in experiment 1 (2012) in Sweden (Fig. 5.5a). Results were similar to those obtained in Spain with no significant increase in catch when the binary combinations of bark beetle pheromones were added to the *M. sutor* pheromone. Neither did smoke volatile blend (S) increase the attraction to the *M. sutor* pheromone. The *M. sutor* pheromone alone, and all combinations with bark beetle pheromones, caught significantly higher numbers of males and females than unbaited control.

Experiment 2 (2013) demonstrated that bark beetle pheromone chalcogran released alone was not attractive to *M. sutor* (F=3.35, P=0.07, d.f.=1 for males and F=2.11, P=0.15, d.f.=1, for females) (not shown in Fig 5.5). Mean catches in traps baited with chalcogran were 0.3 ± 0.15 males and 0.0 ± 0.0 females and in unbaited controls 0.0 ± 0.0 males and 0.2 ± 0.2 females. The low catches were not a result of low activity of *M. sutor* because the experiment was conducted at the same time as experiment 1.

In experiment 3 (2013) the combination of chalcogran and *M. sutor* pheromone caught significantly lower numbers of males than the pheromone alone, while there was no significant difference for females (Fig 5.5b) (F=3.16, P=0.08, d.f.=1 for males and F=0, P=1, d.f.=1, for females).

In experiment 4 (2013), comparing attraction to single bark beetle pheromones with *M. sutor* pheromone and unbaited traps, significant differences in catches were found between treatments (F=14.06, P<0.001, d.f.=5 for males and F=78.95, P<0.001, d.f.=5, for females) (Fig 5.5c). Only *M. sutor* pheromone and Ipsenol were significantly different from unbaited traps. For males there was no significant difference between *M. sutor* pheromone and Ipsenol while for females the catches were higher for the pheromone than for Ipsenol.

In experiment 5 (2013), comparing attraction to four combinations of *M. sutor* pheromone and single bark beetle pheromones with *M. sutor* pheromone alone and Ipsenol alone, treatments differed significantly (F=7.99, P<0.001, d.f.=5 for males and F=9.03, P<0.001, d.f.=5 for females) (Fig. 5.5d). Only the combination of *M. sutor*

pheromone and Ipsenol caught significantly higher numbers of beetles, both for males and females, than *M. sutor* pheromone alone. In this case the combination (P+I) resulted in a synergistic effect since catches obtained by the combination (6.2 ± 1.46 and 9.7 ± 1.62 insects/trap for males and females respectively by P+I) were significantly higher (t=2.82, P = 0.012, d.f.=17.08) than the addition of catches obtained individually (1.6 ± 0.45 and 3.4 ± 0.87 insects/trap for males and females respectively by P and 1.1 ± 0.31 and 0.6 ± 0.42 insects/trap for males and females respectively by I).

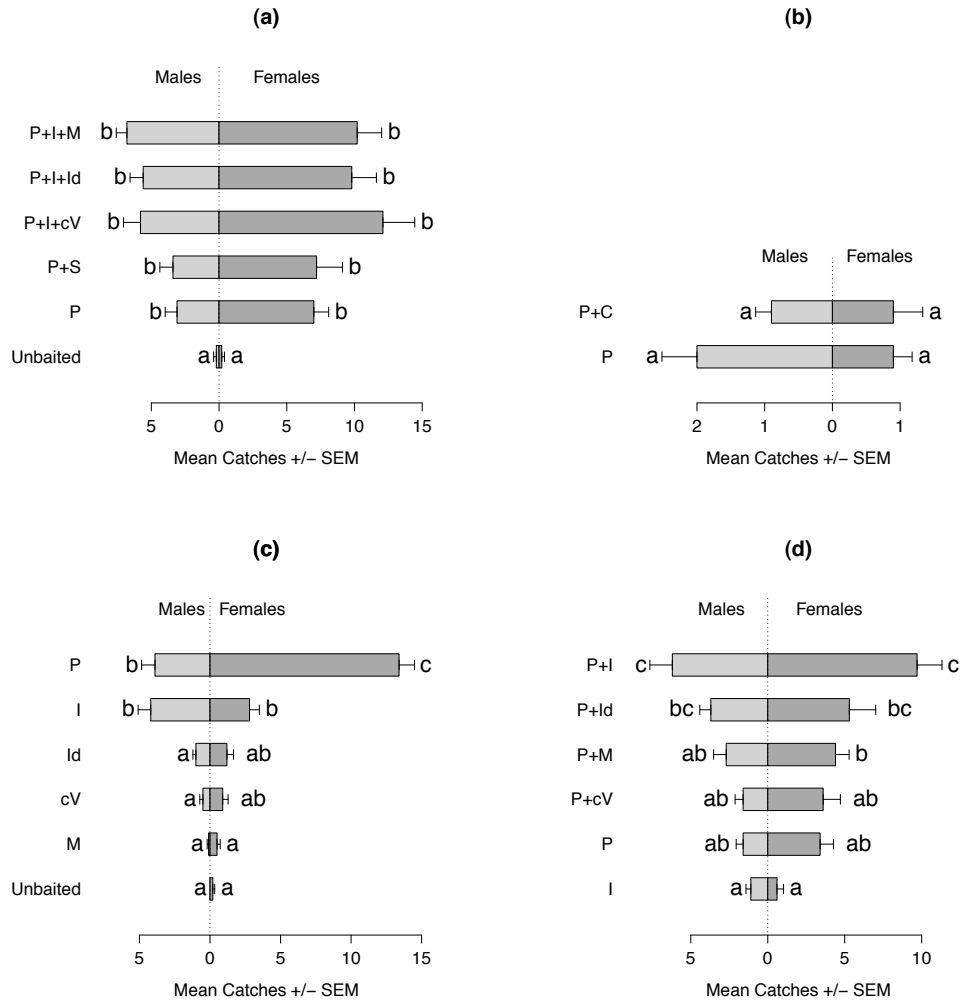


**Figure 5.5.** Mean catches (+ SE) of *Monochamus sutor* between 21 June and 23 August 2012 (a), 11 and 30 July 2013 (b), 20 May and 4 July 2013 (c) and 4 July and 6 September (d) 2013 in Sweden, using multifunnel traps. Treatments were (a) *M. sutor* pheromone (P), pheromone plus blend of 6 smoke volatiles (P+S), pheromone plus ipsenol and *cis*-verbenol (P+I+cV), pheromone plus ipsenol and ipsdienol (P+I+Id), pheromone plus ipsenol and 2-methyl-3-buten-2-ol (P+I+M) and unbaited control (n = 10 replicates); (b) *M. sutor* pheromone (P) and pheromone plus chalcogran (P+C) (n = 10 replicates); (c) *M. sutor* pheromone (P), ipsenol (I), ipsdienol (Id), *cis*-verbenol (cV) , 2-methyl-3-buten-2-ol (M) and unbaited control (n = 10 replicates); (d) *M. sutor* pheromone (P), ipsenol (I), pheromone plus ipsenol (P+I), pheromone plus ipsdienol (P+Id), pheromone plus *cis*-verbenol (P+cV) and pheromone plus 2-methyl-3-buten-2-ol (P+M) (n = 10 replicates). Means with different letters are significantly different (Tukey's HSD test after Bonferroni correction: P<0.05).

*Field test in Austria*

Experiment 1 in Dürrenstein, Austria (2012), found no significant differences among the three pheromone-kairomone blends tested (F=0.03, P=0.96, d.f.=2 for males and F=0.88, P=0.46, d.f.=2 for females) (Fig 5.6a). The combination of the *M. sutor* pheromone plus the two bark beetle kairomones ipsenol and 2-methyl-3-buten-2-ol (P+I+M) obtained the highest mean catches (4 ± 1.73 and 7.25 ± 2.5 insects/trap for males and females, respectively), which were not improved by the addition of α-pinene (α) alone or in combination with the smoke volatile blend (S).

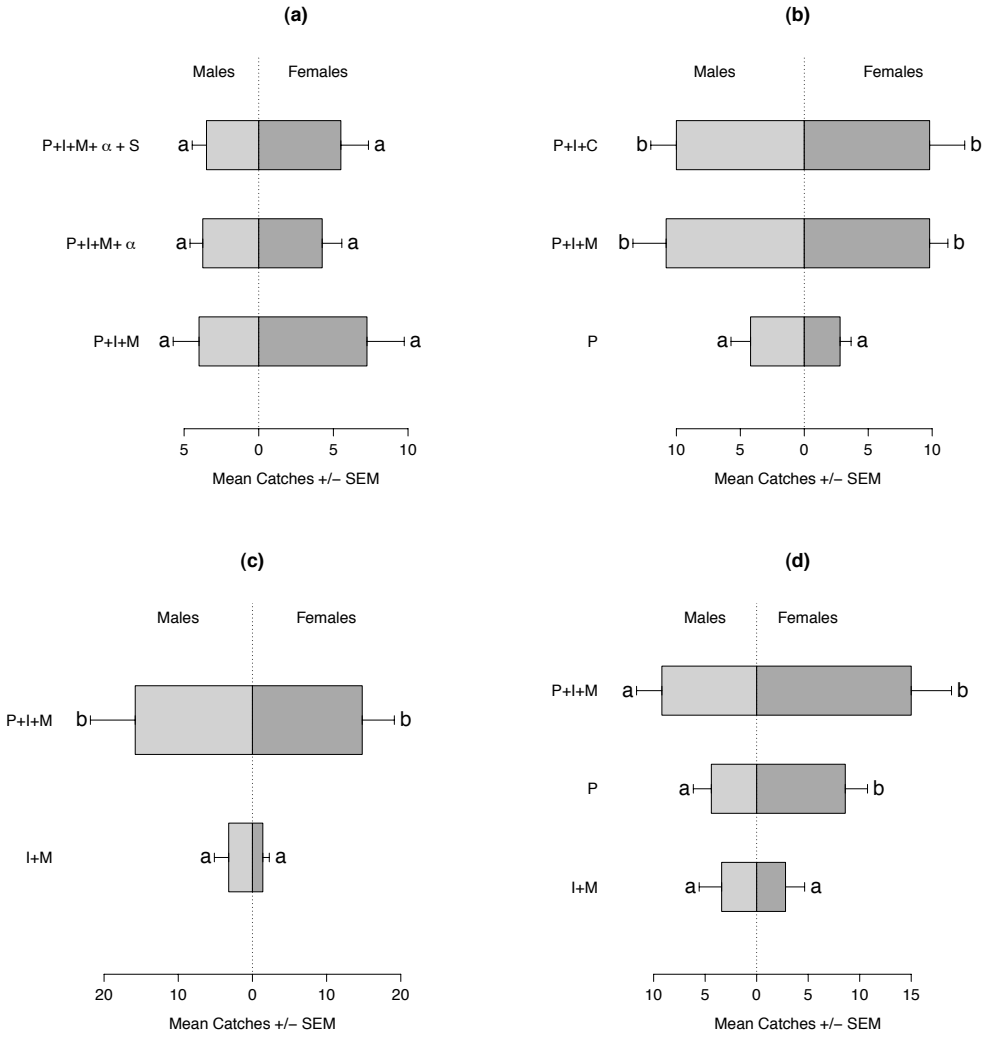


**Figure 5.6.** Mean catches (+ SE) of *Monochamus sutor* between 10 July and 19 August 2012 (a), 8 July and 19 August 2013 (b), 2 July and 13 August 2013 (c), and 3 July to 15 August 2014 (d) in Austria in multifunnel traps. Treatments were (a) *M. sutor* pheromone plus ipsenol and 2-methyl-3-buten-2-ol (P+I+M), the later plus (±)-α-pinene (P+I+M+α), the later plus a 6 smoke volatile blend (P+I+M+α+S) (n = 4 replicates); (b) *M. sutor* pheromone (P), pheromone plus ipsenol and 2-methyl-3-buten-2-ol (P+I+M), pheromone plus ipsenol and chalcogran (P+I+C) (n = 5 replicates); (c) *M. sutor* pheromone plus ipsenol and 2-methyl-3-buten-2-ol (P +I+M) and the bark beetle kairomones alone (I+M) (n = 5 replicates); (d) *M. sutor* pheromone (P), ipsenol and racemic 2-methyl-3-buten-2-ol (I+M), and a combination of the three (P+I+M) (n = 5 replicates). Means with different letters are significantly different (Tukey's HSD test after Bonferroni correction: P<0.05).

A field trial conducted at the same site in 2013 (Experiment 2, Fig 5.6b) tested chalcogran as a potential substitute for 2-methyl-3-buten-2-ol in the pheromone-bark beetle kairomone triple blend. Both blends were significantly more attractive than the pheromone alone (F=12.9, P<0.01, d.f.=2 for males and F=7.26, P=0.01, d.f.=2 for females), but replacement of 2-methyl-3-buten-2-ol (P+I+M) (10.8 ± 2.6 males/trap and 9.8 ± 1.43 females/trap) with chalcogran (P+I+C) did not increase the number of catches (10 ± 2 males/trap and 9.8 ± 2.75 females/trap).The 2013 field trial at Oiswald (Experiment 3; Fig. 5.6c) showed again (F=3.94, P=0.08, d.f.=1 for males and F=9.20, P=0.01, d.f.=1 for females) that the triple blend (P+I +M) resulted significantly far more attractive for both sexes (15.8 ± 6.04 males/trap and 14.8 ± 4.32 females/trap) than the bark beetle kairomones alone (I +M) (3.92 ± 1.93 males/trap and 1.4 ± 0.87 females/trap). Similar results were reported for Experiment 4 at Nasswald (2014) (Fig 5.6d). Though differences were again significant for females (F=10.32, P<0.01, d.f.=2) but not for males (F=2.81, P<0.11, d.f.=2), the catches obtained with the triple pheromone-kairomone blend (P+I +M) were higher (9.2 ± 2.46 males/trap and 15 ± 3.41 females/trap) than the sum of catches with the pheromone (P) (4.4 ± 1.75 males/trap and 8.6 ± 2.16 females/ trap) or the kairomones (I+M) (3.4 ± 2.18 males/trap and 2.8 ± 1.85 females/trap) separately, indicating a truly synergistic effect of the ipsenol and 2-methyl-3-buten-2-ol blend on the *M. sutor* pheromone.


**Figure 5.7.** Forest plot of effect sizes based on log response ratio of means for the number of catches across different experiments, along with their 95% confidence interval (CI) widths. *M. sutor* aggregation pheromone (P) has been set as the reference treatment. Confidence interval widths overlapping ratio = 0 do not represent a significant improvement in number of catches relative to the reference treatment.

*Summary of pheromone vs pheromone plus ipsenol*

Figure 5.7 summarizes results of all experiments comparing the pheromone alone, in combination with ipsenol or blended with ipsenol+2-methyl-3-buten-2-ol. The plot shows that in all cases the kairomones had a positive effect on catches, and the effects were significant in all but two experiments. Since the 95% confidence intervals for the log ratio values of all experiments overlapped, it cannot clearly be affirmed that the (P+I+M) combination performed better than the (P+I) lure.

## Discussion

*Attraction to bark beetle kairomones*

Bark beetle kairomones have been shown to be attractive to both sexes of *M. sutor* (Pajares *et al*., 2012), and to other *Monochamus* sawyers (Allison *et al*., 2001, 2003; Pajares *et al*., 2004; Miller & Asaro, 2005; Miller *et al*., 2011). Some authors have argued that pine sawyers may exploit weakened or recently dead hosts that were located first by bark beetles or improve their own larval nourishment through intraguild predation of bark beetle larvae (Dodds *et al*., 2001; Allison *et al*., 2003). In previous work, a blend of four bark beetle volatiles comprising all the main pheromone components emitted by the *Ips* species, which colonize pines and spruce in Europe (ipsenol, ipsdienol, 2-methyl-3-buten-2-ol and cis-verbenol), proved attractive to both sexes of *M. sutor* in the Pyrenees (Spain) (Pajares *et al*, 2012). A reduced blend of ipsenol plus methy-buthenol, the pheromone components of *Ips typographus* (Linneo) and *I. acuminatus* (Gyllenhaal), which breed in the Norway spruce and Scots pine stands where *M. sutor* occurs, also attracted the species in Sweden (Pajares *et al*, 2012). These kairomones, however, were not individually tested. Other bark beetle semiochemicals that might be relevant to pine sawyers, such as chalcogran, the main pheromone of the *Pityogenes chalcographus* (L.) bark beetle that infests Norway spruce, have not been studied previously.

In our study, GC-EAG recordings showed that *M. sutor* males and females clearly responded to ipsenol and ipsdienol; females also responded to 2-methyl-3-buten-2-ol. No response to cis-verbenol was observed in either sex, though females weakly responded to its oxydated derivative verbenone. However, chalcogran elicited a solid response in *M.*

*sutor* female antennae. Field tests were partially in agreement with antennal responses: ipsenol was by far the most attractive kairomone to both sexes of *M. sutor*, confirming its role as a strong kairomone for this and other *Monochamus* sawyers (Allison *et al*. 2003; De Groot and Nott 2004; Miller and Asaro 2005; Ibeas *et al*; 2007). In contrast, the other kairomones tested (ipsdienol, cis-verbenol and 2-methyl-3-buten-2-ol) showed very weak attraction capacity in the field (Experiments 2 and 4 in Sweden; Fig 5.3c). 2-methyl-3-buten-2-ol had been described as a key component for synergizing the attraction of *M. galloprovincialis* to ipsenol (Ibeas *et al*., 2007). Since we were seeking the best kairomone blend for synergizing the *M. sutor* pheromone, response to kairomone blends alone was not studied further. Chalcogran was unattractive in field tests (Exp. 2 in Sweden) and did not increase catches when added to the pheromone (Exp. 2 in Spain and Exp. 3 in Sweden), contrary to expectations raised by the EAG tests. This volatile bark beetle pheromone has not been tested previously as a kairomone for pine sawyers. Though widespread in many of the spruce forests where *M. sutor* occurs, it might have no role in the chemical ecology of the pine sawyer.

*Attraction to the aggregation pheromone*

The 2-undecyloxy-1-ethanol pheromone has been identified as the male-specific pheromone for *M. sutor* (Pajares *et al*, 2013) and several other *Monochamus* species that colonize conifers, such as *M. galloprovincialis* in Europe (Pajares *et al*., 2010), *M. carolinensis* (Olivier), *M. titillator* (Fabricius) (Allison *et al*., 2012) and *M. scutellatus* (Say) in North America (Fierke *et al*., 2012), and *M. alternatus* in Asia (Teale *et al*., 2011). This compound is chemically related to straight-chain ether pheromones found in other members of the Laminae subfamily, as an example of the parsimony that seems to exist among the pheromones of many of the Cerambycidae (Hanks & Millar, 2013). Our experiments have confirmed that this male-produced pheromone can be properly considered an aggregation pheromone of *M. sutor*, since it significantly attracted both sexes (Shorey, 1973). However, higher numbers of females were caught in six out of the nine experiments (two in Spain, three in Sweden, one in Austria). In average, female catches were 60 % higher than male catches (range: 33% to 243%). In previous work, 2-undecyloxy-1-ethanol was more attractive to females than to males in Sweden and China, but not in Spain (Pajares *et al*, 2013). Since female-biased catches were consistent in the different countries and years, it can be assumed that *M. sutor* females are more attracted

than males to the pheromone, and that cases of male-biased catches may be linked to differences in the sex ratios of local populations.

*M. galloprovincialis* was reported to have responded with equal intensity to its pheromone and to the ipsenol plus 2-methyl-3-buten-2-ol blend (Pajares *et al*, 2010; Álvarez-Baz *et al*; 2015a). Previous studies on *M. sutor* found that the numbers of males and females attracted to the pheromone (released at 2mg/day) were similar to those attracted to the blend of four-bark beetle compounds (2.5 mg/day each of ipsenol, ipsdienol and cis.verbenol, and 10 mg/day of 2-methyl-3-buten-2-ol) but significantly higher than those attracted to the ipsenol-2-methyl-3-buten-2-ol blend (Pajares *et al*: 2013). In our work, using the same dosages, the kairomones tested were significantly less attractive to females than the pheromone alone (Exp. 4 and 5 in Sweden) or blended with ipsenol+2-methyl-3-buten-2-ol (Exp. 4 in Austria). However, equal numbers of *M. sutor* males were caught in the ipsenol traps, the ipsenol+2-methyl-3-buten-2-ol traps and the pheromone traps.

*Attraction to pheromone-kairomone blends*

In many *Monochamus* species, attraction to the pheromone can be increased by combining it with host plant volatiles, bark beetle kairomones, or both (Pajares *et al*, 2010; Hanks & Millar, 2013; Álvarez Baz *et al*., 2015a). Previously, a blend of four bark beetle kairomones (ipsenol, ipsdienol, cis-verbenol and 2-methyl-3-buten-2-ol) and two host kairomones, α-pinene and 3-carene, were reported to significantly increase attraction of *M. sutor* to the pheromone in Spain (Pajares *et al*., 2013). In our work, we have further explored the role of each of these *Ips* spp kairomones and chalcogran when combined with the pheromone. Most *Ips* kairomones increased catches of both sexes, but this was significant only for ipsenol (exp 5 in Sweden, fig 5.5d) and for combinations that included ipsenol and *M. sutor* pheromone (Exp. 1 in Spain, fig 5.4a; Exp. 2 but not in Exp 4 in Austria, fig 5.6b and 5.6d respectively). On the other hand, chalcogran did not increase the catches when combined with *M. sutor* pheromone (Exp. 2 in Spain, fig 5.4b; Exp. 3 in Sweden, fig 5.5b). Neither did it increase the catch when added to a combination of *M. sutor* pheromone and ipsenol compared with this combination plus methyl-buthenol (exp 2 in Austria, fig 5.6b). Thus, ipsenol not only was shown the strongest individual kairomone for *M. sutor* but it resulted also the best single kairomone to be combined with the

pheromone. Its effect can be considered truly synergistic, as the increase was more than additive (Exp. 5 in Sweden, fig 5.5d), similarly to found for *M. galloprovincialis* (Pajares *et al*, 2010)

Two kairomone blends consisting of ipsenol plus one of the other kairomones, tested in combination with the pheromone, gave similar results and caught the same amount of beetles as the ipsenol+pheromone combination (Exp. 1 and 2 in Spain, Fig. 5.4a and 5.4b; Exp. 1 in Sweden, Fig. 5.5a). The ipsenol+2-methyl-3-buten-2-ol blend obtained the highest catches of males and females in Spain. It was tested again in Austria (Exp. 2, 3, and 4, Fig. 5.6b, c and d), where increased catches were reported for both sexes (significant for females) compared to the pheromone alone. When all these results were plotted together (Figure 5.7), there was a positive effect of the kairomones on catches in all experiments. The overlapping of the 95% confidence intervals, however, did not allowed to tell if the (P+I+M) blend performed better than the (P+I) blend. The (P+I+M) combination is currently considered the standard lure for trapping *M. galloprovincialis* in Europe (Pajares *et al*, 2010; Álvarez-Baz *et al*: 2015a) and is commercially available to forest managers. Thus, even though 2-methyl-3-buten-2-ol may not be contributing at the same level as for *M. galloprovincialis*, this combination can be operationally and effectively used for *M. sutor* management.

Pine volatile α-pinene has been reported to increase attraction to the pheromone in several European (i.e *M. galloprovincialis*; Álvarez-Baz *et al*; 2015a), North American (i.e *M. carolinensis* and *M. titillator*; Allison *et al*. 2012) and Asian (*M. alternatus*; Teale *et al*., 2011) pine sawyer species. However, this volatile alone or blended with 3-carene was unattractive to *M. sutor* and attraction did not increase when it was combined with the pheromone (Pajares *et al*; 2103). Though EAG responses to this terpene were not studied, it was field tested twice in combination with the pheromone-bark beetle kairomone blend (Exp. 1 in Spain, Fig. 5.4a and Exp. 1 in Austria, Fig. 5.6a), with null effect. This unspecific monoterpene was found to be attractive to a wide range of non-targeted subcortical beetles, (Schroeder and Weslien, 1994; Erbilgin and Raffa, 2000; Etxebeste *et al*., 2012; Hofstetter *et al*., 2012; Miller *et al*,. 2013, Enrique Macias-Samano *et al*., 2014), such as *Thanasimus formicarius* (Coleoptera: Cleridae), a natural enemy of the bark beetle that is commonly found in areas occupied by *M. sutor*, (Schroeder, 2003; Pajares *et al*., 2004; Gallego *et al*, 2008; Panzavolta *et al*., 2014). Thus, any advantage of incorporating this

terpene into an attractant blend would be outweighed by the detrimental effects of luring non-target organisms.

Schütz *et al*. (1999) reported that some species that colonize fire-damaged trees are attracted to volatiles released from burnt wood. These authors identified the smoke compounds that may attract *Melanophila acuminata* Eschscholtz to burnt trees: mainly phenolic compounds similar to the ones we tested. Since some pine sawyers are known to habitually colonize burnt trees (Parmelee, 1941; Ross, 1960; Markalas 1997; Forsslund, 1934; Ehnström *et al*., 1995), they may also use smoke volatiles as cues to locate them. However, our two tests in Spain (Exp. 1, Fig. 5.4b) and Austria (Exp. 1, Fig. 5.6a) to evaluate the synergistic effect of combined smoke compounds from pines (methoxyphenols) on *M. sutor* attraction to a pheromone-kairomone blend gave negative results, indicating that tested smoke cues are unlikely to be involved in host finding by this species.

Pine wood nematode (PWN), *Bursaphelenchus xylophilus*, is rightly regarded as a major threat to European forests, particularly since its establishment and spread throughout Portugal. The well-documented, extensive tree mortality that has occurred in all countries where pine wilt disease has been recorded in Europe and Asia justifies such alarm. This threat has created an urgent need for tools that help detect and monitor both the nematode and the insects vectoring it. Even though *M. sutor* has not yet been reported to transmit PWN, it is widely assumed to be the most likely vector for spreading PWN if the pathogen is introduced into Northern Europe. Recent progress in the development of improved traps and lures for *M. galloprovincialis* is allowing managers to control the spread of pine wilt disease in Spain, and trapping programs are now at the core of control programmes there. The combination of chemical analysis, electro-antennogram studies and field bioassays has made it possible to evaluate candidate attractant compounds, and identify an effective pheromone-kairomone blend that can be readily used for monitoring and managing *M. sutor* if required.

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# Chapter 6

# Potential of native *Beauveria pseudobassiana* strain for biological control of Pine Wood Nematode vector *Monochamus galloprovincialis*

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## Introduction

The Pine Wood Nematode (PWN), *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle (Nematoda; Aphelenchoididae) has become one of the most harmful foreign organisms worldwide over the last century. Regarded as a weak pathogen in its native North America, it causes Pine Wilt Disease (PWD), a pathology characterized by rapid wilting and death in pines and other susceptible conifers (Wingfield, 1982). After spreading across Southeast Asia for several decades and causing serious economic and environmental damage (Shin, 2008; Zhao, 2008), PWD was first reported in Portugal in 1999 (Mota *et al*., 1999). Despite containment efforts, PWD has spread throughout that country, devastating large pine stands (Daub, 2008; Rodrigues, 2008). It eventually crossed into Spain in 2008 (Zamora *et al*, 2015), where four infection foci have been declared and strong eradication measures are currently being implemented.

PWN requires insect vectors to infect new hosts, and sawyer beetles of the *Monochamus* Dejean genus (Coleoptera: Cerambycidae) serve the purpose (Linit, 1988; Linit and Akbulut, 2008). This genus comprises about 130 wood-boring species worldwide and usually colonizes woody plants that are dead, dying or severely stressed by fire, drought or other organisms. Young adults feed on the bark of pine shoots in the crown of

healthy trees, initially for sexual maturation and later for maintenance. Mated females chew oviposition wounds to lay eggs through the bark of dying or dead trees. Healthy trees are thus inoculated with nematodes during shoot feeding (Mamiya and Enda, 1972), but nematode transmission can also occur when females oviposit into susceptible dying or dead trees (Evans *et al*., 2008). So far, *Monochamus galloprovincialis* (Olivier) is the only known vector for PWN in Europe (Sousa *et al*., 2001). This species colonizes mainly pines, but also fir and spruce trees (Vives, 2000) and is widely distributed throughout the continent (except the United Kingdom, Ireland, Sardinia and Cyprus), and Northern Africa. Control of PWD has proven difficult worldwide (Kamata, 2008). Preventive control of the pathogen by nematicide trunk injection may be feasible for ornamental trees, but it has proven difficult to apply in large areas (Shin, 2008). So long as direct attempts to control the pathogen remain unfeasible, the best method for preventing this disease is to control the insect vectors. Chemical insecticides have been used in some countries (Shin, 2008; Zhao, 2008), but are considered unsuitable due to their questionable effectiveness and potential hazard to environmental and human health.

To replace the use of chemicals, biological control of *Monochamus* sawyers has also been attempted for integrated PWD management in Asia (Shimazu, 2008). Good rates of parasitism by *Dastarcus helophoroides* (Coleoptera: Bothrideridae) and *Scleroderma guani* (Hymenoptera: Bethylidae) on *M. alternatus* were reported in China, especially if parasitoids occurred together with the *Beauveria bassiana* fungus (Shimazu, 2008; Xu, 2008). The entomopathogenic nematodes *Contortylenchus genitalicola* and *Steinernema carpocapsae* have shown good potential for biological control of *M. alternatus*, but practical applications remain undeveloped (Phan, 2008; Shimazu, 2008). Entomopathogenic fungi that infect their host through the cuticle carry great potential as biocontrol agents. They have been used successfully for dealing with important forest and agricultural pests. In addition to directly killing the insects, entomopathogenic fungi have also demonstrated sub-lethal reproductive effects with important implications on host population dynamics (Quesada-Moraga *et al*., 2004; 2008). Some entomopathogenic fungal species have been isolated from their respective hosts and used as pest control agents in other countries (Shin *et al*., 2011). Several *Beauveria bassiana* strains, some of them highly virulent, have been isolated from *M. alternatus* populations in Asia, for example (Shimazu, 2008; Shin *et al*., 2009). However, practical application of these biological agents in forest insect control is limited, particularly in the cryptic larval stages of

the sawyer beetle (Kishi, 1995). Different methods for releasing the entomopathogenic fungi have been attempted, such as direct spraying, inoculation by hand under tree bark or vectoring using bark beetles (Shimazu *et al*., 1995; Shimazu and Sato, 2003; Shimazu, 2008). However, none proved effective enough at killing adults and thereby preventing *B. xylophilus* inoculations in healthy trees. Shimazu *et al*., 1995 developed a method for applying *B. bassiana* to *M. alternatus* by means of non-woven fabric strips, which effectively killed adults (Okitsu, 2000; Shimazu, 2004a) and young larvae (Shimazu *et al*., 1995; Shimazu and Sato, 2003).

In Europe, four *B. bassiana* soil isolates and a commercial formulation were tested against *M. galloprovincialis,* resulting in near 100% mortality and a reduction of adult survival times by 7.7 - 9.3 days (Francardi *et al*., 2003). Naves (2008) reported *B. bassiana* as the most important factor in natural pine sawyer mortality in the area of Portugal affected by PWD. This suggests that native *B. bassiana* strains virulent enough to infect *M. galloprovincialis* might be isolated from natural sawyer beetle populations in Europe. The recent unravelling of the chemical ecology of *M. galloprovincialis* (Ibeas *et al*., 2008, 2007; Pajares *et al*., 2004) and subsequent development of highly effective baits (Pajares, 2010) and traps (Álvarez *et al*., 2014) are also opening up opportunities for integrating entomopathogenic fungi into PWD management. Beetle populations could be inoculated with *B. bassiana* strains by means of lure and infect techniques that attract insects and contaminate them with pathogenic organisms in auto-dissemination devices, then allowing them to infiltrate the insect population (Francardi *et al*., 2013; Klein and Lacey, 1999; Yasuda, 1999). Contaminated beetles that leave the infecting devices must be able to transmit the biological agent to other adults during mating (horizontal transmission) or to offspring through oviposition (vertical transmission). In this work, we present the results of 4 years of research aimed at isolating native entomopathogenic fungal strains from *M. galloprovincialis* populations in Spain, which could be used for vector control through auto-dissemination techniques.

## Material and methods

*Insects*

The *M. galloprovincialis* adults used in the assays for pathogenicity, virulence, horizontal and vertical transmission were obtained from two sources: naturally colonized

fire-damaged pine logs, and baited logs (2012, 2013). One hundred twenty *P. sylvestris* logs, 120 cm long and 8-15 cm in diameter, were placed in piles near forest roads and fire breaks in Tabuyo del Monte (Léon province, Castilla y León, Spain, 42º 18' 13.2''N, 6º 11' 27.6'' W) during the summers of 2011 and 2012. These logs were baited with commercial attractant lures (Galloprotect 2D, SEDQ Barcelona, Spain) to induce colonization by wild pine sawyer adults. Colonized logs were removed at the end of the summer and left in an outdoor cage until new adults emerged the following year. All adults were maintained individually in 1l glass jars under a 15:9 h light:dark photoperiod and 22:15 ºC temperature regime; they were provided with fresh pine twigs weekly until they were used experimentally.

Larvae used for the pathogenicity experiment were obtained through controlled laboratory rearing. Mated *M. galloprovincialis* females were allowed to oviposit on fresh *P. sylvestris* logs (12-20 cm diameter x 50 cm long) collected at Saldaña (Palencia province, Castilla y León, Spain, 42º 37' 40.1''N, 4º 47' 30.0'' W) and placed in plastic boxes (60x30x30cm). After a few weeks, egg-laying wounds showing larval frass were carefully debarked and live larvae were collected.

*Isolation and characterization of entomopathogenic fungi*

Two hundred forty logs 1m long containing *M. galloprovincialis* larvae were collected from fire-damaged pine trees at Cuevas del Valle (Ávila province, Castilla y León, Spain, 40º 17' 58.3'' N, 5º 01' 17.2'' W) in February 2010. After adult emergence ended in September, 32 of these logs that had produced significantly fewer adults than expected were selected and carefully cut into 1cm thick slices using a band saw. Forty-five samples of dead larvae, pupae and adults found in the logs were collected and analyzed, along with wood pieces observed to have fungal mycelia within the insect galleries. Similarly, 250 logs were collected from the same location in February 2011, of which 25 low-producing logs were selected and sliced. From these, 39 samples were obtained and analyzed for entomopathogenic fungal strains.

Dead insects were surface sterilized with 1% sodium hypochlorite followed by three rinses with sterile distilled water. Then, they were placed on sterile wet filter paper in sterile petri dishes that were sealed with Parafilm and kept at 25 ºC in the dark for two weeks to

enable the development of fungal outgrowth on the cadavers. Fungi were isolated directly from *M. galloprovincialis* adult and pupa cadavers with fungal outgrowth, using a sabouroad glucose cloramphenicol agar medium, The fungi were cultivated for 2 weeks at 25 ºC in the dark and were then subjected to morphological and molecular identification. The fungal isolates were classified to the genus level by light microscopy (400× magnification) using taxonomic keys (Barnett and Hunter, 2006). For molecular characterization, mycelia for DNA extraction were obtained by inoculating malt extract agar plates covered with a sterile cellophane sheet. Total DNA was extracted from lyophilized mycelia scraped from the cellophane sheets (Raeder and Broda, 1985).

The nuclear gene EF-1α was amplified, sequenced and analyzed in the different samples. A 1100 bp fragment spanning the 3' 2/3 of the EF-1α gene was amplified with primers tef1fw (5'-GTGAGCGTGGTATCACCA-3') (O'Donnell *et al*., 1998) and 1750-R (5'-GACGCATGTCACGGACGGC-3') (Garrido-Jurado *et al*., 2011). Total reaction volume was 50 µl and contained 1.5 µl genomic DNA, 10 µl PCR reaction buffer (5×), 1.5 µl $MgCl_2$ (25 mM), 0.5 µl dNTPs (20 mM each), 1.5 µl each primers (20 mM), and 0.5 µl *Taq* polymerase (5U, Biotools Labs, Madrid, Spain). The amplification program included an initial denaturation cycle of 1 min at 94ºC, followed by 35 cycles of 1.5 min at 94ºC, 2 min at 55ºC and 3 min at 72ºC, with a final extension step of 7 min at 72ºC. Negative (no DNA) and positive (fungal DNA from a pure culture) controls were included in each set of reactions. The PCR products were electrophoresed on 1% agarose gels buffered with 1× TAE and stained with SYBR® Safe (Invitrogen, Paisley, UK). A 100bp molecular weight standard (Solis Biodyne, Tartu, Estonia) was also included. The PCR products were purified from agarose gels using the Geneclean II kit® system (QBiogene, Inc., Carlsbad, CA), following the manufacturer's protocol. All amplified EF-1α products were sequenced from both ends using the amplification primers.

Published sequences for isolates included within the following genera: *Beauveria* Vuillemin, *Metarhizium* Sorokīn, *Lecanicillium* W. Gams and Zare, Pers. and *Purpureocillium* Luangsa-ard, Hywel-Jones, Houbraken and Samson were retrieved from GenBank and included in the alignments. Alignments were generated using the MegAlign program (DNASTAR package, 1989-92, London, UK). The molecular phylogeny of EF-1α was used to infer the phylogenetic diversity and relationships among the isolates. Phylogenetic trees were created using the MEGA 4.0 software program and uninformative

characters were excluded from the analyses. The maximum parsimony (MP) tree was created from the EF-1α data and heuristic searches, using close-neighbor-interchange. The EF-1α sequence from *Cordyceps militaris* (L.) Fr. (HQ881020) was used as an out-group. A bootstrap full heuristic analysis consisting of 1000 replicates was performed, and a 50% majority rule tree was produced. All entomopathogenic fungal isolates obtained were deposited in the culture collection of the Department of Agricultural and Forestry Sciences, ETSIAM, University of Córdoba, Spain.

*Initial pathogenicity assay of isolated entomopathogenic fungi against M. galloprovincialis adults and larvae*

Two bioassays were carried out on *M. galloprovincialis* adults and larvae to determine the pathogenicity of the three entomopathogenic fungal isolates obtained. Conidia obtained from 14-day-old malt extract agar plates were suspended in a sterile aqueous solution of 0.1% Tween 80 for each isolate. This suspension was shaken, sonicated for 5 minutes and filtered through several layers of cheesecloth to remove mycelia mats. The conidia in the suspension were counted with a Malassez chamber (Blau Brand, Germany) at 400X magnification and finally all suspensions were adjusted to $1x10^8$ conidia/ml. This procedure was repeated for the three entomopathogenic fungi tested.

For each treatment, 10 sexually mature 2 to 3 weeks old adult pine sawyers were placed individually in a stainless steel mesh basket (35mm diameter) and immersed in the suspension for 20 seconds. The control insect was immersed in distilled water plus 0.1% Tween 80. After immersion, insects were kept individually in 1l glass jars and provided with fresh pine twigs under 15:9 h light:dark photoperiod and 22:15 ºC temperature regime. Mortality was assessed daily for 30 days and dead insects were surface sterilized with 1% sodium hypochlorite followed by three rinses with sterile distilled water. They were then placed on sterile wet filtered paper in sterile Petri dishes that were sealed with Parafilm and kept at 25 ºC in the dark to be later inspected for development of fungal outgrowth on the cadavers. Five larvae were also treated similarly for each strain. After immersion, larvae that had received the same treatment were introduced together into one *P. sylvestris* log (8-15 cm diameter x 50 cm long) through 3 cm holes bored into the bark. Larvae were allowed to develop and their activity was assessed by the sawdust produced. When activity was observed to cease, larval galleries were carefully opened and dead

larvae were collected and preserved for later analysis. Dead larvae were processed as described above for fungal outgrowth.

*Virulence assay of B. pseudobassiana against M. galloprovincialis adults*

For the virulence assay, four conidial suspensions ($1\times10^5$, $1\times10^6$, $1\times10^7$ and $1\times10^8$ conidia/ml) were prepared as described above. Eighteen young *M. galloprovincialis* adults 2 to 3 weeks old, evenly distributed by age and size, were individually immersed in each of the conidial suspensions for 20 seconds. Eighteen control adults were also immersed in sterilized, distilled water with 0.1% Tween 80. After immersion, the insects were maintained as described under 15:9 h light:dark photoperiod and 22:15 ºC temperature, and mortality was assessed every two days for 30 days. Dead insects were processed for development of fungal outgrowth on the cadavers as described previously.

*Horizontal transmission of B. pseudobassiana between M. galloprovincialis adults*

*Horizontal transmission of B. pseudobassiana by M. galloprovincialis adults inoculated with a conidial suspension*

Mature *M. galloprovincialis* adults (from 10 to 21 days feeding) were immersed either in a $1\times10^8$ conidia/ml aqueous suspension (inoculated) or in sterilized, distilled water with 0.1% Tween 80 (clean insects), as described above. Ten adult pairs were tested for each of 4 combinations: (I) clean males x clean females, (II) inoculated males x clean females, (III) clean males x inoculated females and (IV) inoculated males x inoculated females. Within 30 minutes after treatment, each insect pair was placed in a plastic box and provided with two *P. sylvestris* bolts (15 cm in diameter x 50 cm long) for mating and oviposition. Bolts were checked every 2 days for egg-laying wounds and the insect pair was removed from the boxes when 15 or more wounds/bolt were counted. The adults were subsequently maintained individually in glass jars (15:9 h light:dark photoperiod and 22:15 ºC temperature). Mortality was recorded daily for the following 30 days. Dead insects were processed for development of fungal outgrowth on the cadavers, as described.

*Horizontal transmission of B. pseudobassiana by M. galloprovincialis adults inoculated with dry conidia*

A similar assay was carried out the following year, but this time the adults were treated with a talc-formulated $4.25\times10^9$ conidia/g *B. pseudobassiana* preparation. A pure talc powder preparation was administered to control insects. Mature adults, 10 to 21 days old, were forced to walk over the powder preparation for about 20 seconds to ensure complete body coverage. Within 30 minutes after treatment, insects were paired into 10 couples for each of the same 4 combinations described above. Each pair was placed in a plastic box with one *P. sylvestris* bolt (8-12 cm in diameter x 30 cm long). To avoid larval over-competition, bolts were checked daily for oviposition wounds and replaced with new bolts if 5 or more wounds/bolt were counted. Insect pairs were left to oviposit in the boxes until a minimum of 20 oviposition wounds/pair were observed, then the live insects were removed and kept individually as described earlier (15:9 h light:dark photoperiod and 22:15 ºC temperature). Adult mortality was recorded every two days for the following 30 days. Dead insects found in the boxes or in the jars were processed for development of fungal outgrowth on the cadavers as described above.

*Vertical transmission of B. pseudobassiana between M. galloprovincialis adults*

*Vertical transmission of B. pseudobassiana by M. galloprovincialis adults inoculated with a conidial suspension*

After oviposition, bolts from the horizontal transmission experiment were removed from the boxes and the eggs were left to develop for two weeks. Then, one of the bolts was carefully debarked and oviposition wounds were examined for neonate larvae, dead eggs and vacant wounds, since usually a proportion of the wounds are egg-less. Similarly, two weeks later, (four weeks after oviposition had ceased), the second bolt was debarked and examined for offspring. In both cases, dead eggs and larvae were processed for development of fungal outgrowth on the cadavers as described above.

*Vertical transmission of B. pseudobassiana by M. galloprovincialis adults inoculated with dry conidia*

Oviposited bolts from the horizontal transmission experiment were left to develop for 5 days. Then, egg-laying wounds were non-destructively examined for evidence of larval

activity (frass). Wounds showing no activity were carefully debarked to check for the presence of progeny and any dead eggs or larvae were collected. Active larvae were recorded and left to complete their development and enter the xylem for pupation. After 6 months, the bolts were debarked and larval xylem entries were counted. Eggs laid were calculated as the sum of dead eggs and live and dead larvae. Dead eggs and larvae were processed for development of fungal outgrowth on the cadavers as described above.

*Statistical analysis*

Mortality data were analyzed using a generalized linear model (distribution=binomial; link=logit). Treatment comparisons were performed with $\chi^2$ test ($P<0.05$) (JMP 8.0, 2008 SAS Institute Inc.). Percentage mortality caused by *B. pseudobassiana* in horizontal transmission assays was corrected for mortality in the controls using Abbott's formula (Abbott, 1925). The Kaplan-Meier survival analysis was used to determine the cumulative mortality response over the assessment period (Kaplan *et al*., 1958). The median lethal concentration ($LC_{50}$) values were estimated by probit analysis (Finney, 1971), with concentration response corrected for control mortality by the program. The estimated time to kill 50% of the insects ($LT_{50}$) was determined using the probit analysis method for correlated data (Throne *et al*., 1995). SPSS 15.0 for Windows (SPSS Inc. 2002) was used for all these analyses.

In vertical transmission experiments, egg-laying wounds, eggs, dead and live larvae and larval entries into the xylem were analyzed untransformed by fitting generalized linear models to a Poisson or quasi-Poisson (in case of over-dispersion) error distribution (Crawley, 2007). Mean comparisons were tested by Tukey's honestly significant difference test. These statistical procedures were performed using R 2.11.1 statistical programming (R Core Team, 2012).

## Results

*Isolation and characterization of entomopathogenic fungi*

Three entomopathogenic fungal isolates were obtained from fungal outgrowth on adult and pupa cadavers of *Monochamus galloprovincialis.* Microscopic observation of

colony growth and conidia arrangement showed general and typical characteristics of the *Beauveria, Isaria* and *Lecanicillium* genera. All isolates yielded 1.1 kb PCR products from the EF-1α gene when amplified using the tef1fw and 1750-R primers. These three sequences were then aligned and compared with 29 other sequences from GenBank, representing lineages distinct from the aforementioned genera. The MP phylogeny inferred from the EF-1α sequence alignment grouped the same isolates with the *Beauveria pseudobassiana, Isaria farinosa* and *Lecanicillium attenuatum* species (Figure 6.1).

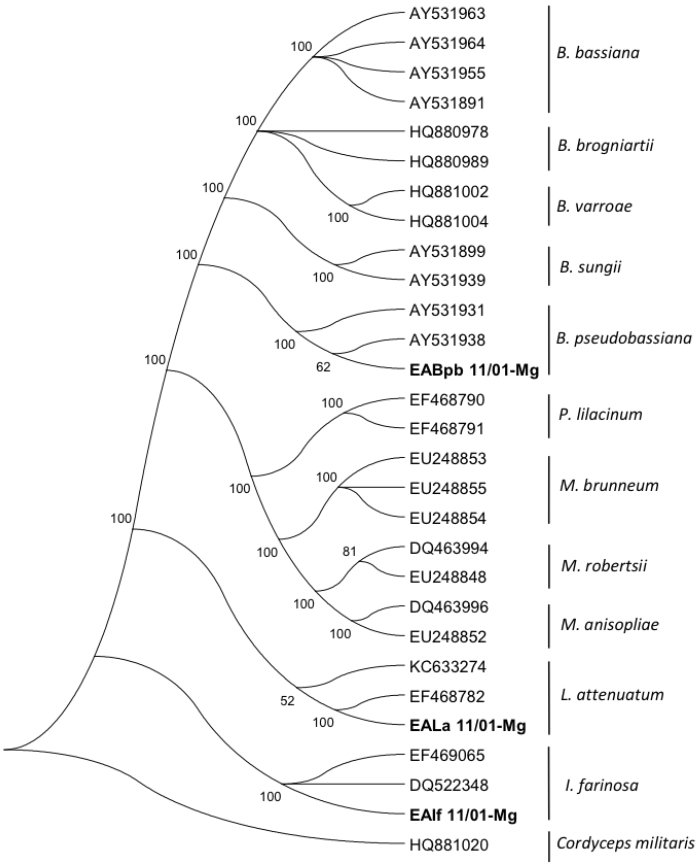


**Figure 6.1.** MP tree generated by parsimony analysis after heuristic searches (close-neighbor-interchange option) using the EF-1α type sequences of the entomopathogenic fungal isolates from *M. galloprovincialis* adults and pupae (EABps 11/01-Mg, EALa 11/01-Mg and EAlf 11/01-Mg). Numbers at the nodes were supported in >50% of bootstrap replicates. Branch lengths are proportional to the number of changes.

### *Initial pathogenicity of isolated entomopathogenic fungi against M. galloprovincialis adults and larvae*

After identification, we evaluated the pathogenicity of the three entomopathogenic fungal strains isolated from dead *M. galloprovincialis* adults and pupae. Only *B. pseudobassiana* proved highly pathogenic against *M. galloprovincialis* adults and larvae ($\chi^2(3)=23.61$, $p<0.001$; $\chi^2(3)=17.54$, $p<0.001$, respectively), causing 100% mortality in both

cases. In contrast, *L. attenuatum* and *I. farinosa* caused 30% and 10% mortality against adults, and 40 and 20% mortality against larvae, respectively (Table 6.1). The average survival time (AST) of adults and larvae treated with *B. pseudobassiana* was significantly lower (13.30 and 8.60 days, respectively) compared to those treated with *L. attenuatum* (26.50 days for adults, 20.60 days for larvae) and *I. farinosa* (29.80 days for adults, 29.6 days for larvae).

*Virulence of B. pseudobassiana against M. galloprovincialis adults*

Mean mortality at 30 days post-inoculation ranged between 44.4 and 100% for $10^5$ and $10^8$ conidia/ml treatments, respectively, whereas control mortality was 16.60%. Probit regression analysis resulted in a regression coefficient (slope ± SE) of 0.70 ± 0.18, and a chi-square value that was not significant ($\chi^2$=1.04, 2 df), indicating a good fit of the regression line, with a $LC_{50}$ value of $2.05\times10^5$ conidia/ml. Probit analysis of cumulative mortality produced non-significant chi-square values for $1\times10^7$ conidia/ml (slope ± SE = 3.23 ± 1.62, $\chi^2$=0.72; 4 df) and $1\times10^8$ conidia/ml (slope ± SE = 4.51 ± 0.84, $\chi^2$=0.88, 2 df), indicating a good fit of the regression lines, with $LT_{50}$ values of 15.03 and 5.07 days for $1\times10^7$ and $1\times10^8$ conidia/ml, respectively.

*Horizontal transmission of B. pseudobassiana between M. galloprovincialis adults*

*Horizontal transmission of B. pseudobassiana by M. galloprovincialis adults inoculated with a conidial susp*ension

AST values for mature *M. galloprovincialis* adults immersed in $1\times10^8$ conida/ml suspension of the *B. pseudobassiana* EABps 11/01-Mg strain were significantly lower (13.80 days for males, 10.00 days for females) than those of untreated adults (24.80 days for males, 23.60 days for females). Similarly, mortality in treated males and females washigher than that of untreated adults (Table 6.2). Inoculated males coupled to clean females did not transmit the fungus. Mortality (0%) and AST (23.60 days) in those females did not differ from those of clean females coupled to clean males. Likewise, inoculated females did not transmit the fungus to clean males. Male mortality (16.66%) and AST (27.20 days) levels in this group were similar to those of males paired with clean females (Table 6.2). Even though the insects treated with the conidial suspensions presented high mortality in all cases, evidence of horizontal transmission was not found.

**Table 6.1.** Pathogenicity of entomopathogenic fungi against *Monochamus galloprovincialis* adults and larvae

| Fungal species | Pathogenicity against adults | | | | | Pathogenicity against larvae | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mortality (%)[b] | | Kaplan-Meier survival analysis | | | Mortality (%)[b] | | Kaplan-Meier survival analysis | | |
| | Total | Fungal outgrowth | AST[a] (mean ±SE) | 95% Confidence interval | | Total | Fungal outgrowth | AST[a] (mean ±SE) | 95% Confidence interval | |
| | | | | Lower | Upper | | | | Lower | Upper |
| Control | 30a | 0a | 26.90±1.83a | 23.30 | 30.49 | 0a | 0a | 30.00±0.00a | 30.00 | 30.00 |
| *Beauveria pseudobassiana* | 100b | 80b | 13.30±2.49b | 8.40 | 18.19 | 100bc | 100b | 8.60±1.47bc | 5.51 | 11.48 |
| *Lecanicillium attenuatum* | 30a | 20a | 26.50±2.18a | 22.21 | 30.78 | 40ac | 20a | 20.60±5.16ac | 10.47 | 30.72 |
| *Isaria farinosa* | 10a | 0a | 29.80±0.19a | 29.42 | 30.17 | 20a | 0a | 29.60±0.35a | 28.89 | 30.30 |

[a] Average survival time (AST) limited to 30 d. Data in the same column followed by the same letter are not significantly different (α = 0.05) according to the log-rank test.
[b] Means within columns with the same letter are not significantly different ($\chi 2$ test, $P<0.05$) according to generalized linear model.

*Horizontal transmission of B. pseudobassiana by M. galloprovincialis adults inoculated with dry conidia*

Mature *M. galloprovincialis* adults that walked over the talc-formula with $4.25\times10^9$ conidia/g of the *B. pseudobassiana* EABps 11/01-Mg strain presented significantly lower AST values (4.60 days for males, 5.00 days for females) than those of clean adults (21.10 days for males, 25.00 days for females). Similarly, inoculated adults had higher mortality than clean adults (Table 6.2). Clear evidence of horizontal transmission of *B. pseudobassiana* between males and females was found. This time, when clean females were paired with inoculated males, the fungus was transmitted from males to females. Mortality (100%) and AST (10.00 days) of clean females paired with inoculated males did not differ from those of inoculated females paired with inoculated males (Table 6.2). Likewise, when clean males were coupled with inoculated females, the fungus was transmitted from the latter to the former. Mortality (100%) and AST (10.40 days) values for clean males paired with inoculated females were virtually analogous to those of inoculated males (Table 6.2). Furthermore, all clean individuals paired with inoculated mates died during the experimental period.

*Vertical transmission of B. pseudobassiana in M. galloprovincialis*

*Vertical transmission of B. pseudobassiana by M. galloprovincialis adults inoculated with conidial suspension*

No clear evidence of vertical transmission was observed. Even though there were significantly more egg-laying wounds by non-treated females than by treated females, differences in number of eggs laid were only significant between the inoculated male x clean female and the inoculated male x inoculated female pairs (Table 6.3). Similarly, no significant differences were found in live offspring (larvae) boring within the bolts 2 weeks after oviposition, though numbers were lower for the infected females (Figure 6.2A).

**Table 6.2.** Transmission of *Beauveria pseudobassiana* strain EABps 11/01-Mg from inoculated *Monochamus galloprovincialis* adults to clean adults of the opposite sex.

| | | | Conidial suspension | | | | | Dry conidia formulation | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Mortality (%)[c, d] | | Kaplan-Meier survival analysis | | | Mortality (%)[c, d] | | Kaplan-Meier survival analysis | | |
| Assay[a] | Inoculated | Clean | Total | Fungal outgrowth | AST[b] (mean ±SE) | 95% Confidence interval | | Total | Fungal outgrowth | AST[b] (mean ±SE) | 95% Confidence interval | |
| | | | | | | Lower | Upper | | | | Lower | Upper |
| I | | ♀ | 0a | 0a | 23.60±3.08a | 17.53 | 29.66 | 0a | 0a | 25.00±2.46a | 20.17 | 29.82 |
| | | ♂ | 0a | 0a | 24.80±2.94a | 19.02 | 30.57 | 0a | 0a | 21.10±3.71a | 13.81 | 28.38 |
| II | | ♀ | 0a' | 0a' | 23.60±3.44A | 16.84 | 30.35 | 100a' | 0a' | 10.00±2.47A | 5.14 | 14.85 |
| | ♂ | | 100b' | 57.14b' | 12.80±1.22B | 10.39 | 15.20 | 100a' | 33.33b' | 5.00±0.00A | 5.00 | 5.00 |
| III | ♀ | | 100A | 42.86A | 11.40±1.44a' | 8.56 | 14.23 | 100A | 40A | 5.50±0.28a' | 4.97 | 6.03 |
| | | ♂ | 16.66B | 0B | 27.20±1.58b' | 24.09 | 30.30 | 100A | 0B | 10.40±2.22b' | 6.04 | 14.75 |
| IV | ♀ | | 100A' | 100A | 10.00±0.60A' | 8.69 | 11.30 | 100A' | 0A' | 5.00±0.36A' | 4.28 | 5.71 |
| | ♂ | | 83.33A' | 42.86A | 13.80±2.07A' | 9.73 | 17.86 | 100A' | 50B' | 4.60±0.45A' | 3.71 | 5.48 |

[a]Assay (I) clean males x clean females, (II) inoculated males x clean females, (III) clean males x inoculated females and (IV) inoculated males x inoculated females.

[b] Within the same assay, data in the same column followed by the same letter are not significantly different (α = 0.05) according to the log-rank test. AST limited to 30 days.

[c] Means within same column and assay followed by the same letter are not significantly different ($\chi 2$ test, $P<0.05$) according to generalized linear model.

[d] Abbott-corrected mortality

**Table 6.3.** Effect of *Beauveria pseudobassiana* strain EABps 11/01-Mg on egg progeny from *Monochamus galloprovincialis* pairs.

| Assay[a] | Inoculated | Clean | Conidial suspension | | Dry conidia formulation | |
|---|---|---|---|---|---|---|
| | | | Oviposition wounds[b,d] (mean ± SE) | Eggs[c,d] (mean± SE) | Oviposition wounds[b,d] (mean± SE) | Eggs [c,d] (mean± SE) |
| I | | ♀ ♂ | 16.35±2.07a | 5.40±1.15ab | 19.89±4.76b | 11.22±2.85b |
| II | ♂ | ♀ | 17.75±1.92a | 5.80±1.04a | 3.22±1.44a | 0.89±0.61a |
| III | ♀ | ♂ | 7.15±1.67b | 3.21±0.94ab | 1.60±0.76a | 0.10±0.10a |
| IV | ♀ ♂ | | 4.95±1.32b | 2.05±0.68b | 1.80±0.79a | 0.80±0.70a |

[a]Assay (I) clean males x clean females, (II) inoculated males x clean females, (III) clean males x inoculated females and (IV) inoculated males x inoculated females.
[b]Wounds made by females through the bark of bolts to oviposit, both containing eggs and egg-less.
[c]Eggs actually laid by females into the oviposition wounds.
[d] Means within same column and assay followed by the same letter are not significantly different (α = 0.05) according to Tukey's Honestly Significant test

Four weeks after egg laying the difference became significant for larvae produced by inoculated females paired with clean males, compared to offspring from clean females paired with inoculated males (Figure 6.2B). Average live larvae in bolts decreased from week 2 to week 4 in all pair combinations. Almost no dead larvae were found in any of the combinations.

Vertical transmission of *B. pseudobassiana* by *M. galloprovincialis* adults inoculated with dry conidia

The results showed that a reduction of progeny induced by horizontal transmission had occurred; control pairs (clean males x clean females) produced significantly larger numbers of offspring than the other pairs. Numbers of egg-laying wounds, eggs (Table 6.3), live larvae after 5 days and larvae entering the xylem after 6 months (Figure 6.3) from control females were significantly higher than from other females. For non-control females,

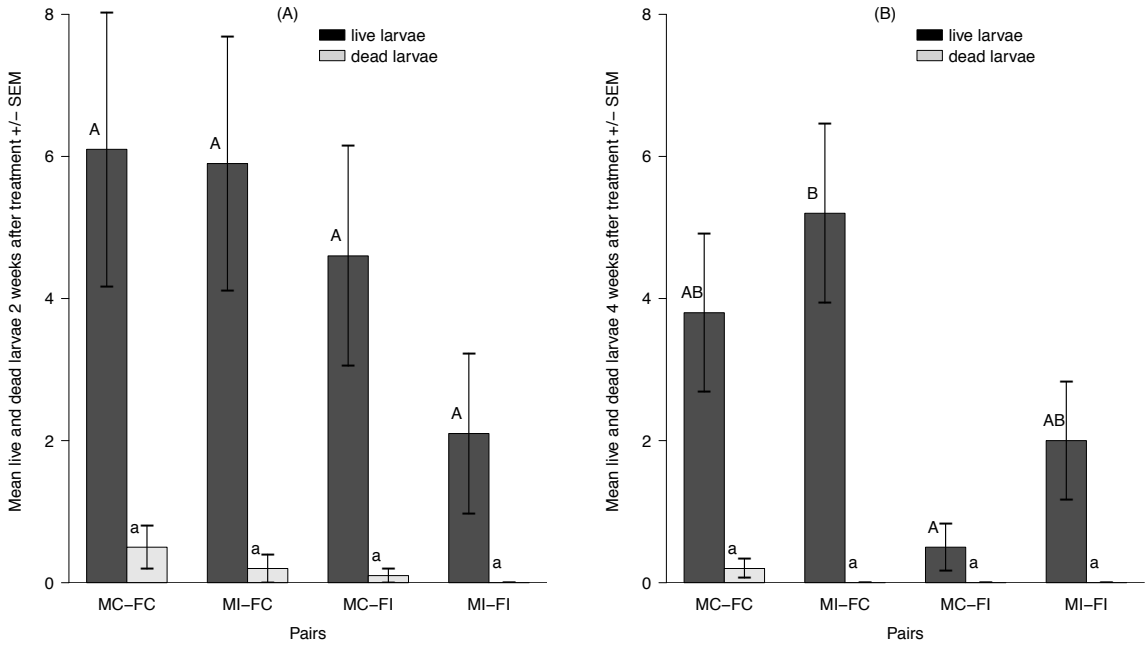


**Figure 6.2.** Mean number of live and dead larvae two (A) and four (B) weeks after egg laying in vertical transmission experiment with conidial suspension. (MC: male clean; MI: male infected; FC: female clean, FI: female infected). Bars for live (F=1.45, P=0.244, df=3) and dead larvae (F=1.29, P=0.292, df=3) in (A) and live (F=5.06, P=0.005, df=3) and dead larvae (F=2.09, P=0.12, df=3) in (B) indicated by the same letter are not significantly different (Tukey's honestly significant difference test).

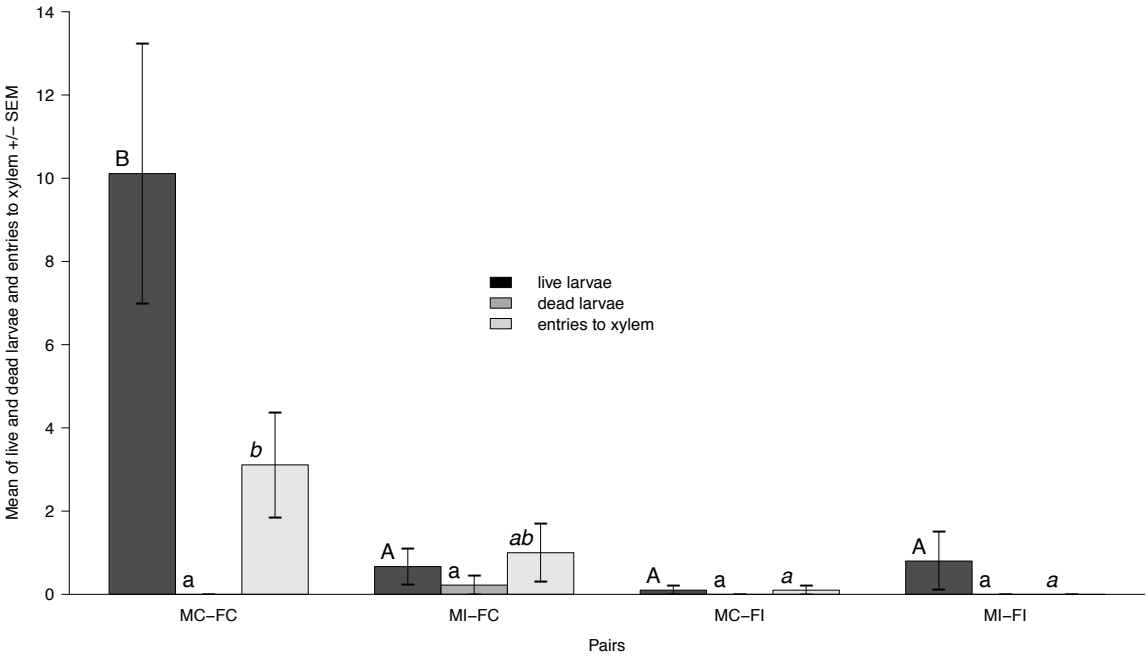


**Figure 6.3.** Mean number of live and dead larvae and larval entries into xylem for vertical transmission experiment with dry conidia. (MC: male clean; MI: male infected; FC: female clean, FI: female infected). Bars for live (F=14.00, P<0.001, df=3) and dead larvae (F=1.08, P<0.37, df=3) and larval entries into xylem (F=8.16, P<0.001, df=3) indicated by the same letter are not significantly different (Tukey's honestly significant difference test)

the low figures for offspring that completed larval development in these pairs was due to the very low number of eggs laid rather than to high egg or larval mortality. Clean females that had mated with inoculated males presented results similar to inoculated females, indicating that they had been infected by their mates.

## Discussion

Entomopathogenic fungi are an important and widespread component of most terrestrial ecosystems and play a key role in regulating insect populations (Jackson *et al*. 2000; Keller & Zimmerman, 1989). A great number of entomopathogenic agents have been isolated from soil, plants and insect cadavers, with varying degrees of pathogenicity and virulence against important agricultural pests (Lacey *et al*., 2001). Though research on fungal biological control against forest pests is still quite limited, some studies have shown entomopathogenic fungi to be common natural pathogens of forest pests such as *Thaumetopoea pityocampa* (Den. & Schiff.), *Lymantria dispar* L., *Anoplophora glabripennis* (Motschlsky), *Monochamus* sp. (Draganova, *et al*., 2013; Peng *et al*., 2011; Sevim *et al*., 2010) and others.

In this work, three entomopathogenic fungi were isolated from *M. galloprovincialis* adults and pupae. We relied on molecular identification methods because morphological features were insufficient to differentiate certain species (Bischoff *et al*., 2009; D'Alessandro *et al*., 2014; Rehner *et al*., 2011). Phylogeny based on EF-1α sequences from the three collected isolates identified *Beauveria pseudobassiana*, *Isaria farinosa* and *Lecanicillium attenuatum* as three distinct entomopathogenic fungi species. *B. bassiana* and *B. brongniartii* have been isolated from *Monochamus sp.* in Asia (Shin *et al*. 2009, 2011), and the former species has been tested for control of PWD vectors in Japan, Korea and Italy (Francardi *et al*. 2003; Shimazu, 1994; Shimazu *et al*. 1995). To our knowledge, this is the first time that isolation of *B. pseudobassiana, I. farinosa* and *L. attenuatum* from *M. galloprovincialis* has been reported. *B. pseudobassiana* proved the best isolate against *M. galloprovincialis*, causing 100% mortality and AST of 13.30 and 8.60 days in adults and larvae, respectively. Similar results (100% mortality and 10-13 days AST) were obtained for *Monochamus saltuarius* and *Mechotypa diphysis* cerambycid beetle adults and larvae treated with the same conidial suspension ($1\times10^8$ conida/ml) of *Beauveria bassiana* (Shin *et al*. 2009).

This study showed the high virulence of *B. pseudobassiana* EABps 11/01-Mg against *M. galloprovincialis*. Adult mortality reached 100% at the highest concentration tested ($1\times10^8$ conidia/ml), with $LT_{50}$ of 5.07 days. Some considerations must be borne in mind regarding the biological control of PWD. First, PWN can be introduced into healthy host trees via feeding wounds from both sexes during beetle shoot feeding (Mamiya and Enda, 1972) or during oviposition, when females transmit nematodes to dying trees via egg-laying wounds (Evans *et al*., 2008). Hypothetically, the effectiveness of *B. pseudobassiana* for suppressing PWN in an affected area would depend on its ability to kill the insects before peak PWN transmission to healthy trees (Shimazu, 2004b). In lab bioassays this window of opportunity corresponds to weeks 2 to 6 after emergence (Naves *et al*., 2007), which corroborates similar findings for *M. alternatus* PWN transmission in Japan (Togashi, 1985).

Results of the present work indicate that the *B. pseudobassiana* EABps 11/01-Mg strain causes high mortality and point to its potential for use in an auto-dissemination strategy targeting *M. galloprovincialis* adults. We observed that *M. galloprovincialis* adults inoculated with the dry conidial formulation of the *B. pseudobassiana* EABps 11/01-Mg strain transmitted the fungus to clean beetles of the opposite sex, irrespective of the sex combination. Prior research has found dry fungal formulations of entomopathogenic fungi to be better adapted for use in auto-dissemination strategies than liquid formulations (Quesada-Moraga el al. 2008). In our study, significant horizontal transmission only occurred when *M. galloprovincialis* adults were inoculated by the dry conidial formulation, indicating that the inoculation method influenced transmission. Higher numbers of conidia were apparently picked up by each single beetle while walking over the dry fungal formulation, which may point to be suitability of the dry formulation and the higher concentration used. Peng *et al*. (2011) reported similar findings from exposing the ventral surfaces of *Anoplophora glabripennis* (Motschulsky) (Coleoptera: Cerambicidae) beetles to *Metarhizium brunneum*: a method comparable to what may happen when insects walk over a fungal band on a tree. Since many conidia are lost to the environment, we hypothesize that through fungal auto-dissemination devices beetles can spread some conidia as they walk over the branches, potentially inoculating other beetles of either sex. Likewise, our bioassays revealed a significant reduction in offspring. This reduction did not result from an increase in egg and larvae mortality but from the low number of eggs laid by clean females that had mated with infected males, similar to that of inoculated females.

These results point to that horizontally-induced reduction of progeny had occurred. Hamilton and Schal, (1990) observed that treated *Blatella germanica* females surviving insecticide exposure showed reduced oothecal production. In the current study, reduction in oviposition wounds and egg production were not significantly different when treated males were paired with clean females compared to when clean males were paired with treated females. Similar results were observed by Quesada-Moraga *et al*. (2004) for *Blatella germanica* inoculated with *Metarihizium anisopliae*. Fungal outgrowth did not occur in some of the *M. galloprovincialis* adults that died in the horizontal transmission assays. This may suggest that mortality was related to other causes such as the release of insecticidal compounds or stress (Butt *et al*., 2013; Fuguet and Vey, 2004; Yousef el al., 2014) and should be considered in future research.

Finally, auto-dissemination strategies may facilitate the practical application of entomopathogenic fungi in forested areas. Specifically, lure and infect tactics seem well suited to that purpose (Francardi *et al*., 2013; Klein and Lacey, 1999; Yasuda, 1999). A very efficient trapping system is currently available for *M. galloprovincialis*, involving commercial pheromone-kairomone lures that attract both sexes equally (Pajares *et al*., 2010) and improved traps that have proven highly effective (Álvarez *et al*., 2014). Beetle populations could be inoculated with the *B. pseudobassiana* EABps 11/01-Mg strain by attracting the insects to traps specifically prepared to contaminate them with the pathogen and allow them to self-release afterwards.

Based on the results of this study, we conclude that *B. pseudobassiana* is an important population regulator of *M. galloprovincialis* in the field; one that could be used in the integrated management of pine wood nematode vectors. This study is the first report of *B. pseudobassiana* being isolated from *M. galloprovincialis* beetles. The isolate caused high mortality in *M. galloprovincialis* adults, especially when the inoculum was applied as dry conidia. Fungal auto-dissemination of this isolate by means of lure and infect tactics may constitute a promising new tool for pine wilt disease management in high risk areas.

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# Chapter 7

# Synthesis

*Traps for M. galloprovincialis live trapping*

Obtained results confirm that there are two main questions affecting the performance of traps: The escape of insects from collector cups (De Groot and Nott 2003; Sweeney *et al*. 2006; Miller and Duerr 2008; Graham and Poland 2012) and attracted insects not falling inside traps. In our study collector cups were elongated and Teflon coated. They were also provided with screened bottom in order to improve the survival of caught insects by increasing the cooling of the collector and favouring the insects to remain quiet so reducing their attempts to escape. This improvement in the collector cup resulted on significantly higher catches of 110%. The capacity of insects to easily hold on trap edges avoiding falling within the trap was minimized by the whole coating of the trap with Teflon. This improvement resulted in higher trap performance, confirming that it is important not only to minimize escape of captured insects but also to maximize the number of those attracted that are actually caught. These findings led in the manufacturing of two proptotypes -respectively based on multiple-funnel and cross-vane standard designs-, which showed a significative higher performance than standard models. Our results indicated that both types of traps were similarly effective in catching and keeping alive pine sawyer beetles..Since analysis of nematode load carried by vectors requires insects to be alive, trap setting and maintenance should be carried out to maximize these. Our model revealed that the number of beetles coexisting in the trap, the elapsed time between consecutive samplings, the mean relative humidity and the maximum radiation were the five most influential variables for insects survival -particularly the last three-. Thus, trap exposure to the sun and samplings as frequently as possible are two factors that would improve the likelihood of having pine sawyers alive and thus provide key information on nematode load.

## *Olfactory receptor neurons in M. galloprovincialis***.**

The sensillae assortment of *M. galloprovincialis* closely resembles that of its sister species *M. notatus* (Drury) and *M. scutellatus* (Say), described by Dyer and Seabrook (1975). Other structures, such as the dome shaped sensillae and the glands associated with stout sensillae chaetica are also similar to those described by the same authors and they probably have a similar, but yet unknown, role in these three species. The most interesting sensillae type in *M. galloprovincialis* are the small sensillae basiconica confined to two sensory fields in each flagellu*m.* There was no remarkable sexual dimorphism with respect to the distribution of this type of sensillae and their abundance along the antenna, suggesting that these sensillae have a similar olfactory role in both sexes.

ORNs tunned to host plant odour, bark beetle pheromones and *M. galloprovincialis* aggregation pheromone were found, which correlates with pevious behaviourally findings (Pajares *et al*., 2004; Ibeas *et al*., 2007; Pajares *et al*., 2010). Despite the behavioural importance of the bark beetle kairomones for *M. galloprovincialis*, we have not identified an ORN "type" that preferentially responds to them, except for the "α-pinene/generalist" cells that in addition of responding to α-pinene and smoke compounds, also responded to *cis*-verbenol. However, the isolated dose-response curve to ipsenol showed that at least some ORNs have high sensitivity to some of these compounds. There is evidence that some *Monochamus* species are attracted to burnt trees (Parmelee, 1941; Ross, 1960; Markalas, 1997). In previous GC-EAG studies we had observed that *M. galloprovincialis* male and female antennae responded to several smoke volatiles, (not published). We found that three groups of neurons responded to smoke compounds as determined by hierarchical cluster analysis. The presence of 2-undecyloxy-1-ethanol-specific ORNs in both sexes, although only one in females compared to five in males, confirms the ecological importance of this attractant for *M. galloprovincialis*.. Given the importance of this ether in the chemical ecology of other *Monochamus* species, we hypothesized that they have also specific receptors for it, most likely located in the basiconic sensillae of the plate areas.

## *Effective lures for trapping M. galloprovincialis*

The experiments confirmed previous findings of *M. galloprovincialis* aggregation pheromone being synergized by host and bark beetle volatiles (Pajares *et al* 2010; Rassati *et al*, 2012). A clear dose-response was observed on *M. galloprovincialis* pheromone, with traps catching two/three times more individuals when pheromone dose was released three/five times the initial amount. This result led to the lure manufacturer (SEDQ, Spain) in 2011 to produce a new standard lure (Galloprotect 2D; SEDQ Spain) releasing a higher rate of the *M. galloprovincialis* pheromone. Previous attempt to replace α-pinene, a conifer compound attracting a wide range of non-targeted beetles including important natural predators such as *Tenmochila. coerulea* or *Thanasimus formicarius* by host compound verbenone were unsuccessful (Pajares *et al*., 2010). Our experiments have demonstrated that incorporating α-pinene to the standard lure does not significantly increase catches but on the other hand has a detrimental effect of attracting predators. These facts makes the uso of this terpene not recommedable.. None of the host terpenes tested in our experiments performed significantly better than α-pinene and some of them proved also attractive to predators. Although previous EAG recordings had shown antennal response by inmature beetles to several host terpenes, our trials found that no immature adults were caught in traps baited with a combination of the standar lure plus host terpenes deployed in forested areas or with a blend of four of these antennally bioactive terpenes in an area deprived of hosts.

## *Effective lures for trapping M. sutor*

Scolytid kairomones have been shown to be attractive to both sexes of *M. sutor* (Pajares *et al*., 2013), and to other *Monochamus* sawyers (Allison *et al*., 2001, 2003; Pajares *et al*., 2004; Miller & Asaro, 2005; Miller *et al*., 2011). In our study, GC-EAG recording showed that *M. sutor* males and females clearly responded to ipsenol and ipsdienol, and females also to 2-methyl-3-buten-2-ol. No response to cis-verbenol was observed by any of the sexes, though females weakly responded to its oxydated derivative verbenone. Chalcogran elicited a neat response to *M. sutor* female antennae. In field tests ipsenol was the most attractive kairomone to both sexes of *M. sutor*, confirming its role as a strong kairomone found for other *Monochamus* sawyers (Allison *et al*. 2003; De Groot and Nott 2004; Miller and Asaro 2005; Ibeas *et al*; 2007). The other kairomones, ipsdienol,

cis-verbenol and 2-methyl-3-buten-2-ol resulted very weakly attractive when released individually at the same dosages. Kairomones were less attractive to females than pheromone. Ipsenol was shown the strongest individual kairomone for *M. sutor* and also resulted synergistic to the pheromone. Pine terpene α-pinene did not increase attraction when combined with the pheromone and kairomones. Even if some pine sawyers are known to colonize burnt trees (Parmelee, 1941; Ross, 1960, Markalas 1997), a blend of smoke volatiles did not increase *M. sutor* attraction when released together with a pheromone-kairomone blend, pointing to that smoke cues are likely not involved in host finding.

## *Entomopathogenic fungi for control of M. galloprovincialis*

Some studies have shown entomopathogenic fungi to be common natural pathogens of forest pests (Draganova, *et al*., 2013; Peng *et al*., 2011; Sevim *et al*., 2010). Three entomopathogenic fungi were isolated for the first time from *M. galloprovincialis* adults and pupae. Molecular methods identified them as *Beauveria pseudobassiana* (EABps 11/01-Mg strain), *Isaria farinosa* and *Lecanicillium attenuatum..* The *B. pseudobassiana* EABps 11/01-Mg strain proved the most virulent isolate against *M. galloprovincialis*, causing 100% mortality in laboratory bioassays. The effectiveness of this fungi in suppressing PWN in an affected area would depend on its ability to kill the insects before peak PWN transmission to healthy trees (Shimazu, 2004b), which has been reported to occur 2 to 6 weeks after emergence (Naves *et al*., 2007). Significant horizontal transmission was proved with adults transmiting the fungus to clean beetles of the opposite sex,. This occurred when dry conidial formulation ($4 \cdot 10^9$ conidia/ml) was used, indicating that the inoculation method (dry or aquous) influenced transmission. Likewise, our bioassays revealed a significant reduction in offspring, not as a result of egg and larvae mortality but from lower female fertility pointing to a horizontally-induced reduction of progeny. Thus, *B. pseudobassiana* can be considered a good candidate for use in auto-dissemination tactics (Francardi *et al*., 2013; Klein and Lacey, 1999; Yasuda, 1999). Since a highly efficient trapping system is currently available for *M. galloprovincialis,* based on improved lures and traps, it may be suitably adapted for use in lure and infect tactics that facilitate the practical application of this entomopathogenic fungus in forested areas.

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# Chapter 8

# Conclusions

## *Traps for M. galloprovincialis live trapping*

1- Teflon coating of multiple-funnel and cross-vane trap surfaces strongly increased catches of pine sawyers.

2- Elongation and ventilation of collector cups increased survival of trapped insects.

3- Newly developed commercial traps *Econex Multifunnel-12*® and *Crosstrap*® resulted highly efficient and equally effective in capturing and providing a suitable sample of live insects for nematode load.

4- Survival of caught insects within the trap can be enhanced if excessive sun exposure of the trap is avoided and a reasonable sampling schedule is implemented.

## *Olfactory receptor neurons in M. galloprovincialis*

5- Antennal sensillae of *M. galloprovincialis* are similar morphologically and electrophysiologically to those described for other species in the same genus.

6- Sensillae basiconica are chemoreceptors that responded to odours from host plants, bark beetles, smoke volatiles and aggregation pheromone.

7- A few of the ORNs could be considered specific to pheromone or to smoke compounds, but a large proportion are generalist or unresponsive, which suggest that many semiochemicals relevant to this species remain still unknown.

8- The presence of smoke-detector ORNs in *M. galloprovincialis* supports the hypothesis that they may use smoke odour to locate suitable breeding hosts.

## *Effective lures for trapping M. galloprovincialis*

9- We have corroborated the strong synergistic effect of bark beetles kairomones to the *M. galloprovincialis* aggregation pheromone in attracting mature individuals of this species.

10- The attractive power of this combination was further improved by increasing the pheromone release rate, leading to the development of a new standard operational lure.

11- Addition of α-pinene or other pine terpenes to the standard lure is not advisable since it will not significantly improve trapping effectiveness of target species but result in high by-catch of non-target species, including natural predators.

12- None of tested terpenes, either in combination with the standard lure on a forested area, or in blends in a non forested area, attracted immature beetles.

## *Effective lures for trapping M. sutor*

13- *M. sutor* males and females were attracted to 2-undecyloxy-1-ethanol, confirming that this ether is its aggregation pheromone.

14- Ipsenol resulted a strong attractive kairomone to this species.

15- Blends of pheromone plus bark beetle pheromones ipsenol alone or with 2-methyl-3-buten-2-ol was shown an efficient lure for management of this species.

16- Neither pine terpene α-pinene nor a blend of smoke volatiles increased attraction to the above pheromone-kairomones blend.

## *Entomopathogenic fungi for control of M. galloprovincialis*

17- Three entomopathogenic fungi were isolated for the first time from *M. galloprovincialis*: *Beauveria pseudobassiana*, *Isaria farinosa* and *Lecanicillium attenuatum*

18- *B. pseudobassiana resulted highly virulent, causing complete mortality and reduced survival times on this pine sawyer.*

19- Horizontal transmission on infected adults by a dry conidial formulation (4 $10^9$ conidia/ml) of this strain to mates was shown. Horizontally-induced reduction of progeny was also observed.

20- This strain represents a suitable candidate for further studies on fungal auto-dissemination by means of lure and infect tactics that take advantage of the semiochemical tools already developed.

# Chapter 9

# Conclusiones

## *Trampas para la captura de M. galloprovincialis vivos*

1- El recubrimiento de Teflón de las superficies de las trampas de intercepción y de multiembudos aumentó considerablemente las capturas de *Monochamus*.

2- La elongación y ventilación de los botes colectores aumentó la supervivencia de los insectos capturados.

3- Las nuevas trampas comerciales desarrolladas Econex Multifunnel-12® y Crosstrap® resultaron muy eficientes e igualmente eficaces en la captura y mantenimiento de insectos vivos para el muestreo de su carga de nematodos.

4- La supervivencia de los insectos atrapados en la trampa puede mejorarse si se evita su exposición excesiva al sol y se muestrea con una periodicidad razonable.

## *Neuronas olfativas receptoras en M. galloprovincialis*

5- Las sensilas antenales de *M. galloprovincialis* son morfológicamente y electrofisiológicamente similares a las descritas para otras especies del mismo género.

6- Las sensilas basiconicas son quimiorreceptores que respondieron a los olores de los árboles hospedantes, de escolítidos, a los volátiles de humo y a la feromona de agregación.

7- Algunas de las neuronas olfativas podrían considerarse específicas de la feromona o de compuestos de humo, pero una gran proporción de ellas resultó generalista o no respondió, lo que indica que aún se desconocen muchos compuestos semioquímicos relevantes para esta especie.

8- La presencia de neuronas olfativas detectoras de volátiles de humo en *M. galloprovincialis* apoya la hipótesis de que podrían utilizar estos olores para localizar sus árboles hospedantes.

### *Cebos eficaces para la captura de M. galloprovincialis*

9- Se ha corroborado el fuerte efecto sinérgico de las cairomonas de escolítidos con la feromona de agregación de *M. galloprovincialis* en la atracción de individuos maduros de esta especie.

10- El poder de atracción de esta combinación ha mejorado aún más mediante el aumento de la tasa de emisión de la feromona, que ha conducido al desarrollo de un nuevo cebo operativo estándar.

11- La adición de α-pineno u otros terpenos de los pinos al cebo estándar no es recomendable, ya que no mejora significativamente la captura de *M. galloprovincialis*, y además provoca una alta captura de especies no objetivo, incluidos algunos depredadores naturales.

12- Ninguno de los terpenos probados, ya sea en combinación con el cebo feromonal-cairomonal estándar en un pinar, o mezclados entre sí en una zona desarbolada, atrajo individuos inmaduros.

### *Cebos eficaces para la captura de M. sutor*

13- Tanto los machos como las hembras de *M. sutor* se vieron atraídos por 2-(undeciloxi) etanol, lo que confirma que se trata de su feromona de agregación.

14- El ipsenol resultó ser una kairomona fuertemente atractiva para esta especie.

15- Las mezclas de feromona con las cairomonas de escolítidos ipsenol solo o con 2-metil-3-buten-2-ol se resultó ser un atrayente eficaz para el manejo de esta especie

16- Ni el terpeno α-pinene ni una mezcla de volátiles de humo aumentaron la atracción cuando fueron añadidos a la anterior mezcla feromonal-cairomonal.

### *Hongos entomopatógenos para el control biológico de M. galloprovincialis*

17- Se aislaron por primera vez de *M. galloprovincialis* los hongos entomopatógenos: *Beauveria pseudobassiana*, *Isaria farinosa* y *Lecanicillium attenuatum*.

18- *B. pseudobassiana* resultó altamente virulenta, causando una mortalidad total y una reducción en el tiempo de supervivencia de los insectos.

19- Se demostró que hubo transmisión horizontal de los adultos infectados a sus parejas con una formulación conidial seca ($4 \cdot 10^9$ conidios/ml) de esta cepa. También se observó una reducción de la progenie inducida horizontalmente.

20- Esta cepa es un candidato adecuado para avanzar sobre la autodiseminación de hongos entomopatógenos mediante técnicas de “atrae e infecta” que aprovechen las nuevas herramientas semioquímicas desarrolladas.