UNIVERSIDAD DE LA FRONTERA

Facultad de Ingeniería, Ciencias y Administración
Programa de Doctorado en Ciencias de Recursos Naturales

OLFACTION IN *Hylastinus obscurus* MARSHAM (COLEOPTERA: SCOLYTIDAE): STUDY OF PHYSIOLOGICAL AND CHEMICAL ASPECTS INVOLVED IN THE RESPONSE TOWARDS SEMIOCHEMICALS PRESENT IN ITS ENVIRONMENT

TESIS PARA OPTAR AL GRADO DE DOCTOR EN CIENCIAS DE RECURSOS NATURALES

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TEMUCO – CHILE
2012

"Olfaction in *Hylastinus obscurus* Marsham (Coleoptera: Scolytidae): Study of physiological and chemical aspects involved in the response towards semiochemicals present in its environment"

Esta tesis fue realizada bajo la supervisión del Director de Tesis, Dr. ANDRÉS QUIROZ CORTEZ, del Departamento de Ciencias Químicas y Recursos Naturales y ha sido aprobada por los miembros de la comisión examinadora.

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Abstract

Hylastinus obscurus (Coleoptera: Scolytidae) is an important pest for red clover crops causing plants decay and reducing the productivity. As other members of the family it completes almost its whole life inside it host which leave to colonize new plants and restart the cycle. Nowadays there is not an effective method to control it except for crop rotation. The stretch relationship between borer and red clover has been subject of investigation and several studies have found chemical compounds in red clover eliciting behavioral responses on insects.

The current work shows evidence about the ability of this insect to perceive the odorant stimuli coming from its environment. Thus, the study of *H. obscurus* antennae using Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM) demonstrated the presence of sensilla concentrated in the club. No significant differences were found between sexes, basiconica and trichodea sensilla were related to chemoreception due to the presence of pores in their surface. The high presence of sensilla chaetica with mechanical function may help to explain the difficulties appeared in the electrophysiological assays.

Solid Phase Microextraction (SPME) was used to collect volatiles from roots of red clover. The elucidation of compounds by Gas Chromatography coupled to Mass Spectrometer (GC-MS) and their testing in behavioral bioassays an electroantennography resulted in two compounds, ethanol and hexanal, not reported before to red clover that elicited responses in borers specially in females. Besides, the trapping of insect volatiles showed the presence of β-pinene which has not been reported among red clover volatiles. Interestingly this compound elicited behavioral responses in males but not in females and electroantennographical assays showed a higher relative response to males than females. Finally, non conclusive results showed the presence of antennospecific proteins similar to those found in other insect species responsible for odour binding. The lacking of genomic information in this group reduce the possibilities to have successful results.

Further studies are required in order to use determine individual compounds or blends which may be used to modify the behavior of this pest and reduce their incidence and damage in field.

Chapter 1

Introduction

General Introduction

The Scolytidae family of insects comprises more than 6000 species described worldwide (Knizek & Beaver, 2004). They are small endophytic beetles, living inside plant tissues protected from environmental factors during their whole life (Sauvard, 2004), excepting short flight periods in their imaginal stage, that serves to disperse the population (Rudinsky, 1962) and to locate suitable breeding sites (Knizek & Beaver, 2004).

They usually live in scattered habitat units, which are suitable for breeding for only a single generation of beetles. (Knizek & Beaver, 2004). Their feeding habits are characteristic of a given species. The most common of them include the consumption of phloem (inner bark) and ectosymbiotic fungi that grow in their galleries, but some species feed on herbaceous plants, fruits and seeds (Atkinson, 1986).

The smell sense in insects depends of olfactory chemoreceptors allocated in the antenna (Klowden, 2005). Scolytidae antenna consist tipically of an elongated scape, a serie of short segments called funicle which supports a flattened oval club (Moeck, 1968) formed by several fused segments and covered with numerous sensilla (Dickens & Payne, 1978). The sensilla are specialized organs formed by cuticular structures, neurons and accessory cells (Klowden, 2005). The presence of pores in the cuticle separates those chemoreceptive sensilla from those with other functions (Klowden, 2005). Thus, the morphological studies provide the foundation for electrophysiological investigations (Whitehead, 1981).

Hylastinus obscurus (Marsham), the clover root borer is a scolytid described as a major pest in red clover crops in Chile in the area comprised by Biobío and Los Lagos regions (Aguilera et al., 1996). The insects cause severe damage by tunnelling into the roots and crown of plants, affecting their health and determining their yield (Steiner & Alderman, 1999). The stretch relationship between *H. obscurus* and red clover has been partially studied. The quantification of damage caused directly by the presence of clover borer in the roots was measured by Pruess & Weaver (1958), and their abundance correlates with the reduction in dry matter yield (Alarcón et al., 2010). *H. obscurus* has demonstrated the ability to select an appropriate host according to its health status (Leath and Byers, 1973). Quiroz et al. (2005) showed that volatiles released by differently–aged plants produce different responses in olfactometric assays. The root extracts from plant five-, seven- and nine-months old were attractive to *H.*

obscurus in laboratory conditions (Manosalva, 2010). This result is consistent with the field observations that plant colonization starts when they are six months old (Alarcón et al., 2010).

The known attraction of the adult stages of Scolytidae to freshly cut or damaged hosts, has been used to make commercially available odorant pheromone lures for many damaging species (Grégoire and Evans, 2004); becoming the trapping methods to attract the adult stages the most commonly employed against Scolytidae (Dodds, 2011). However, little is known about dispersal and host selection except for the stages immediately preceding landing on them (Byers, 1989), and a large variation in the chemicals content has been observed in this group (Schlyter and Birgersson, 1989). Several chemicals released by red clover possibly involved in the behaviour of *H. obscurus* have been reported (e.g. Kamm & Buttery, 1984; Buttery et al., 1984; Tapia et al., 2007), however none of these reports discriminated behaviorally between sexes nor determined the ability of the clover root borer to perceive these stimuli.

When Vogt & Riddiford (1981) reported the finding of a kind of proteins that bind the pheromones to carry them through the sensillar lumen, it supposed an important advance in the study of insect olfaction. These proteins are able to bind pheromones (PBPs) or general odorants compounds (GOBPs) (Field et al., 2000). Their presence have been reported in different insect orders such as Lepidoptera, Coleoptera, Diptera, Hymenoptera, Hemiptera, Orthoptera and Isoptera (Vogt et al., 1991; Nikonov et al., 2002; Ishida, et al., 2002; Calvello et al., 2005; Dickens et al., 1998; Ban et al., 2003; Ishida et al., 2002b). These soluble proteins from 14 to 16 kDa (Jansen, 2005) carry the odorants from the pores in the surface to the odorant receptors (Leal, 2003), changing their structure according to the surrounding pH (Horst et al., 2001). Their affinity for a range of ligands and the possibility of express and purify recombinant PBPs/GOBPs have helped to elucidate novel behaviorally important chemical compounds that could be used in the surveillance and control of damaging insects, a term coined as reverse chemical ecology (Leal et al., 2008).

In Scolytidae, important challenges remain in this field, since the first pheromone isolated and identified in Coleoptera was from a scolytid species (Seybold & Vanderwel, 2003), limited genomic resources are still available, reducing the possibility of use these tools to help monitoring, predict, and manage this group of pest insects (Keeling et al., 2012), and the existence of PBPs/GOBPs have not been confirmed to date.

Hypothesis

Based on these antecedents, the work hypothesis is:

The scolytid *Hylastinus obscurus* has the ability of detecting semiochemicals present in its environment by means of specific structures located on the antennae.

Objectives

General goals

- To determine the electrophysiological response of adult individuals of red clover root borer (*Hylastinus obscurus*) towards semiochemicals identified in their environment.
- To identify the type, distribution and structures of sensilla in the antennae of *Hylastinus obscurus*.
- To evaluate the *Hylastinus obscurus* ability to produce some odor-binding proteins (OBPs) related to the semiochemicals identified in their environment.

Specific goals

- Identify volatiles from clover root borer and its host potentially involved in the insect host-finding and mating behavior.
- Determine the activity of previously identified compounds by means of electrophysiological assays using the antennae of adult insects.
- Describe the type, distribution and structures of sensilla in the antennae of *Hylastinus obscurus*.
- Identify some odor-binding protein candidate in adult clover root borer antennae.

References

Aguilera, A., E. Cisternas, M. Gerding, and H. Norambuena. 1996. Plagas de las praderas. In *Praderas Para Chile*, ed. Ignacio Ruiz N. , p. 310–339.

Alarcón, D., F. Ortega, F. Perich, F. Pardo, L. Parra, and A. Quiroz. 2010. Relationship between radical infestation of *Hylastinus obscurus* (Marsham) and the yield of cultivars and experimental lines of red clover (*Trifolium pratense* L.). *Revista de la Ciencia del Suelo y Nutrición Vegetal* 10(2): 115–125.

Atkinson, T.H. 1986. Biology of the Scolytidae and Platypodidae (Coleoptera) in a tropical deciduous forest at Chamela, Jalisco, Mexico. *Florida Entomologist* 69(2): 303–310.

Ban, L, A. Scaloni, C. D'Ambrosio, L. Zhang, Y. Yahn, and P. Pelosi. 2003. Biochemical characterization and bacterial expression of an odorant-binding protein from *Locusta migratoria*. *Cellular and Molecular Life Sciences* 60(2): 390–400.

Buttery, R.G., J.A. Kamm, and L.C. Ling. 1984. Volatile components of red clover leaves, flowers, and seed pods: possible insect attractants. *Journal of Agricultural and Food Chemistry* 32(2): 254–256.

Byers, J.A. 1989. Chemical ecology of bark beetles. *Experientia* 45(3): 271–283.

Calvello, M, A. Brandazza, A. Navarrini, F.R. Dani, S. Turillazzi, A. Felicioli, and P. Pelosi. 2005. Expression of odorant-binding proteins and chemosensory proteins in some Hymenoptera. *Insect Biochemistry and Molecular Biology* 35(4): 297–307.

Dickens, J.C., F.E. Callahan, W.P. Wergin, C.A. Murphy, and R.G. Vogt. 1998. Intergeneric distribution and immunolocalization of a putative odorant-binding protein in true bugs (Hemiptera, Heteroptera). *Journal of Experimental Biology* 201(1): 33–41.

Dickens, J.C., and T.L. Payne. 1978. Structure and function of the sensilla on the antennal club of the southern pine beetle, *Dendroctonus frontalis* (Zimmerman)(Coleoptera: Scolytidae). *International Journal of Insect Morphology and Embryology* 7(3): 251–265.

Dodds, K.J. 2011. Effects of habitat type and trap placement on captures of bark (Coleoptera: Scolytidae) and longhorned (Coleoptera: Cerambycidae) beetles in semiochemical-baited traps. *Journal of Economic Entomology* 104(3): 879–888.

Field, L.M., J.A. Pickett, and L.J. Wadhams. 2000. Molecular studies in insect olfaction. *Insect Molecular Biology* 9(6): 545–551.

Grégoire, J-C., and H.F. Evans. 2004. Damage and control of BAWBILT organisms, an overview. In *Bark and wood boring insects in living trees in Europe, a synthesis*, eds. Francois Lieutier, Keith Day, Andrea Battisti, Jean-Claude Grégoire, and Hugh Evans. Dordrecht: Elsevier, p. 19–38.

Horst, R, F. Damberger, P. Luginbühl, P. Güntert, G. Peng, L. Nikonova, W.S. Leal, and K. Wüthrich. 2001. NMR structure reveals intramolecular regulation mechanism for pheromone binding and release. *Proceedings of the National Academy of Sciences of the United States of America* 98(25): 14374–14379.

Ishida, Y., A. Cornel, and W.S. Leal. 2002. Identification and cloning of a female antenna-specific odorant-binding protein in the mosquito *Culex quinquefasciatus*. *Journal of Chemical Ecology* 28(4): 867–871.

Ishida, Y., V. Chiang, M. Haverty, and W.S. Leal. 2002b. Odorant-binding proteins from a primitive termite. *Journal of Chemical Ecology* 28(9): 1887–1893.

Jansen, S. 2005. Functional structure of insect odorant binding proteins (OBPs) and chemosensory proteins (CSPs). *Chem. Ecol. Ecotox* 1(167).

Kamm, J. A., and R.G. Buttery. 1984. Root volatile components of red clover: identification and bioassay with the clover root borer (Coleoptera: Scolytidae). *Environmental Entomology* 13(5): 1427–1430.

Keeling, C., H. Henderson, M. Li, M. Yuen, E. Clark, J. Fraser, D. Huber, N. Liao, T. Docking, I. Birol, S. Chan, G. Taylor, D. Palmquist, S. Jones, and J. Bohlmann. 2012. Transcriptome and full-length cDNA resources for the mountain pine beetle,

Dendroctonus ponderosae Hopkins, a major insect pest of pine forests. *Insect Biochemistry and Molecular Biology* 42(8): 525–536.

Klowden, M.J. 2005. *Physiological Systems in Insects*. Second Edition. Academic Press. 688pp.

Knizek, M., and R. Beaver. 2004. Taxonomy and systematics of bark and ambrosia beetles. In *Bark and wood boring insects in living trees in Europe, a synthesis*, eds. F. Lieutier, K. Day, A. Battisti, J-C. Grégoire, and H. Evans. Dordrecht: Springer, p. 41–54.

Leal, W.S. 2003. Proteins that make sense. In *Insect Pheromone Biochemistry and Molecular Biology*, eds. G.J. Blomquist and R.G. Vogt. London: Elsevier Academic Press, p. 447–476.

Leal, W.S, R. Barbosa, W. Xu, Y. Ishida, Z. Syed, N. Latte, A. Chen, T. Morgan, A.J. Cornel, and A. Furtado. 2008. Reverse and conventional chemical ecology approaches for the development of oviposition attractants for *Culex* mosquitoes. *PloS one* 3(8): e3045.

Leath, K.T., and R.A. Byers. 1973. Attractiveness of diseased red clover roots to the clover root borer. *Phytopathology* 63: 428–431.

Manosalva, L. 2010. Estudio de la composición química de extractos de raíces de líneas y cultivares de plantas jóvenes de trébol rosado (*Trifolium pratense* L.) y su relación con la respuesta conductual del insecto *Hylastinus obscurus* (Marsham). Tesis Magister en Ciencias de Recursos Naturales, Universidad de La Frontera. 62pp.

Moeck, H.A. 1968. Electron microscopic studies of antennal sensilla in the ambrosia beetle *Trypodendron lineatum* (Olivier) (Scolytidae). *Canadian Journal of Zoology* 46(3): 521–556.

Nikonov, A.A., G. Peng, G. Tsurupa, and W.S. Leal. 2002. Unisex pheromone detectors and pheromone-binding proteins in scarab beetles. *Chemical senses* 27(6): 495–504.

Pruess, K.P., and C.R. Weaver. 1958. Estimation of red clover yield losses caused by the clover root borer. *Journal of Economic Entomology* 51(4): 491–492.

Quiroz, A., F. Ortega, C.C. Ramírez, L.J. Wadhams, and K. Pinilla. 2005. Response of the beetle *Hylastinus obscurus* Marsham (Coleoptera: Scolytidae) to red clover (*Trifolium pratense* L.) volatiles in a laboratory olfactometer. *Environmental Entomology* 34(3): 690–695.

Rudinsky, J.A. 1962. Ecology of Scolytidae. *Annual Review of Entomology* 7(1): 327–348.

Sauvard, D. 2004. General biology of bark beetles. In *Bark and wood boring insects in living trees in Europe, a synthesis*, eds. F. Lieutier, K. Day, A. Battisi, J-C. Grégoire, and H. Evans. Dordrecht: Elsevier, p. 63–88.

Schlyter, F. and G. Birgersson. 1989. Individual variation in bark beetle and moth pheromones-a comparison and an evolutionary background. *Holartic Ecology* 12(4): 457–465.

Seybold, S.J., and D. Vanderwel. 2003. Biosynthesis and endocrine regulation of pheromone production in the coleoptera. In *Insect Pheromone Biochemistry and Molecular Biology*, eds. Gary J. Blomquist and Richard G. Vogt. San Diego, CA: Elsevier, p. 137–200.

Steiner, J.J. and S.C. Alderman. 1999. Red clover seed production. V. Root health and crop productivity. *Crop Science* 39: 1407–1415.

Tapia, T., F. Perich, F. Pardo, G. Palma, and A. Quiroz. 2007. Identification of volatiles from differently aged red clover (*Trifolium pratense*) root extracts and behavioural responses of clover root borer (*Hylastinus obscurus*) (Marsham) (Coleoptera: Scolytidae) to them. *Biochemical Systematics and Ecology* 35(2): 61–67.

Vogt, R.G., R. Rybczynski, and M.R. Lerner. 1991. Molecular cloning and sequencing of general odorant-binding proteins GOBP1 and GOBP2 from the tobacco hawk moth Manduca sexta: comparisons with other insect OBPs and their signal peptides. *The Journal of Neuroscience* 11(10): 2972–84.

Vogt, R.G., and L.M. Riddiford. 1981. Pheromone binding and inactivation by moth antennae. *Nature* 293(5827): 161–163.

Whitehead, A.T. 1981. Ultrastructure of sensilla of the female mountain pine beetle, Dendroctonus ponderosae Hopkins (Coleoptera: Scolytidae). *International Journal of Insect Morphology and Embryology* 10(1): 19–28.

Chapter 2

Type and distribution of sensilla in the antennae of the red clover root borer *Hylastinus obscurus* Marsham.

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Manuscript submitted to Journal of Insect Science

Type and distribution of sensilla in the antennae of the red clover root borer *Hylastinus obscurus* Marsham.

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Abstract

In order to determine the type, distribution and structures of senses, the antennae of *Hylastinus obscurus* were examined by light and electron microscopy (both scanning and transmission). Thus, the study did not reveal differences in the relative abundance of antennal sensilla between males and females. Four types of different sensilla were identified in the club, plus one type of chaetica found in the scape and funicle of both male and female individuals. In the club the most abundant sensilla were chaetica and basiconica. They were present in the three sensory band described, totalizing ca. 80% of sensilla in the antennal club of *H. obscurus*. Chaetica were prominently mechanoreceptors; although gustatory function could not be discarded. Basiconica instead, showed characteristics typical of olfactory sensilla.

Trichodea were not found in proximal sensory band, exhibiting abundant pores confering them olfactory abilities. Styloconica were the less abundant, and their shape was similar to those reported as hygro and thermoreceptor functions. Finally, the sensillar configuration revealed the chemoreceptive abilities as well as the abundance of receptors in the antenna of *H. obscurus* prepared for not chemical stimulus.

Keywords: Scolytinae, chemoreception, scanning electron microscopy, transmission electron microscopy.

Introduction

Scolytinae is an important insect group that comprises ca. 6,000 species (Grégoire & Evans 2004), characterizated because many of them attack healthy trees that they girdle and kill or feed on fungi that they cultivate in tunnels (Gillott 2003). These beetles occupy a wide range of niches among various herbaceous and woody plants (Zúñiga et al. 2002). The red clover root borer (*Hylastinus obscurus* Marsham) is a scolytin that feeds on red clover roots (*Trifolium pratense*), a widespread leguminous forage crop, being the main cause of its decline (Quiroz et al. 2005). As other Scolytinae species, the importance of chemical stimuli have been studied to figure out their behavior (see Byers 1989). Tapia et al. (2007) reported the preference of *H. obscurus* for volatiles extracted from red clover roots of 1.5 years old instead of those extracted from roots of 2.5 years old, suggesting that *E*-2-hexenal attracted the insect, whereas the same dose of limonene repelled the red clover borer. Recently, Manosalva et al. (2011) noted the attractive effect upon these beetles of several long-chain fatty acids found in roots of nine months old plants.

Is well known such the olfactory system is the primary sense that insects use in analyzing the environment, in crucial tasks such as finding food, nesting, mating and in conspecifics (Picimbon 2003). The antennae concentrate the olfactory chemoreceptors (Klowden 2007), conferring to the insects the ability for discriminating a myriad of physiologically irrelevant chemical compounds in the environment from essential chemical signals (Leal 2003). In fact, the antennal morphology of different Scolytinae species motivated an important number of researches in the past. Moeck (1968) elegantly described the antennal sensilla of

Trypodendron lineatum and Payne et al. (1973) noted clear differences among genus after studying the antennae of sixteen Scolytinae species. The utility of this information exceeds the purely morphological concerns, because provide the foundation for electrophysiological studies (Whitehead 1981) and the use of chemosensory system as an odor detector for determining relevant compounds in chemical ecology (Larsson & Svensson 2004).

Although our knowledge about the chemical signals involved in the behavior of *H. obscurus* has increased in the recent years, still remain some questions about its antennal morphology which could be very helpful not only to get a better overview on its biology but particularly to performing electrophysiological researches.

Materials and Methods

Insects

Adult individuals of *H. obscurus* were isolated from red clover roots collected from red clover plots allocated in Regional Research Center INIA-Carillanca, Araucanía, Chile. The insects were separated and stored in petri dishes. Then they were frozen at -20°C per 10 hours before sample preparation. The whole heads and antennae were cut using scalpel and sex of individuals was determined under light microscope to save the tissue separately following the methodology reported by Carrillo et al. (1978).

Microscopy

Scanning Electron Microscopy (SEM) was carried out following the methodology employed in the Electronic Microscopy Laboratory of Universidad de Concepción, Chile. Heads and antennae of previously frozen beetles were immersed in 15% v/v ethanol solution and sonicated per 30 seconds. Then samples were dehydrated keep them in 30, 50, 70, 90 and 99% v/v ethanol solutions per 3 minutes each, and they were leave to dry overnight at room temperature. Finally the samples were put on a sample holder and treated in a critical point dryer per 20 minutes and gold-coated to be observed using a LEO 420 microscope (Carl Zeiss SMT, Oberkochen, Germany).

The samples for Transmission Electron Microscopy (TEM) were obtained in the same way than those used in SEM. The tissues were immersed in 3% v/v glutaraldehyde solution per 24 hours, transferred to 0.1% w/v cacodylate buffer per 4 hours at room temperature. Then they were fixed in 1% w/v osmium tetroxide solution and sequencially dehydrated in acetone to be

embedded in a resine composed by polypropylene oxide and araldite. Finally they were mounted on capsules and oven-dried per 48 hours before to be cut using microtome. The samples were observed using a JEM-1200 EX II microscope (JEOL, Japan) at 80 kV.

Image and data analysis

The images obtained by SEM and TEM were processed using ImageJ software v. 1.44p (National Institute of Health, USA), and datum were analyzed by t-Student test using StatsDirect v.2.7.8 (StatsDirect Ltd., UK).

Results

The antenna

The antenna of *H. obscurus* consisted of scape, seven-segmented funicle and club (Figure 1A). The average length of female antenna was 547 μm, which is slightly longer than 512 μm of mean to the male antenna. The scarce number of sensilla upon the scape and funicle contrasted to the abundantly-covered club (Table 2), where the sensilla were distributed in three transversal sensory bands.

Each one of these bands (Figure 1B) was formed by a wide belt of chaetica sensilla in the proximal part, such as the upper part of those sensilla covered the distal narrow belt placed next to them and formed by basiconica sensilla mainly.

The type and size of sensilla covering the different parts of antenna were similar to male and female and no apparent sexual dimorphism was found in these sensitive structures (Table 1).

Types of sensilla

The number of sensilla in the scape and funicle was significantly lower than the club of the antenna, and there was not significant difference between male and female (Table 2). A decreasing number of the two most abundant types of sensilla in the antennal club was found from the band 1 (Sb1) to band 3 (Sb3) (Table 3). Chaeticum was the most abundant type of sensillum found in the antennal club, and there was significant differences between male and female in the number of sensilla of the three types located in Sb2 and Sb3 (Table 3). In the scape and funicle, the sensilla were clearly distinguishable by their lenght from 28 to 61 μm

and by their orientation ca. 45° respect to the surface (Figure 1C), and they were equipped with a socket (Figure 1C) and basal diameters of 1.3 to 2.3 μm and 0.2 μm in the apex. Bilaterally branched, from seven to twenty pegs were alternately distributed on the smooth surface of the sensillum. Pores were not observed in the surface.

In the club the shape of chaetica sensilla were similar to those found in scape and funicle. They were supported in a socket (Figure 2A), and from 26 to 37 μm in length, the basal diameter was 1.5 to 1.7 μm and 0.3 μm in the apex and there were 5 to 9 pegs per sensillum. Transmission microscopy showed circular solid cross sections with no pores in the wall (Figure 2C and 2D).

Sensilla basiconica (Figure 3) were found in the club only and forming a stretched belt in the sensory band in a distal position respect to the chaetica sensilla. They did not show socket but showed oblique insertion, the form varied from straight in the proximal to bent in the distal sensory band. The external shape was slightly rough, and the length varied from 9 to 21 μm, meanwhile the basal diameter was 1.3 to 1.7 μm to finish in a sharp apex of 0.1 μm. Internally was observed a thick wall of 0.2 μm disrupted by pores in the upper section which linked the outside part to the sensillar lumen. The number of dendrites observed oscilated from two to three, and the number of dendritic segments reached sixteen.

Sensilla trichoidea appeared dispersed in the sensory bands among chaetica and basiconica sensilla without a clear pattern of distribution. The length oscilated between 20 and 38 μm and its basal diameter was 1.3-1.9 μm clearly reduced up to finishing in a rounded tip of 0.3 μm wide. The density of this type of sensillum varied in the different sensory bands being more

abundant in the distal than proximal one (Table 3). Besides of presence of a socket in the base, externally was notorious the rough surface forming longitudinal strips (Figure 4). Transmission images showed a thick wall of 0.2 μm interrupted by numerous pores, whilst the center of sensillar lumen was filled by two dendrites comprising up to eighteen dendritic segments (Figure 4).

The type styloconicum (Figure 5) was registered in scarce number just in the distal sensory band (Sb3). The length varied from 6 to 8 μm and basal diameter from 1.3 to 1.5 μm. With absent socket and smooth surface the upper half was formed by ca. 10 finger-shaped pegs looking like a bud.

Discussion

Although the antennae of adult insects present various types of sensilla with different functions playing an important role in a number behaviors during adult life (Hu et al. 2009),. The more researches reported these structures, the more names are used to name them, making difficult to compare the results even among antennal systems of closely related species. As noted by Merivee et al. (1999), this type of confusion in sensilla terminology is apparently due to the lack of fixed criteria for discrimination.

Even though the number of antennal sensilla varies greatly in different insect species, the relative count and diversity of sensilla found in *H. obscurus* is close in magnitude to those reported previously to other members of Scolytinae like *Scolytus multistriatus* (Borg & Norris 1971), *Dendroctonus frontalis* (Dickens & Payne 1978) and *D. ponderosae* (Whitehead 1981).

Those species for which micrographs are available for both sexes show no apparent differences in the antennae between the sexes for the type, relative number, location and size of sensilla. There are considerable variations in the range of lengths of the sensilla (Table 1). However, in general, the variations in sensillum length are not directly related to sex, species, or genus, and the lengths for the various sensilla appear to have no correlation with the size of the antenna (Payne et al. 1973).

Characterized by their spine-like form (Snodgrass, 1993), chaetica in the club represented more than 40% of the total sensilla (Table 3) and being compared to those in the scape and funicle, just the single presence of a socket at the base differenced the chaetica type II from the type I. According to Borg & Norris (1971) who studied the antennal club of *Scolytus*

multistriatus,chaetica function as mechanoreceptors, enabling the insect to determine the position of the antenna with respect to its surroundings (Payne et al. 1973). The presence of two rows of pegs projected outside and forming an angle of ca. 120° (Figure 2B) may be considered modifications which allow the uptake and transmission of most diverse mechanical stimuli (Keil 1997).

Internally they have a thick wall and no pores were observed in surface. Borg & Norris (1971) described the dendrites do not penetrate inside the seta but remain at the base because of their function. Chen et al. (2010), who studied antennal sensilla of *Dendroctonus valens* noted a stretched central area lacking of sensillar lumen. In the other hand, the images of different sections of this type of sensillum in *H. obscurus* (Figure 2C and 2D) showed the lumen is not an empty area, in concordance with those observed in *Psylliodes chrysocephala*, which structure typifies a sensillum with a combined gustatory/mechanosensory function (Isidoro et al. 1998).

The relative abundance of basiconicum was comparable to chaeticum but disposed compactly forming a sort of palisade; in the same manner to those described previously by Payne et al. (1973) to the antennae of *Pseudohylesinus sp.* and several *Dendroctonus* species. Internally the sensilla are notable by their thick wall (Snodgrass 1993) that showed pores in the upper part only, suggesting the olfaction as the likely function of this type of sensillum (Klowden, 2007). The description agrees with the type of sensillum called basiconica 1, the most numerous in the antenna of *Dendroctonus valens* (Chen et al. 2010)

Trichodeum was the third most abundant type of sensillum. Due to both its length and presence, it was notorious in the distal sensory band (Figure 5) though it was not registered in the proximal sensory band (Table 3). The abundant pores formed radial channels connecting the surface to the lumen (Figure 5) that may confer olfactive functions. The description resembles sensilla trichodea 3 descripted by Chen et al. (2010) in *Dendroctonus valens*.

Sensilla styloconica resulted quite scarce and difficult to find within the sensory bands of male and female antenna. It was not possible to get any TEM image which could help to lucubrate about their function. However their shape and size resemble those reported by Dickens & Payne (1978) as fluted sensilla in *Dendroctonus frontalis*, or also called sensilla basiconica type II in *Semiadalia undecimnotata* (Jourdan et al. 1995), or grooved peg in *Trogossita japonica* (Usha Rani & Nakamuta 2001) and *Callosobrochus chinensis* and *C. maculatus* (Hu et al. 2009). Bartlet et al. (1999) and Steinbrecht (1989) reported it as thermo- and hygrosensitive receptors, but Hu et al. (2009) added chemoreception as another possible function. The reduced number of this type of sensors in insects is noted by Altner & Loftus (1985) compared to chemo- and mechanoreceptors.

In conclusion, as well as other Scolytinae species, males and females of *Hylastinus obscurus* did not show clear differences in the type, number and length of antennal structures. Additionally the presence of porous sensilla reflected the aptitude of antennae to perceive chemical stimuli, and they can be subject of study for the better figuring out of the *H. obscurus* ecology where the chemicals maybe involved. However the relatively high abundance mechanoreceptors, specially in the club, should be considered as potential source of bias for the electroantennographical works in this borer.

Acknowledgments

Authors thank the support to this research from The National Comission for Scientific and Technologic Development (Conicyt) through Fondecyt Project #1100812.

R. Palma thanks the thesis support funding from The National Comission for Scientific and Technologic Development (Conicyt) through the project #AT24090126.

References

Altner H, Loftus R. 1985. Ultrastructure and function of insect thermo- and hygroreceptors. *Annual Review of Entomology*, 30(1): 273-295.

Bartlet E, Romani R, Williams IH, Isidoro N. 1999. Functional anatomy of sensory structures on the antennae of *Psylliodes chrysocephala* L. (Coleoptera: Chrysomelidae). *International Journal of Insect Morphology and Embryology*, 28(4): 291-300.

Borg TK, Norris DM. 1971. Ultrastructure of sensory receptors on the antennae of *Scolytus multistriatus* (Marsh.). *Cell and Tissue Research*, 113(1): 13–28.

Byers JA. 1989. Chemical ecology of bark beetles. *Experiencia*, 45(3): 271-283.

Carrillo R, Matamala J, Mundana N. 1978. Descripción de los órganos reproductores y determinación del número de generaciones anuales de *Hylastinus obscurus* (Marsham) (Coleoptera: Scolytidae). *AgroSur*, 6(2): 79-82.

Chen H-bo, Zhang Z, Wang H-bin, Kong X-bo. 2010. Antennal morphology and sensilla ultrastructure of *Dendroctonus valens* LeConte (Coleoptera: Curculionidae, Scolytinae), an invasive forest pest in China. *Micron*, 41(7): 735-741.

Dickens J, Payne T. 1978. Structure and function of the sensilla on the antennal club of the southern pine beetle, *Dendroctonus frontalis* (Zimmerman) (Coleoptera: Scolytidae). *International Journal of Insect Morphology and Embryology*, 7(3): 251–265.

Grégoire JC, Evans HF. 2004. Damage and control of BAWBILT organisms, an overwiew. In: Lieutier F, Day KR, Battisti A, Grégoire JC, Evans H, Editors. *Bark and Wood Boring Insects in Living Trees in Europe, a Synthesis*, pp. 19-37. Springer.

Hu F, Zhang G-N & Wang J-J. 2009. Scanning electron microscopy studies of antennal sensilla of bruchid beetles, *Callosobruchus chinensis* (L.) and *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae). *Micron*, 40(3): 320-326.

Isidoro N, Bartlet E, Ziesmann J, Williams IH. 1998. Antennal contact chemosensilla in *Psylliodes chrysocephala* responding to cruciferous allelochemicals. *Physiological Entomology*, 23(2): 131-138.

Jourdan H, Barbier R, Bernard J, Ferran A. 1995. Antennal sensilla and sexual dimorphism of the adult ladybird beetle *Semiadalia undecimnotata* Schn. (Coleoptera: Coccinellidae). *International Journal of Insect Morphology and Embryology*, 24:307–322.

Keil TA. 1997. Functional morphology of insect mechanoreceptors. *Microscopy research and technique*, 39(6): 506-531.

Klowden MJ. 2007. *Physiological Systems in Insects*. 2nd edition. Academic Press.

Larsson MC, Svensson GP. 2004. Methods in insect sensory ecology. In: Christensen TA, Editor. *Methods in insect sensory neuroscience*, pp 27-58. CRC Press.

Leal WS. 2003. Proteins that make sense. In: Blomquist GJ, Vogt RG, Editors. *Insect Pheromone Biochemistry and Molecular Biology*, pp. 447-476. Elsevier Academic Press.

Manosalva L, Pardo F, Perich F, Mutis A, Parra L, Ortega F, Isaacs R, Quiroz A. 2011. Behavioral responses of clover root borer to long-chain fatty acids from young red clover (*Trifolium pratense*) roots. *Environmental Entomology*, 40(2): 399-404.

Merivee E, Rahi M, Luik A. 1999. Antennal sensilla of the click beetle, *Melanotus villosus* (Geoffroy) (Coleoptera: Elateridae). *International Journal of Insect Morphology and Embryology*, 28(1-2): 41-51.

Moeck HA. 1968. Electron microscopic studies of antennal sensilla in the ambrosia beetle *Trypodendron lineatum* (Olivier) (Scolytidae). *Canadian Journal of Zoology*, 46(3): 521-556.

Payne T, Moeck H, Willson C, Coulson R, Humphreys W. 1973. Bark beetle olfaction—II. Antennal morphology of sixteen species of Scolytidae (Coleoptera). *International Journal of Insect Morphology and Embryology*, 2(3): 177-192.

Picimbon J-F. 2003. Biochemistry and evolution of OBP and CSP proteins. In: Blomquist GJ, Vogt RG, Editors. *Insect Pheromone Biochemistry and Molecular Biology*, pp. 539-566. Elsevier Academis Press.

Quiroz A, Ortega F, Ramírez CC, Wadhams LJ & Pinilla K. 2005. Response of the beetle *Hylastinus obscurus* Marsham (Coleoptera: Scolytidae) to red clover (*Trifolium pratense* L.) volatiles in a laboratory olfactometer. *Environmental Entomology*, 34(3): 690-695.

Steinbrecht RA. 1989. The fine structure of thermo-/hygrosensitive sensilla in the silkmoth *Bombyx mori*: Receptor membrane substructure and sensory cell contacts. *Cell and Tissue Research*, 255(1): 49-57.

Snodgrass RE. 1935. *Principles of Insect Morphology*. Cornell University Press.

Tapia T, Perich F, Pardo F, Palma G, Quiroz A. 2007. Identification of volatiles from differently aged red clover (*Trifolium pratense*) root extracts and behavioural responses of clover root borer (*Hylastinus obscurus*) (Marsham) (Coleoptera: Scolytidae) to them. *Biochemical Systematics and Ecology*, 35(2): 61-67.

Usha Rani P, Nakamuta K. 2001. Morphology of antennal sensilla, distribution and sexual dimorphism in *Trogossita japonica* (Coleoptera: Trogossitidae). *Annals of the Entomological Society of America*, 94(6): 917–927.

Whitehead AT. 1981. Ultrastructure of sensilla of the female mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera: Scolytidae). *International Journal of Insect Morphology and Embryology* 10(1): 19–28.

Zúñiga G, Cisneros R, Hayes JL, Macias J, Zu G. 2002. Karyology, geographic distribution, and origin of the genus *Dendroctonus* Erichson (Coleoptera: Scolytidae). *Annals of the Entomological Society of America*, 95(3): 267-275.

Table 1. Average length (μm ± SD) and width at the base (μm ± SD) of the different types of sensilla found in the antennae of males and females of *H. obscurus.*

Type of sensillum	**Female**		**Male**	
	lenght	**width**	**length**	**width**
Chaeticum I	41.5 ± 12.3	1.7 ± 0.3	35.0 ± 5.2 ^{ns}	1.9 ± 0.2 ^{ns}
Chaeticum II	32.7 ± 4.1	1.7 ± 0.1	31.2 ± 3.2 ^{ns}	1.6 ± 0.1 ^{ns}
Basiconicum	17.3 ± 3.9	1.3 ± 0.1	17.3 ± 2.7 ^{ns}	1.4 ± 0.2 ^{ns}
Trichodium	29.6 ± 4.5	1.5 ± 0.1	31.5 ± 5.7 ^{ns}	1.6 ± 0.2 ^{ns}
Styloconicum	6.6 ± 1.3	1.4 ± 0.0	7.2 ± 1.2 ^{ns}	1.4 ± 0.1 ^{ns}

^{ns} Not significative differences were found between both sexes according to t-Student test ($P \leq 0.05$).

Table 2. Average number of sensilla (± SD) in the different sections of the *H. obscurus* antenna.

Section of the antenna	**Female**	**Male**
scape	12 ± 3	11 ± 2 [ns]
funicle	37 ± 5	35 ± 1 [ns]
club	496 ± 23	522 ± 62 [ns]

[ns] Not significative differences were found between both sexes according to t-Student test ($P \leq 0.05$).

Table 3. Average number of the three most abundant type of sensilla (± SD) in the antennal club of *H. obscurus* by sex, sensory band and type of sensillum.

Sensory band	Type of sensillum	Female	Male
Sb1	chaeticum II	106 ± 6	100 ± 31 [ns]
	basiconicum	96 ± 6	121 ± 21 [ns]
Sb2	chaeticum II	80 ± 1	54 ± 5*
	basiconicum	90 ± 1	87 ± 4 [ns]
	trichoideum	20 ± 1	23 ± 2*
Sb3	chaeticum II	51 ± 5	59 ± 13 [ns]
	basiconicum	32 ± 7	49 ± 6*
	trichoideum	22 ± 2	28 ± 4 [ns]

* Indicates significative difference between sexes according to t-Student test ($p \leq 0.05$)

[ns] Not significative.

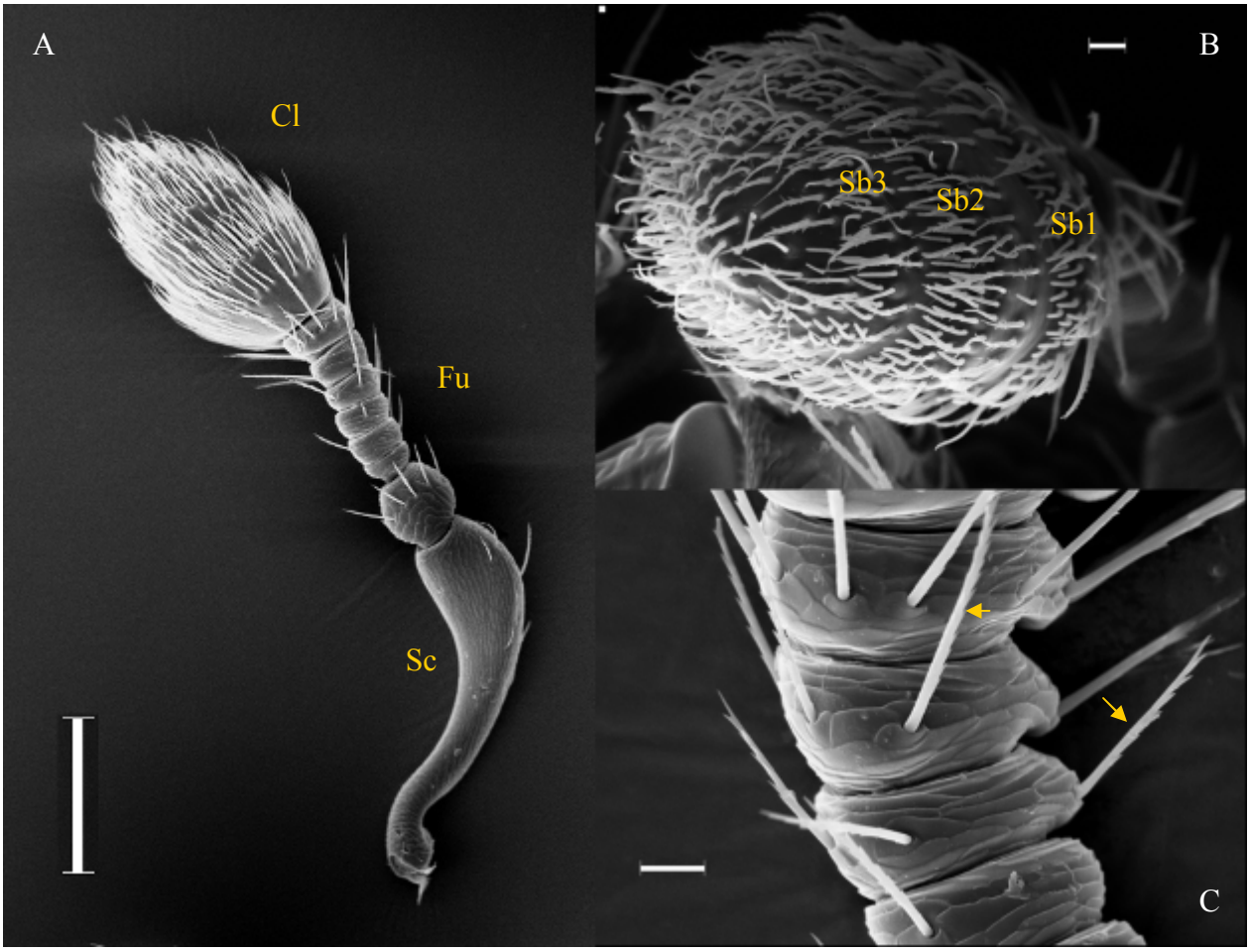

Figure 1. Antenna of *H. obscurus*. **(A)** General overview showing the scape (Sc), the funicle (Fu) formed by seven segments and the club (Cl). **(B)** The spatial distribution of sensilla which form three sensory bands (Sb1, Sb2 and Sb3). **(C)** Close up upon the antenites forming the funicle showing some chaetica sensilla type I (arrows) emerging from cuticle. Scale bar: A=100 µm; B=20 µm; C=10 µm.

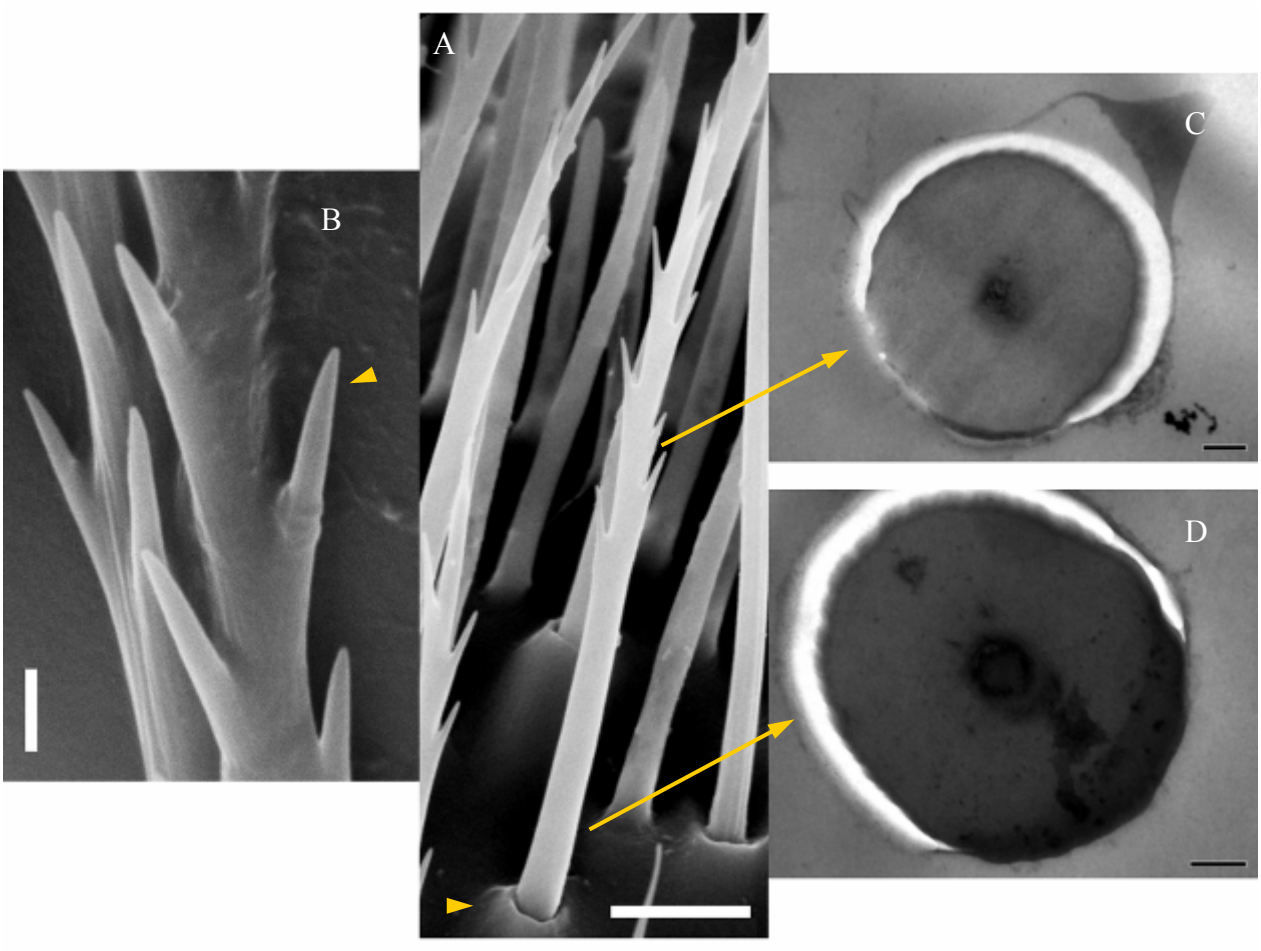

Figure 2. (A) Overview of a sensillum chaeticum type II supported in socket (arrowhead). (B) Detail of the laterally alternated branches (arrowhead). (C) TEM image of a transversal cut in the upper part of the sensillum, while (D) show the image to the lower part. Scale bars: A=5 µm, B=1 µm, C=D=0.2 µm.

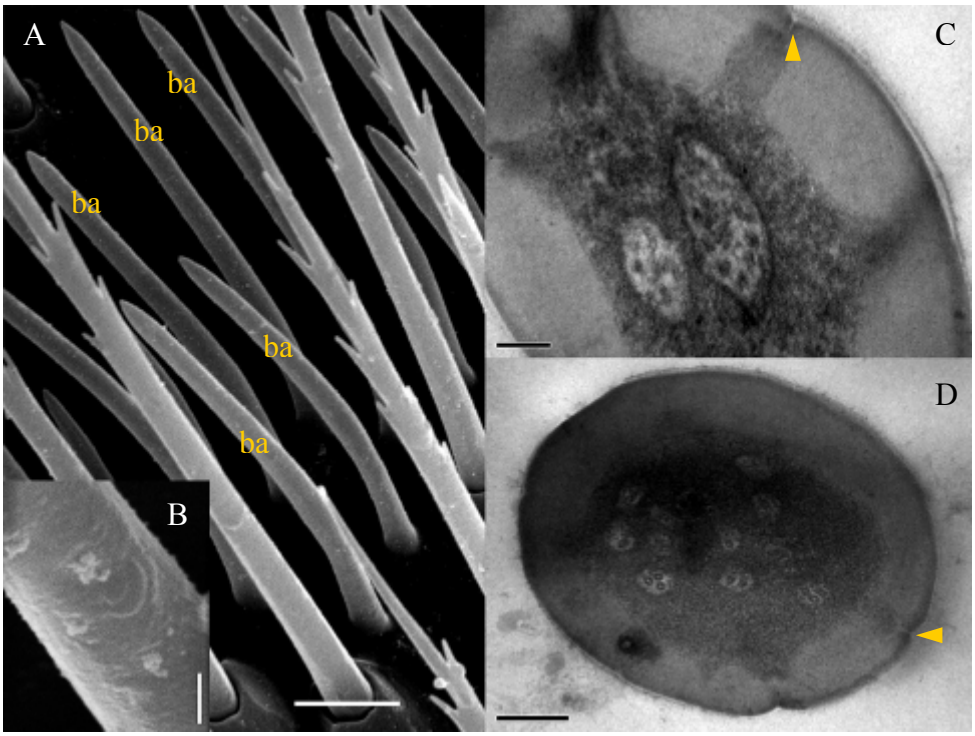

Figure 3. (A) Sensillum basiconicum (ba) in a sensory band. (B) shows the slightly grooved surface of this type of sensilla. (C) In the cross-section the thick wall is interrupted by spaced pores (arrowhead) which communicate outside with three dendrites in the lumen. (D) Several dendrites segments and pores (arrowhead) formed in the upper part of basiconicum. Scale bars: A=5 µm, B=0.5 µm, C=0.2 µm and D=0.1 µm.

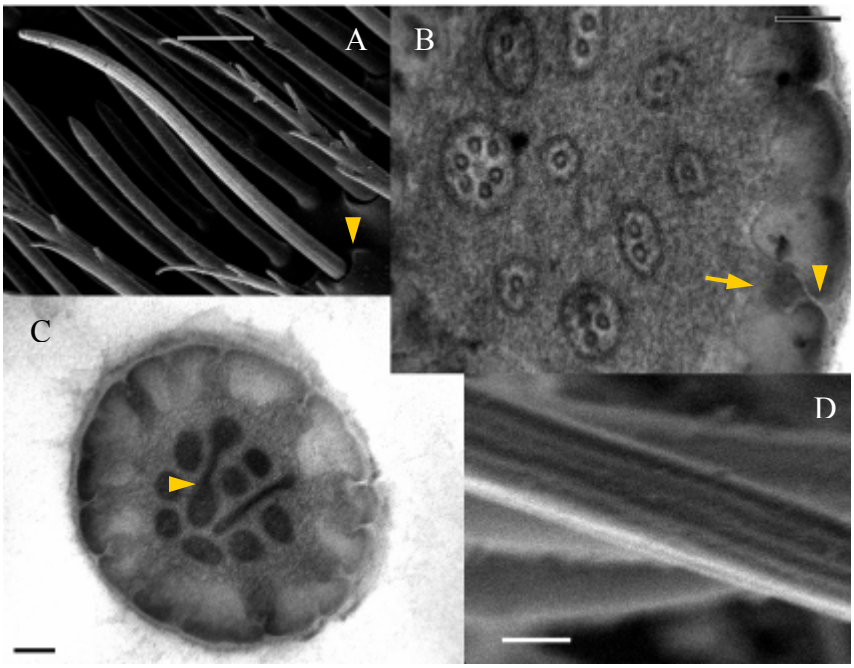

Figure 4. Sensillum tricodeum (A) supported in socket (arrowhead) showing its grooved surface (D). (B) Pores formed by short and stretched channels (arrowhead) communicate the outside to the sensillar lumen (arrow) which is filled by dendrite segments. (C) Incompletely formed dendrite segments (arrowhead) appeared in the upper part, while the high density of pores characterized this type of sensillum. Scale bars: A=5 µm, B=0.1 µm, C=0.1 µm and D=1 µm

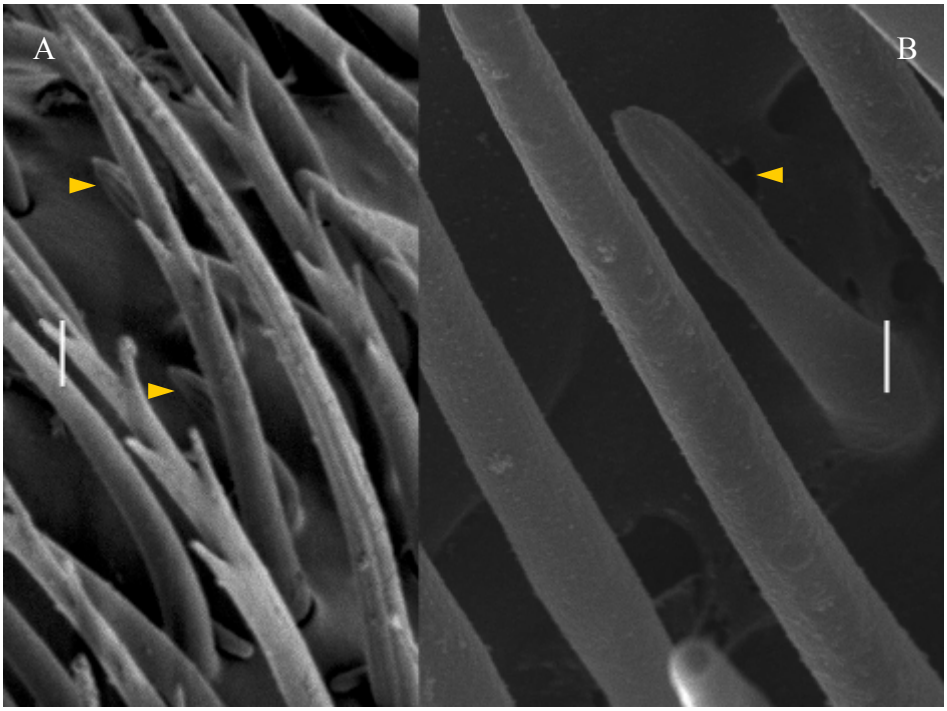

Figure 5. (A) Sensilla styloconica found in the second sensory band in a male antenna indicated by arrowheads. (B) The finger-shaped pegs in the top of this type of sensillum resemble a bud (arrowhead). Scale bars: A=2 µm; B=1 µm.

Chapter 3

Behavioral and electrophysiological response of *Hylastinus obscurus* towards volatiles identified from roots of *Trifolium pratense* L.

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Article submitted to the Journal of Soil Science and Plant Nutrition

BEHAVIORAL AND ELECTROPHYSIOLOGICAL RESPONSE OF *Hylastinus obscurus* TOWARDS VOLATILES IDENTIFIED FROM ROOTS OF *Trifolium pratense* L.

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ABSTRACT

Root volatiles coming from field-collected red clover plants of five different ages were trapped by Solid Phase Microextraction (SPME) and analyzed by Gas Chromatography-Mass Spectrometry (GC-MS). Thus, ethanol, *E*-2-hexenal, hexanal, 3-octanone, limonene and α-pinene were identified. Then, the electroantennographic (EAG) and olfactometric responses of clover root borer, *Hylastinus obscurus*, towards the identified compounds were studied. The GC-MS results demonstrated the utility of SPME as a valuable free-solvent collecting method, adding ethanol and hexanal to the list of compounds reported previously in this forage specie. Besides, EAG experiment showed that all the tested compounds were active, but mixed

responses were found in behavioral assays. For females, ethanol and *E*-2-hexenal resulted to be attractive in at least one of their doses; meanwhile hexanal, 3-octanone, *R*- and *S*-limonene resulted to be repellent in at least one of tested doses. Comparatively, females responded to a larger number and doses of tested compounds than males. This behavior could mean a more active role of females in host finding and colonization. The current work contributes to fill a gap in the chemical ecology of red-clover/borer system, relating the releasing of volatiles from roots and the age of plants when the infestation is actually occurring.

KEYWORDS

Root volatiles, red clover, root borer, electroantennography, olfactometry, SPME.

INTRODUCTION

Hylastinus obscurus (Coleoptera: Curculionidae: Scolytinae), commonly named red clover root borer, is an important cosmopolitan pest that attack red clover crops and having agronomical effects because reduces their persistence and productivity, reaching infestation of 70 to 100% of plants in pastures of two and three years old (Aguilera et al., 1996). In fact, Pruess and Weaver (1958) reported that just 1.5 borer per plant caused 5.5% of reduction in forage yield. Meanwhile, secondary effects appear because the borer opens the entrance to new pests and diseases. Thus, is considered the most important cause for red clover decay (Steiner and Alderman, 1999; 2003) and pesticides applications have not been successful on its control (Quiroz et al., 2005). After copulate, adults flight from October to November (spring in South Hemisphere) to infesting new plants. Then new individuals complete their entire cycle inside the root, leaving the plants only to spread to other areas on next season.

Being red clover described as the most important host of this pest, the close plant-insect relationship has been subject of several works. Those mainly have reported that chemicals released by red clover plants play an important role in the root borer behavior. Thus, Leath and Byers (1973) noted the attractiveness of the disease-roots leachate and diseased roots pieces in behavioral assays. Quiroz et al. (2005) established the attraction of *H. obscurus* towards aerial parts volatiles, specifically in plants of 1.5, 2.0 and 2.5 years old. Previously Buttery et al. (1984) listed the compounds found in leaves, flowers and seed pods, suggesting possible insect attractants but red clover borer is not mentioned. Tapia et al. (2007) also determined this effect and identificated *E*-2-hexenal and methyl benzoate as causing attractive response in red clover root extracts in plants of 1.5 years old. Besides, Tapia et al. (2005) reported attraction also, but they did not find differences in the response of clover borers exposed to healthy and diseased root extracts from 60 days old plants. Recently, Manosalva (2010) studying extract of roots of young plants of red clover, noted the attractiveness for *H. obscurus* to volatiles of plants of 5, 7 and 9 months, whereas field studies have demonstrated that plants are colonized when they are six months old (Alarcón et al., 2010).

Although the evidence point out to a group of compounds involved in this insect-plant interaction, their electrophysilogical activities and ubiquities have not been yet proved, specifically for plants which ages are close to the beginning of infestation. In addition all the compounds identified in roots have been collected using solvent-dependant methodologies, such solvent can coelute with valuable compounds of low polarity.

Solid Phase Micro Extraction (SPME) has been reported as a simple and fast method to obtain fingerprints of plant headspace (Cornu et al., 2001). It uses a fused silica fiber coated with

polymeric coating to extract organic compounds from their matrix and directly transfer analytes into a gas chromatograph (GC) by thermal desorption in a GC injector (Zhang and Pawliszyn, 1995). As a solvent-free technique it offers the possibility to analyze highly volatiles and/or trace compounds that cannot be easily disclosed by GC when organic solvents are used by the co-elution with the solvent or loss during concentration (Rochat et al., 2000).

The aim of the current work was to clarify the link between the volatiles released by red clover and their ability to elicit response in *Hylastinus obscurus*; studying the electrophysiological and behavioral response of clover borers to individual compounds identified from roots of different ages.

MATERIALS AND METHODS

Insects

Red clover plants were collected from different plots, but allocated in the same place than plants used to SPME trapping. In the laboratory, wearing gloves and using tweezers, the insects were isolated from their substrate and placed in glass Petri dishes with fresh root pieces on humid filter paper, which were held at 7 °C until their utilization. To perform the bioassays, the insects were removed from the root pieces 12 hours before of their use, and allocated in humid chambers consisted of glass Petri dishes with pieces of filter paper humidified with distilled water and held at 7°C. Then, one hour before the experiments, the humid chambers were changed to room temperature. Individuals were used once, and their sex was determined after the bioassay, following the description made by Matamala (1976).

Volatiles collection

Different-aged clover plants (cv. Redqueli) were carefully collected from plots located in the Regional Research Center INIA-Carillanca in Vilcún, Araucanía, Chile and brought to the laboratory. There, the aerial part was separated from the roots, which were gently washed with distilled water to taking off the soil and then they were air dried. Two grams of fresh roots of at least six plants were weighted and put them in a 40 mL glass vial with a PTFE septum at room temperature 30 minutes before to start the volatiles collection. Then, during 60 minutes volatiles in the flask were trapped using a Solid Phase Microextraction (SMPE) holder containing a 65 μm polidimethylsiloxane/ divinylbencene fiber (Supelco, USA). The volatiles from roots of red clover plants were collected the same day they were brought from field.

Chemical analysis and compounds determination

The collected volatiles were analized by gas chromatography-mass spectrometry (GC-MS), injecting the fiber in a Thermo-Finnigan chromatograph (Milan, Italy) with electron impact ionization (70eV), equipped with a BP-1 capillary column (30 m length by 0.22 mm by 0.25 μm; SGE, Victoria, Australia). Helium was used like carrier gas and the oven was programmed at 40 °C as starting temperature, the ramp was 5 °C per minute up to 240 °C held by 3 minutes. The temperature in the injector and transfer line were 250 °C

The volatiles collected were identified by comparison of their mass spectra with those of commercial standards and library database spectra using NIST mass spectral search program (ver. 2.0), Pherobase (http://www.pherobase.com) and NIST webbook (http://webbook.nist.gov/chemistry) cited by Babushok et al. (2007)

Chemical standards

All the chemicals used in the bioassays corresponded to chromatographic grade standards; ethanol (Merck, Darmstadt, Germany, 99.9% purity); *R*-α-pinene (Merck, Munich, Germany, 97% purity); *S*-α-pinene (Merck, Munich, Germany, 97% purity); *S*-limonene (Sigma Aldrich, Steinheim, Germany, 96% purity); *R*-limonene (Sigma Aldrich, Steinheim, Germany, 97% purity); *E*-2-hexenal (Merck, Hohenbrunn, Germany, 96% purity); hexanal (Aldrich, Steinheim, Germany, 98% purity); and 3-octanone (Aldrich, Steinheim, Germany, 98% purity).

Behavioral bioassays

A Y-shape glass tube was used as arena; its arms and central tube were 55 mm length and 9 mm i.d. To move the air from the arms to the base, vacuum was applied at the end of central tube at 0.2 L min^{-1}. At the end of both arms an odor cartridge was connected. These consisted of a glass tube of 9 mm o.d. containing a paper strip (8 mm by 60 mm). Each strip was impregnated under the fume hood with 50 µL of solution or hexane (control) and air-dried per 30 s before to put them inside the cartridge. Behind the odor cartridge, a charcoal filter was connected to ensure just clean air was entering in to the arena. Finally, an adult *H. obscurus* individual was introduced in the base of the Y-tube and the vacuum tube connected. Finally, the arm chosen by the borer was noted after 5 minutes or less. The assay was considered successful when the insect passed 10 mm forward the Y tube bifurcation, but when the insect did not choose any arm, the assay was discarded. After the olfactometric assay, insects were stored individually in labeled microcentrifuge tubes with ethanol up to determine their sex. At least 15 replications per sex were performed by compound and dose, plus a complete set using hexane in both arms.

Electroantennography

The heads of adult insects were cut using scalpel and tweezers under microscope. The electrodes consisted of silver wires inside of glass capillaries filled with 1M KCl and 0.1% PVP solution (Syed and Leal, 2007). The indifference electrode was inserted in the base of head and the recording electrode was contacted with the tip of one of the antennae (Mendesil et al., 2009). The preparation was made on Syntech MP-12 micromanipulator (Hilversum, The Netherlands), connected to 10X amplifier. The signal was received by Syntech IDAC-02 interface (Hilversum, The Netherlands) which was linked to a personal computer where the EAG software interpreted and collected the information. The preparation was allocated under a humidified and charcoal-filtered air flux, ca. 5 mm from the glass pipe outlet of 25 mm i.d. an hole of 2.5 mm at 50 mm from the outlet was used to connect the odor cartridges. The tested compounds were applied on filter paper strips (8 mm by 50 mm) using 10 µL of standard. Then they were put inside of 5 mL disposable polypropylene syringes (Syed et al., 2003; Jeanbourquin and Guerin, 2007). The stimulus controller Syntech CS-05 (Hilversum, The Netherlands) was set to continuous flow of 0.6 L min^{-1}, while the stimuli were applied for 1 second at 0.7 L min^{-1}. At least 45 s were allowed between puffs, and the stimulations of different odorants were applied in random order, but starting and finishing with ethanol puffs. In total six replications were done per sex and compound using ethanol as control.

Analysis of data

Electroantennographic relative responses were compared by Kruskal-Wallis test, followed by Conover-Inman to group separation (Conover, 1999) using StatsDirect statistical software v.

2.7.8 (StatsDirect Ltd., UK). Meanwhile frecuencies obtained in olfactometric assays were analized by G-test for goodness of fit (Sokal and Rohlf, 2005).

RESULTS

The GC-MS analysis of samples obtained by SPME showed the presence of six compounds (Table1); ethanol, hexanal, *E*-2-hexenal, α-pinene, 3-octanone and limonene. No one root sample showed all the six compounds. *E*-2-hexenal appeared in 5-months old roots only and limonene in 16- and 24-months old roots. 3-octanone appeared in the different ages tested except 24-months old as well as ethanol was present in all the treatments, but was not detected in 16-months old roots. Hexanal appeared in the three youngest age treatments and α-pinene was determined intermittently at 9- and 24-months old.

<Insert Table 1 near here>

Males and females of *H. obscurus* responded similarly to the stimulus in EAG assays, but clear differences appeared in behavioral assays. The Figure 1 shows the typical EAG deflection obtained from ethanol compared to those obtained from air; meanwhile the relative electroantennographic response of all remaining compounds is reported in Figure 2 using ethanol as reference. In males the relative responses can be separated in three different groups. *E*-2-hexenal and hexanal showed the highest response, and with the exception of *S*-α-pinene, the remaining compounds resulted to be significantly different from air. In females, the responses followed the same pattern, but all the tested compounds were significantly different from air, being *E*-2-hexenal and hexanal the most active compounds.

Behaviorally, males and females of *H. obscurus* responded differently (Fig. 3). Females appear to be more sensitive to hexanal, *R*- and *S*-limonene than males, and these did not response to *R*- isomer of limonene. Meanwhile, ethanol only elicited response in females varying from attractant to repellent depending on dose. In total, females responded to six of eight compounds in at least one of the tested doses. In contrary, males just responded to three of the eight tested compounds.

DISCUSSION

From the compounds found by SPME trapping, *E*-2-hexenal has been reported in red clover plants as volatile released from aerial parts (Buttery et al., 1984), and roots (Tapia et al., 2007) together with α-pinene and limonene, whereas the presence of 3-octanone in roots was established by Kamm and Buttery (1984).

>Insert Figure 1 near here>

Although the volatiles found in the current study are similar to those reported in previous works, clear differences appeared, even when plants of the same age are compared. Partially at least, these differences may be attributed to the methods employed to collect the samples. An example is ethanol, which retention time (Table 1) match up with the retention time of solvent used in other studies, explaining why this compound was not registered before. In the other hand, Kigathi et al. (2009), noted the differences in volatile profile comparing the compounds emitted by red clover plants after hervibory as well laboratory as field conditions, and they attributed those results to the differences in growing conditions and sampling time.

Considering the previous reports, this appear to be the first record of ethanol in roots of red clover, and its potential value as semiochemical is clear considering the EAG response and behavioral assays. Ethanol has been noted as a fermentation indicator during host colonization by bark beetles (Byers, 1989). In fact, scolytids which not use aggregation pheromone to attack their hosts frequently are strongly attracted to the hosts by their monoterpenes, ethanol, or a combination of both (Vrkocová et al., 2000). Stressed branches of Douglas-fir (*Pseudotsuga menziesii*) containing two or three orders of magnitude larger amounts of ethanol than non-stressed branches, attracted significantly more *Scolytus unispinosus* (Kelsey and Joseph, 2001). In addition, Joseph et al. (2001) showed the effect of ethanol in the effectiveness of traps lured to collect bark beetles, and high release rates of ethanol increased the number of *Dendroctonus valens*, *Hylurgops sp.* and *Hylastes sp.* captured in combination with other kairomones in pine forests.

<Insert Figure 2 near here>

Hexanal was released from 5-, 9- and 11-months old roots, but it was not detected in 16- and 24-months old roots. This compound was behaviorally inactive for males, but all the tested doses showed to repel females as well as *R*- and *S*-limonene did. Pointed as a green-leaves volatile by Thiéry and Marion-Poll (1998), its inclusion was not able to reduce significantly the attractiveness of traps baited with the pheromone of *Conophtorus resinosae* (de Groot and MacDonald, 1999) and *Ips pini* (Huber et al., 2001). In fact, Jaffé et al. (1993) showed how hexanal synergized a mix of different pineapple and coconut volatiles, which attracted palm weevils, *Rhynchophorus palmarum* in laboratory assays, but it did not do the same in field tests. Before, the presence of hexanal was noted in alfalfa by Buttery et al. (1982), and it was

tested in laboratory as a possible attractant to *Hylastinus obscurus*, but no positive results were found (Kamm and Buttery, 1984).

3-octanone appeared in all the roots sampled, excepting in 24-months old plants. Mixed effects have been attributed to this compound. Thus, is reported as a secondary odorant to the strawberry blossom weevil *Anthonomus rubi* (Bichão et al., 2005). Whereas, Ramachandran et al. (1991) noted that 3-octanone released from the larval frass of *Pseudoplusia includens* when it fed on soybean plants, attracted its natural parasitoid *Micropilis demolitor*. In the current study, this compound which is electrophysiologically perceived by male and females of *H. obscurus* (Fig.2), did not elicit behavioral activity excepting in females exposed to the highest dose. This result agrees with the report of Pfeil and Mumma (1993), who observed that high doses of 3-octanone seemed to produce deterrency in females of the phorid fly, *Megasella halterata*.

<Insert Figure 3 near here>

Tapia et al. (2007), noted the repellent activity of limonene, although isomers and sexes were not discriminated. This report corroborates that result, but establishes a clear difference between males and females respect to the enantiomers of limonene (Figure 3). Meanwhile all doses of both *R*- and *S*-limonene tested elicited a repellent response from females, just *S*-limonene produced the same effect in males in two of three tested doses. Wibe et al. (1998) reported the ability of receptor neurons in *Hylobius abetis* to respond differently to both isomers of limonene and Mutis et al. (2010) showed that just *R*-limonene enantiomer was electroantennographically active to males of *Aeghorinus superciliosus* and able to attract them

in laboratory bioassays, in the same manner that females volatiles extract did it. In field conditions, males and females of *Conophthorus coniperda* were attracted to traps containing both enantiomers of limonene (Miller, 2007). Moreover, the release rate of limonene was directly proportional to the numbers of beetles caught in traps baited with their sexual pheromone.

In the current report, with the exception of the lowest dose of *S*-α-pinene on males, both isomers were not olfactometrically active. In this case, the apparent opposition between behavioral and electrophysiological responses resembles the report of White and Hobson (1993), who studying the response of *Dendroctonus ponderosa* towards chiral monoterpenes from ponderosa pine, found opposite responses in the EAG bioassays respect to the field results.

The attractant role of *E*-2-hexenal, has been reported previously in different scolytids (Huber et al., 2000), and is similar to the report of Tapia et al. (2007) for red clover borer, although they did not discriminated the effect by sex of individual. The EAG showed large relative response, and the attraction behavior elicited on males and females of *Hylastinus obscurus* was established in behavioral assays.

Finally, we can not ignore the fact that particular responses to individual volatiles components varies among scolytid species (Poland and Haack, 2000); and odor components emitted may be unique for a given plant species, and some insects can perceive these species-specific variations in the relative amounts and employ them to discriminate between host and non-host

plants (Schoonhoven et al., 2005). Further studies are required to test the compounds blend effects on clover borer activity.

CONCLUSIONS

According to the current results, the application of SPME technique made possible add ethanol and hexanal to the range of compounds with promising activity that should be confirmed in field. The stronger response of females to the stimuli compared to males in the behavioral assays could be explained because they are responsible to find a suitable host to their offsprings.

AKNOWLEGMENTS

Rubén Palma thanks the support of Conicyt project AT24090126 and Andrés Quiroz thanks the funding received through the Fondecyt project 1100812 which made this research possible.

REFERENCES

Aguilera, A., Cisternas, E., Gerding, M., Norambuena, H. 1996. Plagas de las praderas. In: I. Ruiz (ed.), Praderas para Chile, Ministerio de Agricultura, Chile, pp. 310-339.

Alarcón, D., Ortega, F., Perich, F., Pardo, F., Parra, L., Quiroz, A. 2010. Relationship between radical infestation of *Hylastinus obscurus* (Marsham) and the yield of cultivars and experimental lines of red clover (*Trifolium pratense* L.). Revista de la Ciencia del Suelo y Nutrición Vegetal 10, 115–125.

Babushok, V. I., Linstrom, P. J., Reed, J. J., Zenkevich, I. G., Brown, R. L., Mallard, W. G., Stein, S. E. 2007. Development of a database of gas chromatographic retention properties of organic compounds. Journal of Chromatography. A 1157, 414-21.

Bichão, H., Borg-Karlson, A.-K., Araújo, J., Mustaparta, H. 2005. Five types of olfactory receptor neurons in the strawberry blossom weevil *Anthonomus rubi*: selective responses to inducible host-plant volatiles. Chemical Senses 30, 153-170.

Buttery, R. G., Kamm, J. A., Ling, L. C. 1982. Volatile components of alfalfa flowers and pods. Journal of Agricultural and Food Chemistry 30, 739–742.

Buttery, R. G., Kamm, J. A., Ling, L. C. 1984. Volatile components of red clover leaves, flowers, and seed pods: possible insect attractants. Journal of Agricultural and Food Chemistry 32, 254–256.

Byers, J. A. 1989. Chemical ecology of bark beetles. Experientia 45, 271-283. **Conover, W. 1999.** Practical Nonparametric Statistics. Wiley, New York, 587 p.

Cornu, A., Carnat, A. P., Martin, B., Coulon, J. B., Lamaison, J. L., Berdagué, J. L. 2001. Solid-phase microextraction of volatile components from natural grassland plants. Journal of Agricultural and Food Chemistry 49, 203–209.

de Groot, P., MacDonald, L. M. 1999. Green leaf volatiles inhibit response of red pine cone beetle *Conophthorus resinosae* (Coleoptera: Scolytidae) to a sex pheromone. Naturwissenschaften 86, 81–85.

Huber, D. P., Borden, J. H., Stastny, M. 2001. Response of the pine engraver, *Ips pini* (Say) (Coleoptera: Scolytidae), to conophthorin and other angiosperm bark volatiles in the avoidance of non-hosts. Agricultural and Forest Entomology 3, 225-232.

Huber, D. P., Gries, R., Borden, J. H., Pierce Jr., H. D. 2000. A survey of antennal responses by five species of coniferophagous bark beetles (Coleoptera: Scolytidae) to bark volatiles of six species of angiosperm trees. Chemoecology 10, 103-113.

Jaffé, K., Sánchez, P., Cerda, H., Hernández, J. V., Jaffé, R., Urdaneta, N., Guerra, G., Martínez, R., Miras, B. 1993. Chemical ecology of the palm weevil *Rhynchophorus palmarum* (L.) (Coleoptera: Curculionidae): Attraction to host plants and to a male-produced aggregation pheromone. Journal of Chemical Ecology 19, 1703-1720.

Jeanbourquin, P., Guerin, P. M. 2007. Sensory and behavioural responses of the stable fly *Stomoxys calcitrans* to rumen volatiles. Medical and Veterinary Entomology 21, 217-24.

Joseph, G., Kelsey, R. G., Peck, R. W., Niwa, C. G. 2001. Response of some scolytids and their predators to ethanol and 4-allylanisole in pine forests of central Oregon. Journal of Chemical Ecology 27, 697-715.

Kamm, J. A., Buttery, R. G. 1984. Root volatile components of red clover: identification and bioassay with the clover root borer (Coleoptera: Scolytidae). Environmental Entomology 13, 1427-1430.

Kelsey, R, Gladwin, J. 2001. Attraction of *Scolytus unispinosus* bark beetles to ethanol in water-stressed Douglas-fir branches. Forest Ecology and Management 144, 229–238.

Kigathi, R. N., Unsicker, S. B., Reichelt, M., Kesselmeier, J., Gershenzon, J., Weisser, W. W. 2009. Emission of volatile organic compounds after herbivory from *Trifolium pratense* (L.) under laboratory and field conditions. Journal of Chemical Ecology 35, 1335-1348.

Leath, K., Byers, R. 1973. Attractiveness of diseased red clover roots to the clover root borer. Phytopathology 63, 428–431.

Manosalva, L. 2010. Estudio de la composición química de extractos de raíces de líneas y cultivares de plantas jóvenes de trébol rosado (*Trifolium pratense* L.) y su relación con la respuesta conductual del insecto *Hylastinus obscurus* (Marsham). Tesis de Magíster, Universidad de La Frontera, Chile, 62 p.

Matamala, J. 1976. Biología, niveles de infestación, daño y combate químico de *Hylastinus obscurus* (Marsham) (Coleoptera: Scolytidae). Bachelor thesis, Universidad Austral de Chile, 51 p.

Mendesil, E., Bruce, T. J. a, Woodcock, C. M., Caulfield, J. C., Seyoum, E., Pickett, J. 2009. Semiochemicals used in host location by the coffee berry borer, Hypothenemus *hampei*. Journal of Chemical Ecology 35, 944-950.

Miller, D. R. 2007. Limonene: attractant kairomone for white pine cone beetles (Coleoptera: Scolytidae) in an eastern white pine seed orchard in western North Carolina. Journal of Economic Entomology 100, 815-822.

Mutis, A., Parra, L., Manosalva, L., Palma, R., Candia, O., Lizama, M., Pardo, F., Perich, F., Quiroz, A. 2010. Electroantennographic and behavioral responses of adults of raspberry weevil *Aegorhinus superciliosus* (Coleoptera: Curculionidae) to odors released from conspecific females. Environmental Entomology 39, 1276-1282.

Pfeil, R. M., Mumma, R. O. 1993. Bioassay for evaluating attraction of the phorid fly, *Megaselia halterata* to compost colonized by the commercial mushroom, *Agaricus bisporus* and to 1-octen-3-ol and 3-octanone. Entomologia Experimentalis et Applicata 69, 137-144.

Poland, T. M., Haack, R. 2000. Pine shoot beetle, *Tomicus piniperda* (Col., Scolytidae), responses to common green leaf volatiles. Journal of Applied Entomology 124, 63-69.

Pruess, K. P., Weaver, C. R. 1958. Estimation of red clover yield losses caused by the clover root borer. Journal of Economic Entomology, 51, 491-492.

Quiroz, A., Ortega, F., Ramírez, C. C., Wadhams, L. J., Pinilla, K. 2005. Response of the beetle *Hylastinus obscurus* Marsham (Coleoptera: Scolytidae) to red clover (*Trifolium pratense* L.) volatiles in a laboratory olfactometer. Environmental Entomology 34, 690-695.

Ramachandran, R., Norris, D. M., Phillips, J. K., Phillips, T. W. 1991. Volatiles mediating plant-herbivore-natural enemy interactions: soybean looper frass volatiles, 3-octanone and guaiacol, as kairomones for the parasitoid *Microplitis demolitor*. Journal of Agricultural and Food Chemistry 39, 2310–2317.

Rochat, D., Ramirez-Lucas, P., Malosse, C., Aldana, R., Kakul, T., Morin, J. P. 2000. Role of solid-phase microextraction in the identification of highly volatile pheromones of two Rhinoceros beetles *Scapanes australis* and *Strategus aloeus* (Coleoptera, Scarabaeidae, Dynastinae). Journal of Chromatography A 885, 433–444.

Schoonhoven, L., van Loon, J., Dicke, M. 2005. Insect-Plant Biology. Oxford University Press, New York, 421 p.

Sokal, R., Rohlf, F. 2005. Biometry. W. Freeman, New York, 887 p.

Steiner, J., Alderman, S. 1999. Red clover seed production. V. Root health and crop productivity. Crop Science 39, 1407-1415.

Steiner, J., Alderman, S. 2003. Red clover seed production. VI. Effect and economics of soil pH adjusted by lime application. Crop Science 43, 624-630.

Syed, Z, Guerin, P. M., Baltensweiler, W. 2003. Antennal responses of the two host races of the larch bud moth, *Zeiraphera diniana*, to larch and cembran pine volatiles. Journal of Chemical Ecology 29, 1691-1708.

Syed, Zainulabeuddin, Leal, W. S. 2007. Maxillary palps are broad spectrum odorant detectors in *Culex quinquefasciatus*. Chemical Senses 32, 727-738.

Tapia, S., Pardo, F,. Perich, F., Quiroz, A. 2005. Clover root borer *Hylastinus obscurus* (Marsham) (Coleoptera: Scolytidae) has no preference for volatiles from root extracts of disease infected red clover. Acta Agriculturae Scandinavica B 55, 158-160.

Tapia, T., Perich, F, Pardo, F, Palma, G., Quiroz, A. 2007. Identification of volatiles from differently aged red clover (*Trifolium pratense*) root extracts and behavioural responses of clover root borer (*Hylastinus obscurus*) (Marsham) (Coleoptera: Scolytidae) to them. Biochemical Systematics and Ecology 35, 61-67.

Thiéry, D., Marion-Poll, F. 1998. Electroantennogram responses of Douglas-fir seed chalcids to plant volatiles. Journal of Insect Physiology 44, 483-490.

Vrkocová, P., Valterová, I., Vrkoc, J., Koutek, B. 2000. Volatiles released from oak, a host tree for the bark beetle *Scolytus intricatus*. Biochemical Systematics and Ecology 28, 933-947.

White, P. R., Hobson, K. R. 1993. Stereospecific antennal response by red turpentine beetle, *Dendroctonus valens* to chiral monoterpenes from ponderosa pine resin. Journal of Chemical Ecology 19, 2193-2202.

Wibe, A., Borg-Karlson, A. K., Persson, M., Norin, T., Mustaparta, H. 1998. Enantiomeric composition of monoterpene hydrocarbons in some conifers and receptor neuron discrimination of α-pinene and limonene enantiomers in the pine weevil, *Hylobius abietis*. Journal of Chemical Ecology 24, 273–287.

Zhang, Z., Pawliszyn, J. 1995. Quantitative extraction using an internally cooled solid phase microextraction device. Analytical Chemistry 67, 34-43.

Table 1. Percentage of area and retention time to volatiles collected by SPME and identified by GC-MS from red clover roots of different ages.

Compound	Retention time (min)	5 months	9 months	11 months	16 months	24 months
Ethanol	1,07	2,28	0,49	13,64	n.d	2,44
Hexanal	3,28	22,15	4,9	10,56	n.d	n.d
***E*-2-Hexenal**	4,19	8,97	n.d	n.d	n.d	n.d
α-pinene	6,5	n.d	0,63	n.d	n.d	4,82
3-Octanone	7,53	5,15	7,58	2,12	3,81	n.d
Limonene	8,87	n.d	n.d	n.d	0,25	6,3

n.d.: not detected

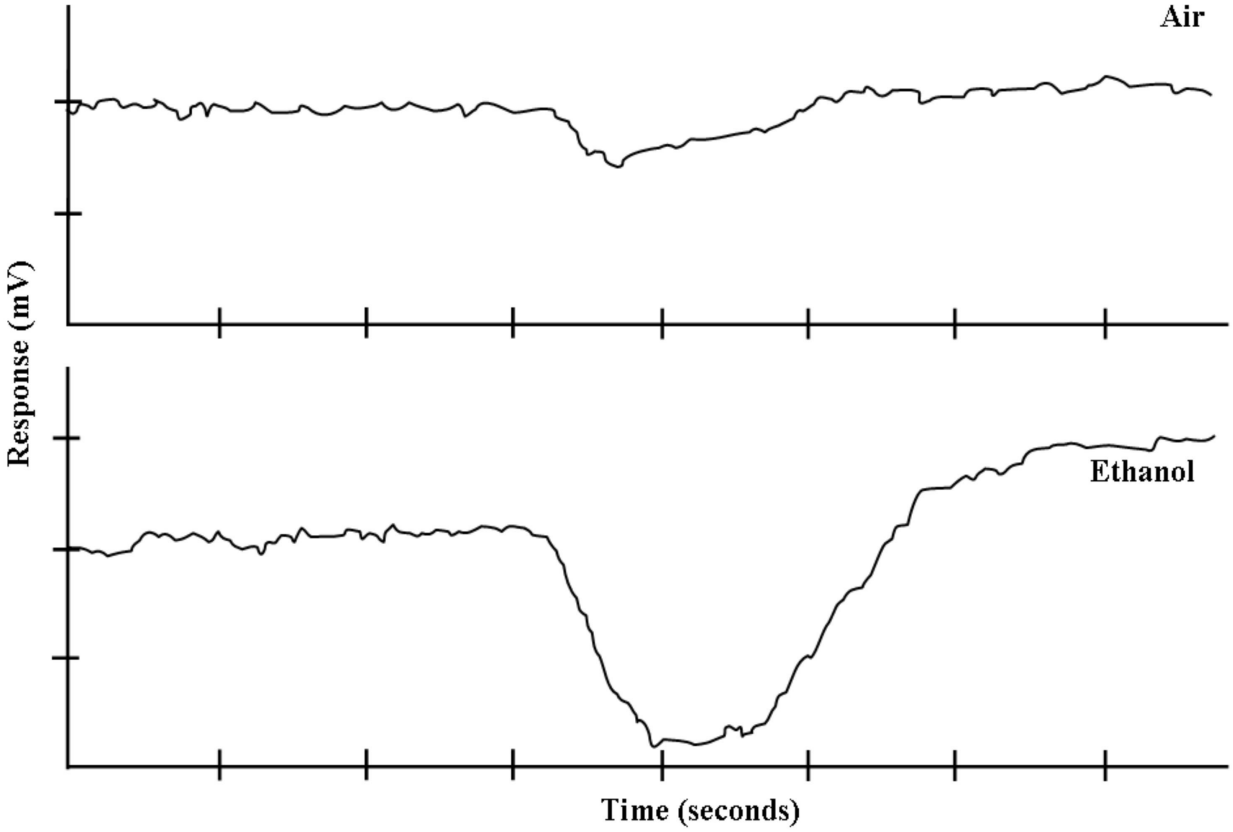

Figure 1. Example of deflections obtained from *H. obscurus* stimulated by air and ethanol in EAG assays.

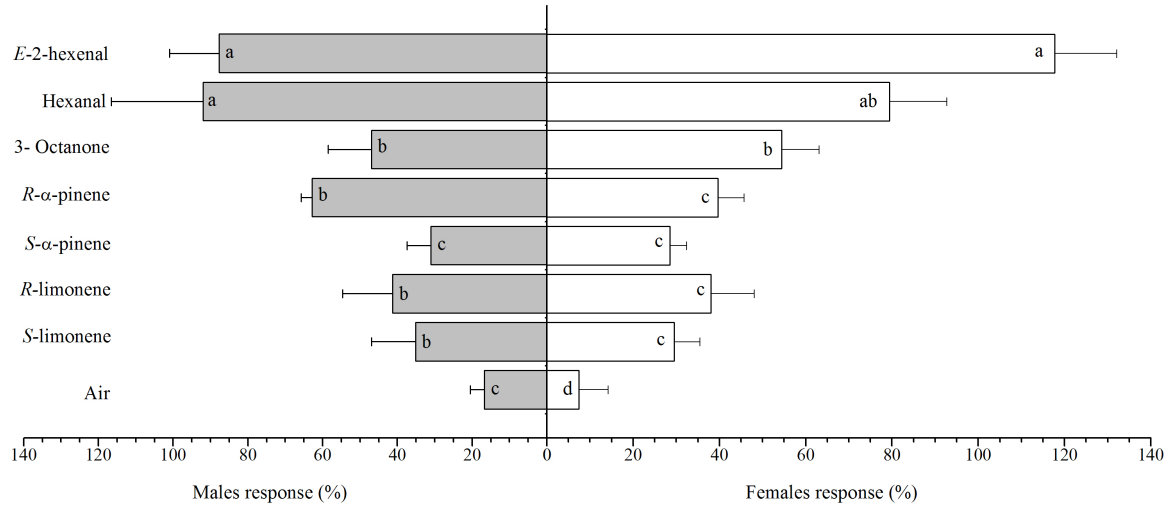

Figure 2. Electroantennographic response of *Hylastinus obscurus* to different compounds found in roots of red clover. Values are expressed as relative percentage (+SE) respect to ethanol response, used as reference. Different letters to the same sex indicates statistical differences ($p<0.05$) to Conover-Inman non-parametric test for multiple comparision for n=5.

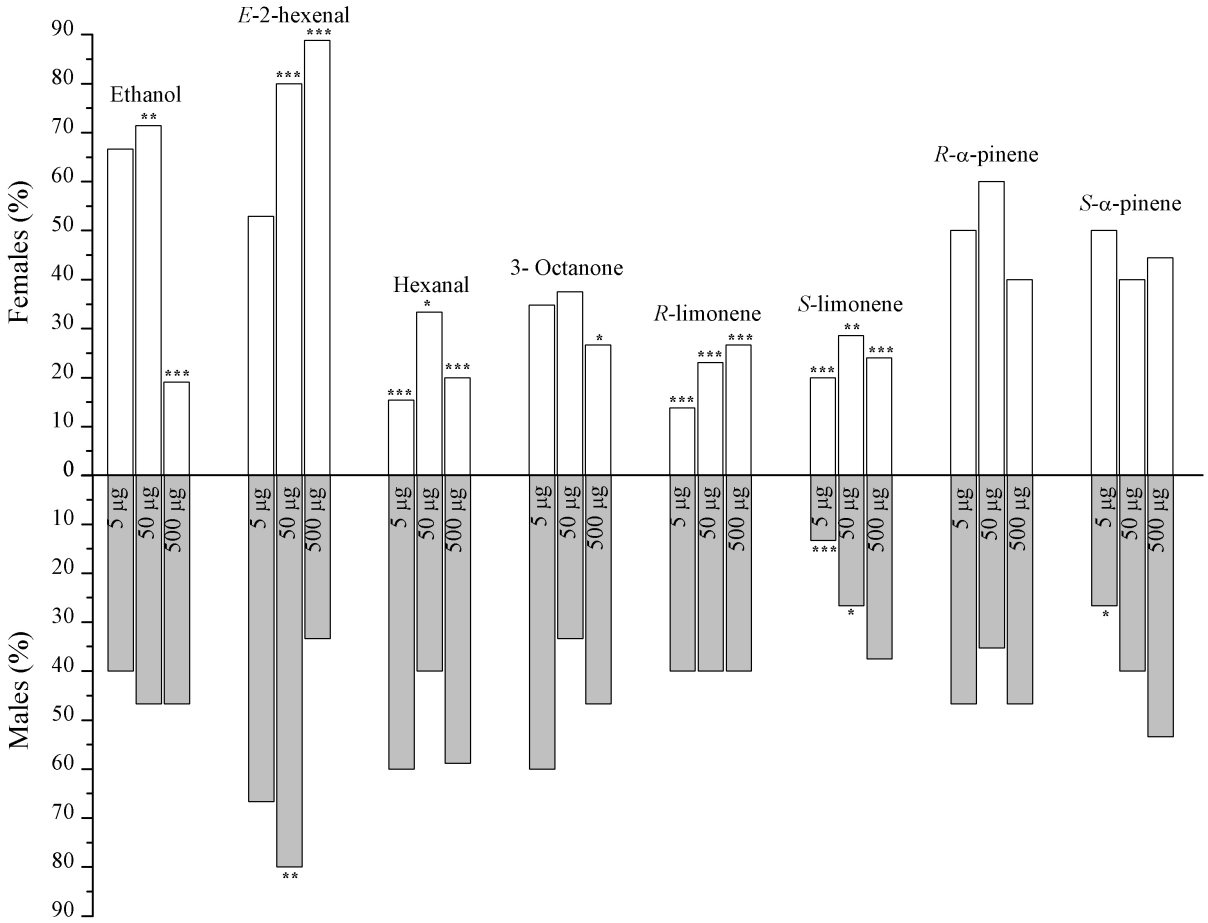

Figure 3. Response (%) of *H. obscurus* males and females towards three doses of different odorant stimuli in a Y-tube olfactometer. One, two and three asterisks indicate statistical significance according to G-Test for $P<0.1$; $P<0.05$; and $P<0.01$, respectively with $n\geq15$.

Chapter 4

Behavioral and electrophysiological responses to conspecific volatiles from adult *Hylastinus obscurus* L. (Coleoptera: Scolytidae)

Behavioral and electrophysiological responses to conspecific volatiles from adult *Hylastinus obscurus* L. (Coleoptera: Scolytidae)

Abstract

Volatile compounds were captured from the head space of adult clover root borers by using solid phase microextraction (SPME) and a dynamic system for volatile trapping. Three compounds were identified: α-pinene, β-pinene and limonene. The response of *H. obscurus* toward these compounds in a Y-tube olfactometric bioassays and the electroantennographic response towards β-pinene were studied. Olfactometric results showed that females were active to a larger number of compounds and doses than males. Some doses of *S*-limonene, *(-)*-α-pinene and *(-)*-β-pinene were repellent for males, meanwhile the highest dose of *(+)*-β-pinene resulted in an attractive response. Electroantennographic assays demonstrated a dose-dependent activity. This is the first report which relates the behavioral and electroantennographical activity of *Hylastinus obscurus* toward volatiles coming from conspecific individuals.

Introduction

Hylastinus obscurus (Coleoptera: Scolytidae), commonly named red clover root borer, is an important cosmopolitan pest that attack red clover crops, reducing their persistence, productivity and opening the entrance to new pests and diseases (Matamala, 1976). Is considered the most important cause for red clover decay (Steiner & Alderman, 1999) and pesticides applications have not been successful on its control (Quiroz et al., 2005). During the spring in the South Hemisphere, adults flight from October to December infesting new plants (Carrillo & Mundaca, 1974). Agronomical actions to reduce their damage have included insecticides applications and increase crop rotation but the problem persist (Aguilera et al., 1996).

Remarkable reviews (see Byers, 1989; Byers, 1995) demonstrate the complexity of chemical ecology in scolytids, and explain why few works have reported the participation of red clover odors in the borer ability to recognize its host. Leath & Byers (1973) have reported the attractiveness of the disease-roots leachate and diseased roots pieces in behavioral assays. Tapia et al. (2007) also determined this effect, identifying *E*-2-hexenal and methyl benzoate as

causing the attractive response of red clover root extracts. In contrary, Tapia et al. (2005) did not find differences in the response of red clover borer exposed to volatiles from healthy and diseased roots. In the other hand, Quiroz et al. (2005) established the attraction of *H. obscurus* towards volatiles released from the aerial parts of red clover. Previously, Buttery et al. (1984) listed the compounds found in leaves, flowers and seed pods, suggesting possible insect attractants but red clover borer was not mentioned.

The study of host and conspecific odors is justified by numerous reports to other scolytid species in forest ecosystems, which host selection behavior, is governed largely by chemical cues. Highly intricate chemical communication systems are operating based on complicated interactions between host-tree odours, aggregation pheromones produced by the beetles or associated microorganisms (Schoonhoven et al. 2005). Thus, our aim was determine the behavioral and electroantennographical activity of *Hylastinus obscurus* toward individual volatiles compounds released from adult insects.

Matherials and methods

Insects. Red clover plants were collected from different plots allocated in the Regional Research Center INIA-Carillanca in Vilcún, Araucanía, Chile, and brought to the laboratory. There, the insects were isolated from their substrate and they were placed in glass Petri dishes at 7 °C with fresh root pieces on humid filter paper until their utilization in the bioassays. At least 12 hours before to perform the assays, the insects were placed in a humid chamber without food, consisting in a glass Petri dish with paper towels humidified with distilled water and held at 7 °C. Individuals were used once, and their sex was determined after the bioassay, using the description made by Matamala (1976).

Volatiles collection using Solid Phase Microextraction (SPME). Fifty male and female adults of *Hylastinus obscurus* were introduced separately into two 40 mL glass vials with PTFE septa at room temperature by 30 minutes, and then a SMPE holder containing a 65 µm polidimethylsiloxane/ divinylbencene fiber (Supelco, USA) was introduced into the vial for collecting the volatiles. After 18 hours of trapping, the volatiles sorbed into the fiber were desorbed in the injector of the GC at 250°C for being analyzed by GC-MS.

Volatiles collection using Porapak-Q. Alternatively, volatiles were adsorbed on 100 mg Porapak Q columns (80-100 mesh; Waters Associates), previously cleaned with 1 ml of redistilled diethyl ether (GC grade; Merck, Darmstadt, Germany), and conditioned at 150 °C for 2 h in a stream of nitrogen (70 ml/m). Volatiles were trapped by using a positive/negative pressure air system (Agelopoulos et al. 1999). The air was purified sequentially by activated 5-Å molecular sieves and then charcoal. Volatiles were desorbed from the Porapak-Q by elution with 1 ml of redistilled hexane (GC-MS grade; Optima Scientific, Darmstadt, Germany), which was concentrated to 100 μl under a nitrogen flow.

Chemical analysis and compounds determination. The collected volatiles were analized by gas chromatography-mass spectrometry (GC-MS), injecting the samples in a Thermo-Finnigan chromatograph (Milan, Italy) equipped with a BP-1 capillary column (30 m length by 0.22 mm by 0.25 μm; SGE, Victoria, Australia). Helium was used like carrier gas and the oven was programmed at 40 °C as starting temperature, the ramp was 5 °C per minute up to 240 °C held by 3 minutes. The temperature in the injector and transfer line were 250 °C. The volatiles collected were identified primarily for comparation of their Kovats indices and mass spectra with those of commercial standards and library database spectra (data not shown). The concentrations of these compounds were estimated by the comparison of their areas respect to those obtained by injection of pure standards at known concentration, and their ratios were used to prepare the blends.

Behavioral bioassays. A Y-shape glass tube was used as arena; its arms and central tube were 55 mm length and 9 mm i.d. A constant air flow at 0.2 L min $^{-1}$ was applied for moving the volatiles from the arms to the base of the Y-tube. A cartridge containing semiochemical was connected at the end of one of the arms, and the control consisted in a cartridge containing the respective solvent. Cartridges consisted of glass tubes of 9 mm o.d. containing a paper strip (8mm by 60 mm). Each strip was impregnated under the fume hood with 50 μL of semiochemical solution or hexane (control). The solvent was allowed to evaporate exposing the trips to the laboratory environment at 20°C per 30 s before to put them inside the cartridge. Behind the odor cartridge, a charcoal filter was connected to ensure just clean air was entering

in to the arena. Finally, an adult *H. obscurus* individual was introduced in the base of the Y tube. To facilitate the insect movement into the tube, the inner bottom of arms and central tube were coated with filter paper strips. Finally, the arm chosen by the borer was recorded after 5 minutes or less. The assay was considered successful when the insect passed 10 mm forward the Y-tube bifurcation, but when the insect did not choose any arm, the assay was discarded. At least 15 replications were performed per sex, per compound and per dose, plus a complete set using hexane in both arms. After the olfactometric assay, insects were stored individually in microcentrifuge tubes with ethanol up to determine their sex.

Electroantennography. Insects were anesthetized in cold (-15 °C per 3 minutes) and their heads were cut using scalpel and tweezers under the microscope. The electrodes consisted of Ag-AgCl wires inside of glass capillaries filled with Ringer solution modified and 0.1% PVP solution (Syed & Leal 2007). The indifference electrode was inserted in the base of head and the recording electrode was contacted to the tip of one of the antennae (Mendesil et al. 2009). The preparation was made on Syntech MP-12 micromanipulator (Hilversum, The Netherlands), connected to 10X amplifier. The signal was received by Syntech IDAC-02 interface (Hilversum, The Netherlands) which was linked to a personal computer where the EAG software interpreted and collected the information. The preparation was allocated under a humidified and charcoal filtered air flux, ca. 3 mm from the glass pipe outlet of 4 mm i.d. An hole of 2.5 mm at 50 mm from the outlet was used to connect the odor cartridges. The tested compounds were applied on filter paper strips (4 mm by 60 mm) using 10 µL of solution (in hexane) and air-dried under fume hood for 15 seconds, then they were put inside of glass Pasteur pipettes (Ruiz-Montiel et al. 2008). The stimulus controller Syntech CS-05 (Hilversum, The Netherlands) was set to continuous flow of 0.4 L min^{-1}, while the stimuli were applied for 1 second at 0.4 L min^{-1}. At least 30 s were allowed between stimulations. Linalool at 100,000 ppm was used as standard stimulus, meanwhile 1,000 ppm; 10,000 ppm and 100,000 ppm solutions of odorant were tested on preparations. Each odorant stimulation was preceded and followed by standard stimulus, and after each bioassay the sex of individual was determined according to Matamala (1976).

Data Analysis. Data obtained in olfactometric assays were analyzed using G-test for frequencies suggested by Sokal & Rohlf (2005). The values obtained in electroantennographic assays were compared by Mann-Whitney test (Conover 1999) using StatsDirect software v.2.7.8. (StatsDirect Ltd., UK).

Results

Volatiles identified in the head space of adults male and female borers showed the presence of α-pinene, β-pinene and limonene (Figure 1) from both collection methods in 1:1:1 ratio for females and 5:5:1 for males respectively. The individual assay of these compounds resulted in mixed responses. Thus, olfactometric results from Y-tube bioassay showed that both isomers of limonene were clearly avoided by females, but for males this behavior was elicited by the two lowest dose of *(-)*-limonene (Figure 2). α-pinene and β-pinene did not elicit a significant response from females, but repellency was observed to the lowest dose on males . The most remarkable data were the opposite response of males to the highest dose of both isomers of β-pinene, resulting attractive the *(+)*-β-pinene and repellent the *(-)*-β-pinene.

The behavioral response of adults *H. obscurus* to different blends of the semiochemicals are shown in figure 3 and 4. Two blends sharing racemic mixtures of α-pinene and limonene, but in one adding *(-)*-β-pinene, and the other one *(+)*-β-pinene, resulted to be clearly repellent for males (Fig. 3). Meanwhile, just the blend containing *(-)*-β-pinene was repellent for females. In contrary, the mixes containing racemic α-pinene and an enantiomer of β-pinene resulted repellent for females (Figure 4) and indifferent for males.

The novel presence β-pinene in the volatiles of males and females compared to limonene and α-pinene that were found in red clover before, motivated the electroantennographic assays. The response of *H. obscurus* to both isomers of β-pinene showed dose-dependent curves (Figure 5). The relative deflections showed no differences between both sexes, excepting the highest dose of *(+)*-β-pinene which values was higher to males than females. The air reached relative deflections close to those obtained to the lowest dose of *(+)*-β-pinene and *(-)*-β-pinene.

Using the ratio found in females the bioassays performed using blends of terpenic compounds (Figures 3 and 4) tried to find the possible synergic or detrimental effect by the presence of more than one compound at the same time in the behavior of males and females. Thus the

mixture of three compounds shown to be repellent to males in the combinations with both isomers of β-pinene meanwhile this behavior was observed just to the isomer *(-)* to females.

Discussion

Some compounds identified in our study have been reported previously in red clover. Tapia et al. (2007) noted the presence of limonene and α-pinene in root extracts. Including β-pinene, those compounds are common volatiles emitted by coniphers hosts of different scolytids species (Thiéry & Marion-Poll 1998; Huber et al. 2000) and they take part in the chemical ecology of bark beetles. Both isomers of α-pinene are used as kairomone by the common pine shoot beetle *Tomicus piniperda* (Byers 1989), while both isomers of limonene are attractant to the white pine cone beetle *Conophthorus coniperda* and worked as synergist increasing the attraction to traps baited with sexual pheromone (Miller 2007).

Behaviorally, males and females of *H. obscurus* responded differently (Figure 1). Females appear to be more sensitive to *(+)*- and *(-)*-limonene than males, and not responding to *(+)*-isomer of limonene. Previously, Tapia et al. (2007) noted the repellent activity of limonene coming from red clover roots, although were not discriminated isomers and sexes. This higher sensitivity may be related with their role to find a suitable host to their offsprings. In the case of α-pinene, just the lowest dose of *(-)*-α-pinene on males was repellent and the remaining doses showed no activity in males or females.

The third terpenic compound, β-pinene is first-time reported in volatiles of *H. obscurus.* This compound just showed effect in males but not in females (Figure 2) and its use has been reported as attractant of different wood-boring and bark beetles (Joseph et al. 2001; Brockerhoff et al. 2006). The situation changed testing the blends of three semiochemicals or just two terpenes respect to the effect of single-compound (Figure 3 and 4). Considering limonene resulted in no attraction to males and females used alone, new blends with no limonene were prepared. In the assays both mixtures had no effect in the behavior of males but they were clearly repellents for females.

Although the employ of lures containing β-pinene to trapping and monitoring the red turpentine bark beetle *Dendroctonus valens* has been successful (Zhang et al. 2009), the addition of β-pinene may reduce the attraction of cone beetle *Conophthorus coniperda* to α-pinene (Miller et al. 2003). Besides we can not ignore the fact that odor components emitted

may be unique for a specie, and some insects can perceive these species-specific variations in the relative amounts and employ them to discriminate (Schoonhoven et al. 2005). Finally is known in some bark beetle species how they release some chemicals just when feed in host (Byers, 1995), so it is possible that other semiochemicals could participate in clover borer intraspecifical recognition.

The dose-response curves (Figure 5A and 5B) in the electroantennographic activity of β-pinene, resulted in noticeable differences between male and female response, such as response of males were more affected by amount of compound employed than those of females, suggesting a lower threshold for males than females, which could implicate a larger ability to perceive this compound. Sex-dependent responses to odors have been reported previously; Seabrook et al. (1987) noted larger EAG measures for males than females of *Trichoplusia ni* for two female-released pheromones.

Even though the results for the role of β-pinene in the chemical ecology of red clover borer have not been conclusives, its presence in the volatiles of both males and females, besides the ability of its both enantiomers to affect the males behavior remains as an interesting issue for further studies. The possibility to test β-pinene in conjunction with other behaviorally active compounds found in red clover might be explored.

References

Aguilera, A., Cisternas, E., Gerding, M., & Norambuena, H. 1996. Plagas de las praderas. In I. Ruiz N. (Ed.), Praderas Para Chile (pp. 310–339).

Agelopoulos, N., Hooper, A. M., Maniar, S. P., Pickett, J. A. & Wadhams, L. J. 1999. A novel approach for isolation of volatile chemicals released by individual leaves of a plant in situ. *Journal of Chemical Ecology*, 25(6): 1411-1425.

Brockerhoff, E. G., Jones, D. C., Kimberley, M. O., Suckling, D. M., & Donaldson, T. 2006. Nationwide survey for invasive wood-boring and bark beetles (Coleoptera) using traps baited with pheromones and kairomones. Forest Ecology and Management, 228(1-3): 234–240.

Buttery, R. G., Kamm, J. A., & Ling, L. C. 1984. Volatile components of red clover leaves, flowers, and seed pods: possible insect attractants. Journal of Agricultural and Food Chemistry, 32(2): 254–256.

Byers, J. A. 1989. Chemical ecology of bark beetles. Experientia, 45(3): 271–283.

Byers, J. A. 1995. Host tree chemistry affecting colonization in bark beetles. in R.T. Cardé and W.J. Bell (eds.). Chemical Ecology of Insects 2. Chapman and Hall, New York, pp. 154-213.

Carrillo, R., & Mundaca, N. 1974. Biología de *Hylastinus obscurus* (Marsham); Coleoptera Scolytidae. Agricultura Técnica, 34(1): 29–35.

Conover, W. 1999. Practical Nonparametric Statistics. Wiley, Ed. 3rd Edition. 587pp.

Huber, D. P. W., Gries, R., Borden, J. H., & Pierce, H. D. 2000. A survey of antennal responses by five species of coniferophagous bark beetles (Coleoptera: Scolytidae) to bark volatiles of six species of angiosperm trees. Chemoecology, 10(3): 103–113.

Joseph, G., Kelsey, R. G., Peck, R. W., & Niwa, C. G. 2001. Response of some scolytids and their predators to ethanol and 4-allylanisole in pine forests of central Oregon. Journal of Chemical Ecology, 27(4): 697–715.

Leath, K., & Byers, R. 1973. Attractiveness of diseased red clover roots to the clover root borer. Phytopathology, 63: 428–431.

Matamala, J. 1976. Biología, niveles de infestación, daño y combate químico de *Hylastinus obscurus* (Marsham) (Coleoptera: Scolytidae). Tesis Ingeniero Agrónomo. Universidad Austral de Chile. 51pp.

Mendesil, E., Bruce, T.J., Woodcock, C.M., Caulfield, J.C., Seyoum, E., & Pickett, J.A. 2009. Semiochemicals used in host location by the coffee berry borer, *Hypothenemus hampei*. Journal of Chemical Ecology, 35(8): 944–950.

Miller, D. R. 2007. Limonene: attractant kairomone for white pine cone beetles (Coleoptera: Scolytidae) in an eastern white pine seed orchard in western North Carolina. Journal of economic entomology, 100(3): 815–822.

Miller, D. R., Crowe, C. M., Asaro, C., & Debarr, G. L. 2003. Dose and enantiospecific responses of white pine cone beetles, *Conophthorus coniperda*, to alpha-pinene in an eastern white pine seed orchard. Journal of Chemical Ecology, 29(2): 437–451.

Quiroz, A., Ortega, F., Ramírez, C. C., Wadhams, L. J., & Pinilla, K. 2005. Response of the beetle *Hylastinus obscurus* Marsham (Coleoptera: Scolytidae) to red clover (*Trifolium pratense* L.) volatiles in a laboratory olfactometer. Environmental Entomology, 34(3): 690–695.

Ruiz-Montiel, C., García-Coapio, G., Rojas, J. C., Malo, E. A., Cruz-López, L., del Real, I., & González-Hernández, H. 2008. Aggregation pheromone of the agave weevil, *Scyphophorus acupunctatus*. Entomologia Experimentalis et Applicata, 127(3): 207–217.

Schoonhoven, L., van Loon, J., & Dicke, M. 2005. Insect-plant Biology. 2nd Edition. New York: Oxford University Press. 421pp.

Seabrook, W., Linn, C., Dyer, L., & Shorey, H. 1987. Comparison of electroantennograms from female and male cabbage looper moths (*Trichoplusia ni*) of different ages and for various pheromone concentrations. Journal of Chemical Eecology, 13(6): 1443–1453.

Sokal, R., & Rohlf, F. 2005. Biometry. W. Freeman, Ed. 3rd edition. New York. 887pp.

Steiner, J., & Alderman, S. 1999. Red clover seed production. V. Root health and crop productivity. Crop Science, 39: 1407–1415.

Syed, Z., & Leal, W. S. 2007. Maxillary palps are broad spectrum odorant detectors in *Culex quinquefasciatus*. Chemical Senses, 32(8): 727–738.

Tapia, S., Pardo, F., Perich, F., & Quiroz, A. 2005. Clover root borer *Hylastinus obscurus* (Marsham) (Coleoptera: Scolytidae) has no preference for volatiles from root extracts of disease infected red clover. Acta Agriculturae Scandinavica, Section B - Soil & Plant Science, 55(2): 158–160.

Tapia, T., Perich, F., Pardo, F., Palma, G., & Quiroz, A. 2007. Identification of volatiles from differently aged red clover (*Trifolium pratense*) root extracts and behavioural responses of clover root borer (*Hylastinus obscurus*) (Marsham) (Coleoptera: Scolytidae) to them. Biochemical Systematics and Ecology, 35(2): 61–67.

Thiéry, D., & Marion-Poll, F. 1998. Electroantennogram responses of Douglas-fir seed chalcids to plant volatiles. Journal of Insect Physiology, 44(5-6): 483–490.

Zhang, L., Clarke, S. R., & Sun, J. 2009. Electrophysiological and behavioral responses of *Dendroctonus valens* (Coleoptera: Curculionidae: Scolytinae) to four bark beetle pheromones. Environmental Entomology, 38(2): 472–477.

Tables and figures

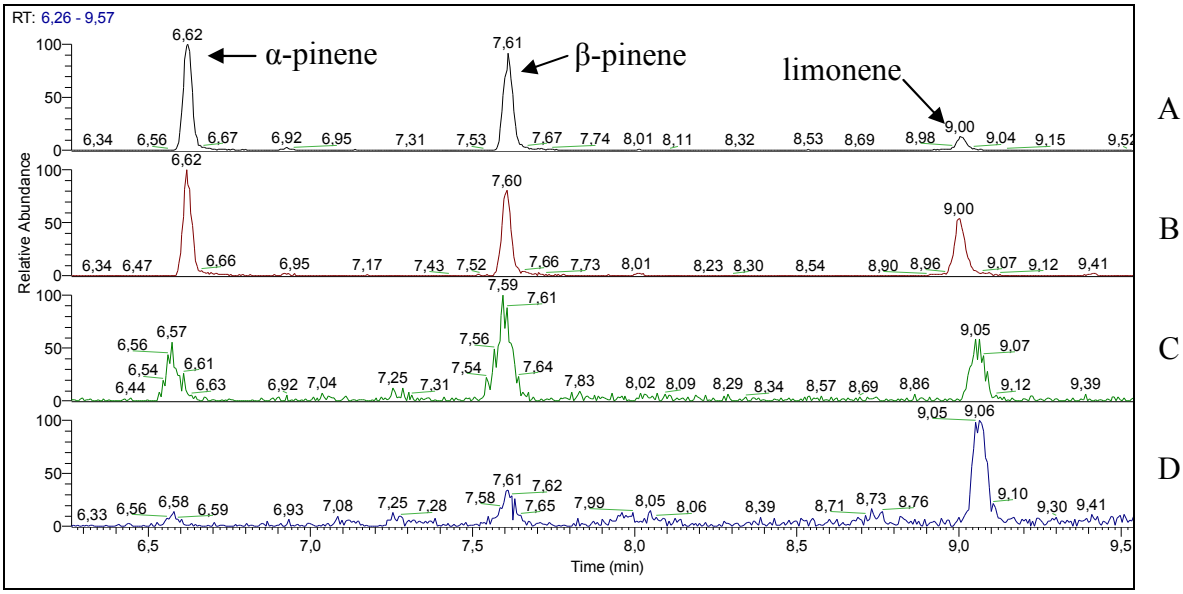

Figure 1. Chromatograms obtained by GC-MS and compounds identified as result of trapping by Porapak-Q for 72 hours;50 females (A) and 50 males (B). Trapping by SPME for 18 hours; 47 females (C) and 47 males (D).

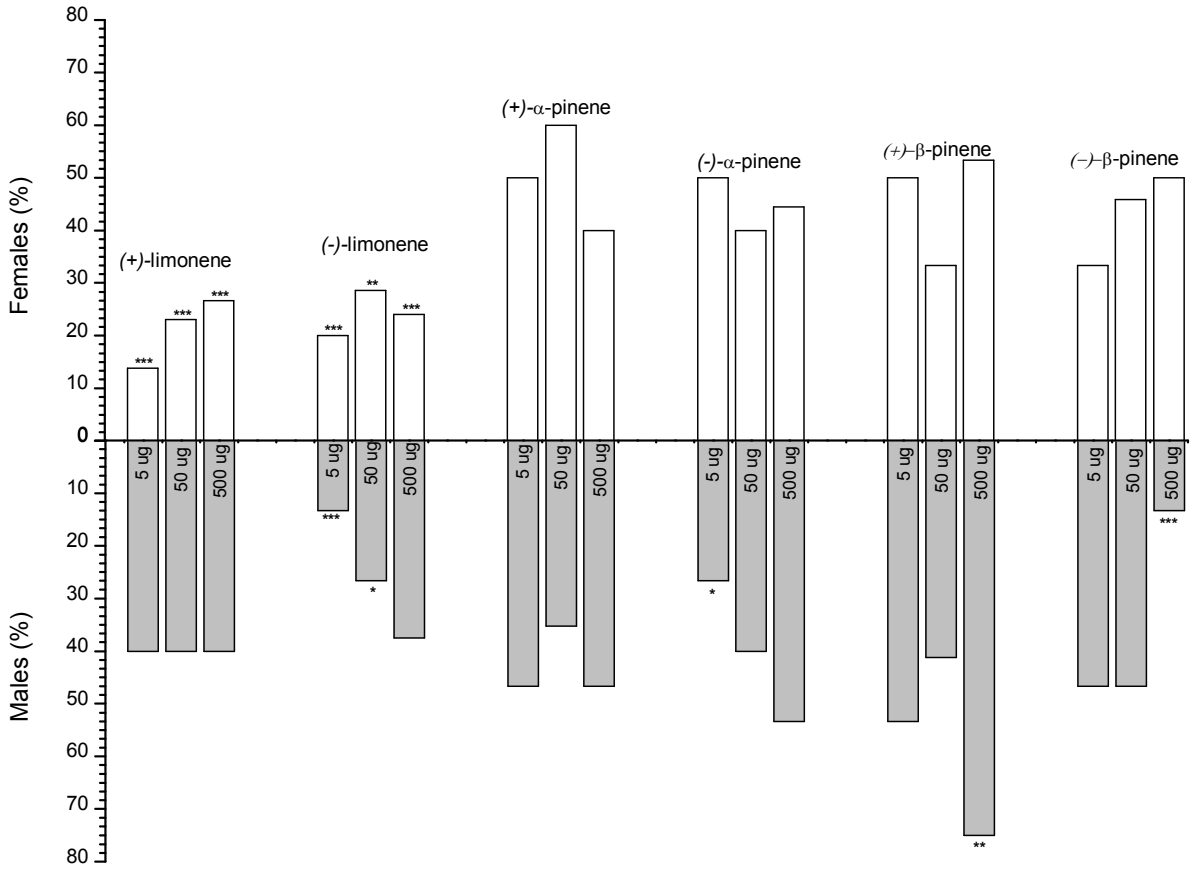

Figure 2. Response (%) of adult insects towards three different doses of odorant stimuli in a Y-tube olfactometer. One, two and three asterisks indicate statistical significance according to G-Test for $P<0.1$; $P<0.05$; and $P<0.01$, respectively using $n \geq 15$.

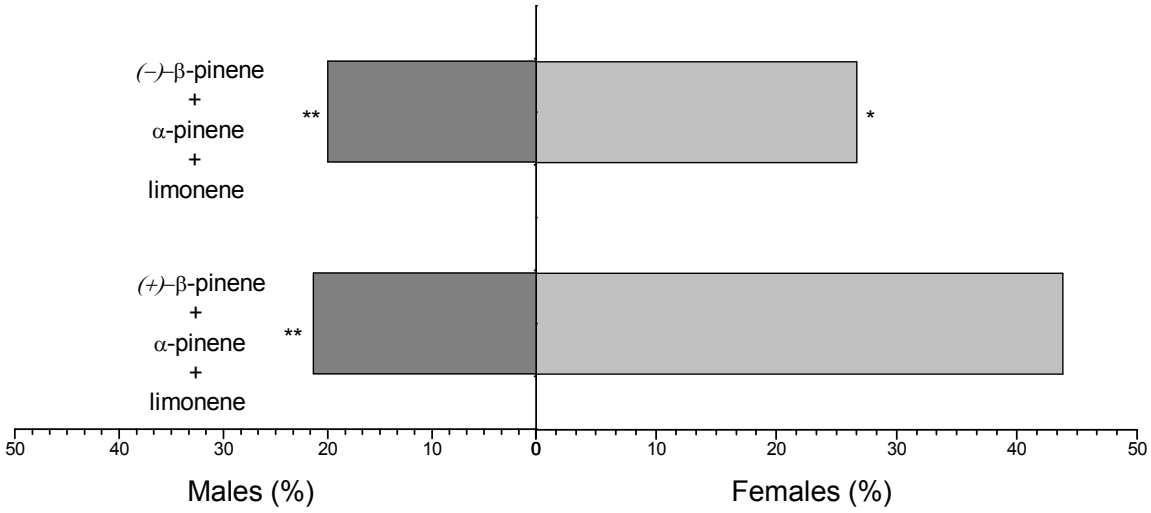

Figure 3. Response (%) of males and females of *H. obscurus* in a Y-tube olfactometer towards two different blends of odorant stimuli resembling their ratio in the collected volatiles. One and two asterisks indicate statistical significance according to G-Test for P≤0.1; P≤0.05, respectively using n≥15.

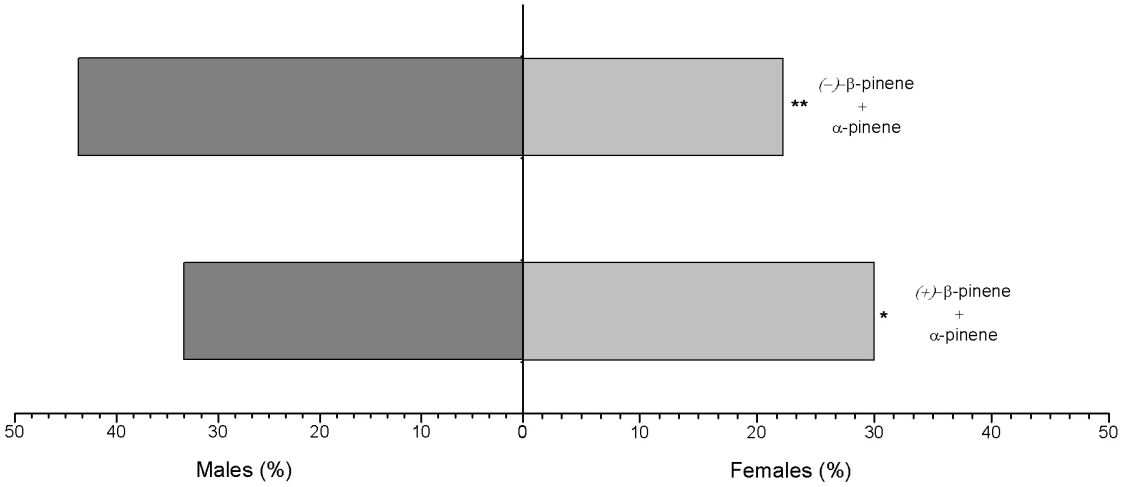

Figure 4. Response (%) of males and females of *H. obscurus* in a Y-tube olfactometer towards two different blends of odorant stimuli resembling their ratio in the collected volatiles. One and two asterisks indicate statistical significance according to G-Test for P≤0.1; P≤0.05, respectively using n≥15.

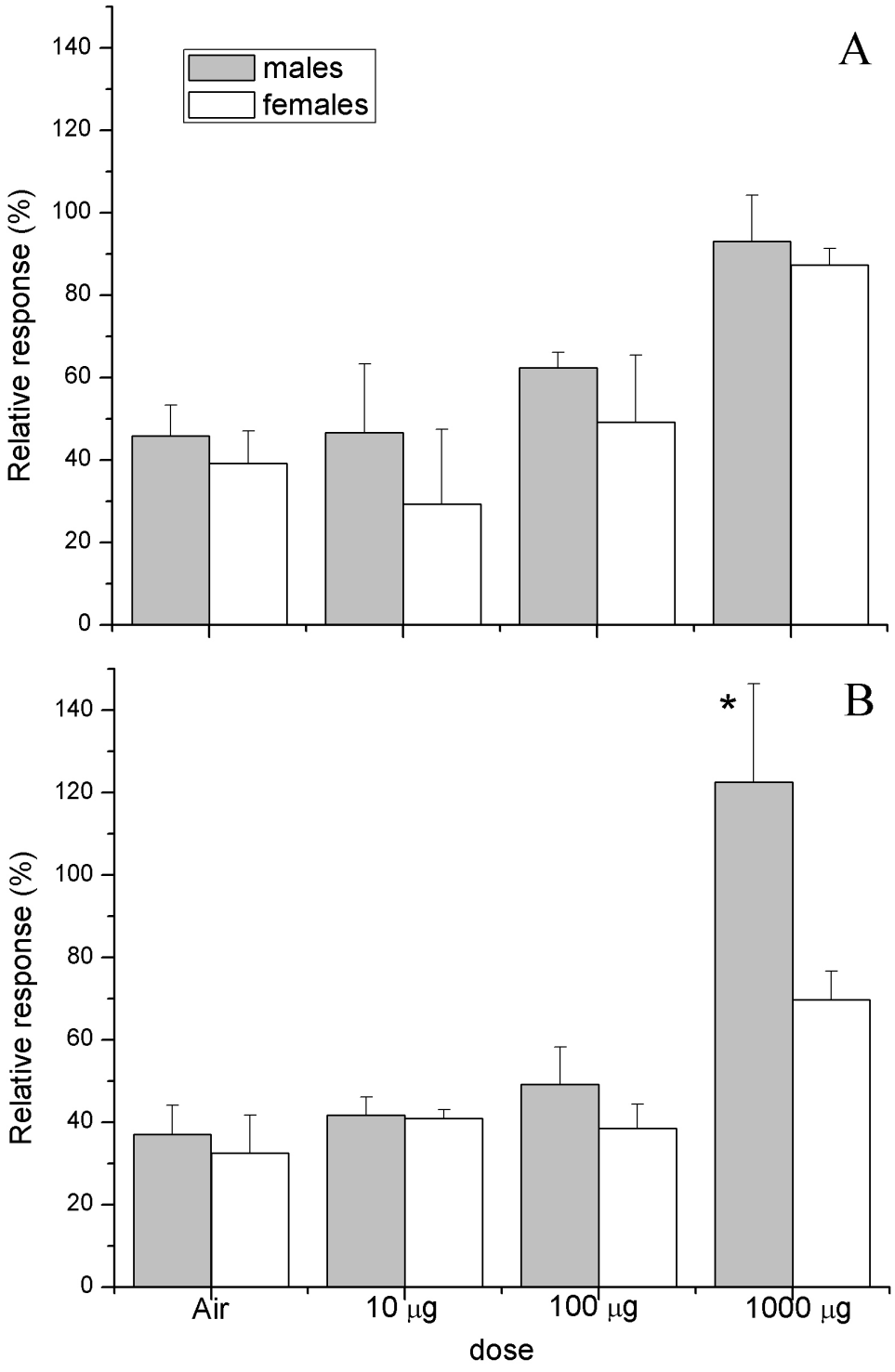

Figure 5. Mean EAG relative response (+SE) of males and females of *Hylastinus obscurus* towards three different doses of (-)-β-pinene [A]; and (+)-β-pinene [B]. Asterisk indicates differences between sexes at same dose ($P<0.05$).

Chapter 5

Evidences of antennal chemoreceptive proteins in *Hylastinus obscurus*

Abstract

Insects depend of their olfaction sense to survive. Thirty years ago was discovered the first protein that binds odor, but until now the accuracy and sensibility of this complex ability is not well understood. The role played by molecular biology to help answer some questions about the olfaction in insects may be very important. Considering the economic importance of some insect families from Coleoptera, it is remarkable that few species have been studied about their ability to produce proteins to bind behaviorally important molecules. Using these tools, a preliminary evidence of the presence of these proteins is reported here, with similar pattern to those reported in other beetle species. However, further analyses are required to elucidate the role of these proteins in order to improve the understanding of smell sense in *H. obscurus*. The recent new findings about the genomic of a couple of scolytids may be used by their phylogenetic proximity to *H. obscurus* to step up the knowledge in the chemical ecology of the clover root borer.

Introduction

To survive, insects depend on their ability to detect chemical signals from the environment (Leal 2001). In fact, chemical signal are probably the most important cues for insects to determine the suitability of feeding and oviposition sites (Vosshall 2000; Picimbon 2003; Larsson & Svensson 2005). Smell may be defined as chemostimulation by compounds in very low concentration but volatile at physiological temperatures (Gillott 2005). Insect antennae are designed to receive and process volatile stimuli (Leal 2003), and the olfactory sensilla are allocated there and it have the ability to transform chemical into electrical signals (Wojtasek 2002; Jacquin-Joly & Merlin 2004) that is carried and processed in the brain (Leal 2005).

The sequence of events begins when a volatile molecule entering by a sensilla pore. The odor-binding protein (OBP) carries the molecule (commonly apolar) through the aqueous sensillar lymph to the odor receptor (OR), placed in the dendrite's membrane (Vogt & Riddiford 1981; Ruebenbauer 2006). The OR gives the chemical message as electrical stimulus to the nervous which transport to the brain (Vogt et al. 1985). Finally the odor-degrading enzime (ODE) destroy the odor molecule, leaving the system ready to receipt another molecule (Jacquin-Joly & Merlin 2004; Rützler & Zwiebel 2005).

Odor-binding proteins (OBPs) are specialized carrier proteins found in antennal sensilla and palpus of insects (Vogt et al. 1991). Their molecular weight is near to 15 kDa and

lower than 150 aminoacids (Callahan et al. 2000; Vogt 2003) and with very well conserved six cysteine residues that form three disulfides bridges (Wojtasek & Leal 1999; Laue 2000), conferring great stability to the protein (Pelosi et al. 2006). They can be classified in two principal groups; pheromone-binding proteins (PBPs) which appear in antenna only (preferently males) and bind a pheromone, and general odor-binding proteins (GOBPs) may be present in palpi as well as antenna and bind a non-pheromone compound (Tegoni et al. 2004). However, some exceptions have been noted to this rule (Callahan et al. 2000; Liu et al. 2010).

The physiological function of OBPs are not well understood (Pelosi et al. 2006), but they can be found so high concentration as 10-20 mM in sensilla (Vogt et al. 1991; Wojtasek 2002), meanwhile *in vitro* studies showed that OBPs are not required to stimulate ORs, althought the presence of OBPs seems to reduce odor concentration necessary to stimulate the ORs (Leal 2003). When PBPs were first reported (Vogt & Riddiford 1981), they were postulated to contribute to the specificity of the olfactory system for binding a single one pheromone compound (Gräter et al. 2006), but newer studies report their ability to bind a range of compounds (Maida et al. 2000; Campanacci et al. 2001; Maida et al. 2003; Liu et al. 2010; Guo et al. 2012). Currently, the most plausible explanation is two-step recognition, combining OBP and OR role. Then, any molecule may be accepted as a suitable ligand to elicit a response (Leal 2003; Leal 2005).

Wojtasek & Leal (1999) found that the conformation of PBP changes according to the pH in the surrounding media. The binding with pheromone ocurrs only at sensillar lymph pH (close to 7) and complex is not formed at acid pH (near 4), which is the dendrite surface condition. This could explain the bind-unbind mechanism and could be applied to other OBPs (Leal 2003). Up to date, odor-binding proteins has been found and described from different Endopterygota species like Lepidoptera, Coleoptera, Diptera, Hymenoptera, Hemiptera, Orthoptera and Isoptera (Vogt et al. 1991; Dickens et al. 1998; Vogt et al. 1999; Nikonov et al. 2002; Ishida et al. 2002; Ishida et al. 2002b; Ban et al. 2003; Calvello et al. 2005), but those reports are minimal considering the abundance of insect species.

Hylastinus obscurus, is an economically important pest which attack and cause the decline in the red clover production (Carrillo & Mundaca 1974). As other Scolytidae species the borers complete their whole life protected from environmental factors inside their host (Rudinsky 1962) and remain with no control excepting crop rotation (Aguilera et al. 1996). Even though the chemical ecology of *H. obscurus* has received some attention, there is not enough information about their olfaction. Thus, every information related to their ability to

find their host and mate may be helpful in order to design an integrated borer management program. The aim of this work was to find any evidence of the existence of OBPs in *Hylastinus obscurus*.

Material and methods

Insects. Red clover plants were collected from plots in Regional Research Center INI,Carillanca, Vilcún, Región de La Araucanía, Chile and carried to the Chemical Ecology Laboratory, Universidad de La Frontera. Wearing gloves and helped by tweezers, the adult borers were isolated and placed in Petri dishes. The individuals were frozen at -80°C inside microcentrifuge tubes to be used in antennal protein extraction, or they were held in glass Petri dishes containing pieces of root at 7°C until to be uses in RNA extraction.

Protein extraction. The tubes containing the frozen borers were manipulated in ice, and using a microscope and fine tweezers 500 antenna of each sex were excised and put separately in a new microcentrifuge tube on ice. At the same time, the sex of each individual was determined according to Matamala (1976). In the same manner hindlegs were cut and used as blank tissue (Ishida et al. 2002), using the ratio three antennae for one leg. The collected tissue was grind in a microcentrifuge tube using a disposable pestle and 70 uL 10mM Tris-HCl buffer, pH 8. Then, it was centrifuged at 4 °C and 13.200 rpm for 20 minutes. The supernatant was recovered and concentrated in a vacuum microcentrifuge for 40 minutes. The samples were loaded and separated by native 15% polyacrilamide gel electrophoresis (PAGE) and stained with Coomassie brilliant blue for 7 minutes. The gel was unstained in a methanol plus glacial acetic acid solution overnight and then it was documented.

Protein sequencing. A new sample prepared with the twice antenna was loaded and separated by PAGE. Then the band of interest were transferred to a PVDF membrane by electroblotting, following the procedures reported by (Ishida et al. 2002) and (Nagnan-Le Meillour et al. 2004). Then, the pieces of membrane containing the majoritary bands were cut and air-dried to be saved in separately in microcentrifuge tubes and sent to The Molecular Structure Facility of the University of California-Davis (USA) and the N-terminal sequence to the first 10 aminoacids was obtained by Edman degradation method.

RNA extraction and cDNA synthesis. Total RNA was extracted from *H. obscurus* antennae using TRIzol (Invitrogen) (Ishida et al. 2002). Alternatively RNA extraction was performed using RNeasy kit (Qiagen) following the manufacturer instructions. The first strand of cDNA was synthesized using Smart RACE cDNA Amplification Kit (Clontech) and reverse transcriptase. 3'-RACE was performed using degenerate primer, designed on the basis of residues at the N-terminal aminoacid sequence (Nagnan-Le Meillour et al. 2004). Besides, degenerate primers designed to actin were used to get a product which will be used as positive control (Ishida et al. 2002). The PCR product was checked in 1.2% agarose gel and stained with ethidium bromide and documented.

Results and Discussion

Gel electrophoresis is the most common technique employed for isolating OBPs; but usually are detected very few antennae-specific proteins (Wojtasek 2002). The separation of proteins in native PAGE gel (Figure 1) showed the presence of four bands which were not detected in legs lane. Odor binding proteins are antennospecific (Ishida et al. 2002; Nagnan-Le Meillour et al. 2004). The pattern is similar to those reported previously for other insect species (Wojtasek et al. 1998; Vogt 2003).

From the observed bands, one was selected due to its intensity to be sequenced. Due to the low staining level, the number of antennae was doubled up to one thousand to be electroblotted to a PVDF membrane. The N-terminal band sequencing for females sample resulted in a 10-aminoacids chain Asp-Gln-Arg-Gln-Lys-Phe-Ile-Asp-Phe-His (DQRQKFIDFH). Using it as template two degenerated primers were designed to perform the 3' RACE: 5'GA(T/C)CA(A/G)AG(A/G)CA(A/G) AA(A/G)TT(T/C)AT(T/C/A)GA(T/C)TT(T/C)CA(T/C)3';5'GA(T/C)CA(A/G)CG(T/C/A/G)CA(A/G)AA(A/G)TT(T/C)AT(T/C/A)GA(T/C)TT(T/C)CA(T/C)3'.

Molecular tools have allowed the discovering of a significant diversity of OBPs in some insect species (Robertson et al. 1999). However, cDNA cloning results cannot be taken as the final step; for instance only 5 OBP´s from *D. melanogaster* were identified by the method above mentioned, but genome screening identified near 30 OBP´s (Vosshall 2000; Karlin et al. 2001).

The results shown that PCR product was not amplified using the degenerate primers, although a couple of band between 400 and 500 bp appeared to the positive control (Figure 2).

Considering that the understanding of molecular components involved in the insect olfaction may aid to develop new strategies to reduce disease transmission and crop damage (Jacquin-Joly & Merlin 2004; Rützler & Zwiebel 2005); the current limited genomic resources available for scolytids restrict the use of genomic-information to improve the tools used in the monitoring and control of these pests (Keeling et al. 2012). The tiny size of antenna and low number of genes that is generally isolated using the conventional tools, contributes to our poor understanding of the olfactory system in nonmodel species (Zhu et al. 2012).

References

Aguilera, A., Cisternas, E., Gerding, M., & Norambuena, H. 1996. Plagas de las praderas. In I. Ruiz N. (Ed.), *Praderas Para Chile* (pp. 310–339).

Ban, L., Scaloni, A., D'Ambrosio, C., Zhang, L., Yahn, Y., & Pelosi, P. 2003. Biochemical characterization and bacterial expression of an odorant-binding protein from *Locusta migratoria*. *Cellular and Molecular Life Sciences*, *60*(2): 390–400.

Callahan, F. E., Vogt, R. G., Tucker, M. L., Dickens, J. C., & Mattoo, A. K. 2000. High level expression of "male specific" pheromone binding proteins (PBPs) in the antennae of female noctuiid moths. *Insect Biochemistry and Molecular Biology*, *30*(6): 507–514.

Calvello, M., Brandazza, A., Navarrini, A., Dani, F. R., Turillazzi, S., Felicioli, A., & Pelosi, P. 2005. Expression of odorant-binding proteins and chemosensory proteins in some Hymenoptera. *Insect Biochemistry and Molecular Biology*, *35*(4): 297–307.

Campanacci, V., Krieger, J., Bette, S., Sturgis, J. N., Lartigue, A., Cambillau, C., Breer, H., et al. 2001. Revisiting the specificity of *Mamestra brassicae* and *Antheraea polyphemus* pheromone-binding proteins with a fluorescence binding assay. *The Journal of Biological Chemistry*, *276*(23): 20078–20084.

Carrillo, R., & Mundaca, N. 1974. Biología de *Hylastinus obscurus* (Marsham); Coleoptera Scolytidae. *Agricultura Técnica*, *34*(1): 29–35.

Dickens, J. C., Callahan, F. E., Wergin, W. P., Murphy, C. A., & Vogt, R. G. 1998. Intergeneric distribution and immunolocalization of a putative odorant-binding protein in true bugs (Hemiptera, Heteroptera). *Journal of experimental biology*, *201*(1): 33–41.

Gillott, C. 2005. *Entomology*. Third Edition. Springer. 834pp.

Gräter, F., Xu, W., Leal, W. S., & Grubmüller, H. (2006). Pheromone discrimination by the pheromone-binding protein of *Bombyx mori*. *Structure*, *14*(10): 1577–1586.

Guo, H., Huang, L.-Q., Pelosi, P., & Wang, C.-Z. 2012. Three pheromone-binding proteins help segregation between two *Helicoverpa* species utilizing the same pheromone components. *Insect Biochemistry and Molecular Biology*, *42*(9): 708–716.

Ishida, Y., Cornel, A. J., & Leal, W. S. 2002. Identification and cloning of a female antenna-specific odorant-binding protein in the mosquito *Culex quinquefasciatus*. *Journal of Chemical Ecology*, *28*(4): 867–871.

Ishida, Y., Chiang, V. P., Haverty, M. I., & Leal, W. S. 2002b. Odorant-binding proteins from a primitive termite. *Journal of Chemical Ecology*, *28*(9): 1887–1893.

Jacquin-Joly, E., & Merlin, C. 2004. Insect Olfactory Receptors: Contributions of Molecular Biology to Chemical Ecology. *Journal of Chemical Ecology*, *30*(12): 2359–2397.

Karlin, S., Bergman, A., & Gentles, A. J. 2001. Annotation of the *Drosophila* genome. *Nature*, *411*(6835): 259–260.

Keeling, C. I., Henderson, H., Li, M., Yuen, M., Clark, E. L., Fraser, J. D., Huber, D. P. W., Liao, N., Docking, T.R., Birol, I., Chan, S.K., Taylor, G.A., Palmquist, D., Jones, S., & Bohlmann, J. 2012. Transcriptome and full-length cDNA resources for the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, a major insect pest of pine forests. *Insect Biochemistry and Molecular Biology*, *42*(8): 525–536.

Larsson, M. C., & Svensson, G. P. 2005. Methods in insect sensory ecology. In T. A. . Christensen (Ed.), *Methods in Insect Sensory Neuroscience* (pp. 25–55). London: CRC Press.

Laue, M. 2000. Immunolocalization of general odorant-binding protein in antennal sensilla of moth caterpillars. *Arthropod structure & development*, *29*(1): 57–73.

Leal, W.S. 2001. Molecules and macromolecules involved in chemical communication of scarab beetles. *Pure and Applied Chemistry*, *73*(3): 613–616.

Leal, W. S. 2003. Proteins that make sense. In G. J. Blomquist & R. G. Vogt (Eds.), *Insect Pheromone Biochemistry and Molecular Biology* (pp. 447–476). London: Elsevier Academic Press.

Leal, W.S. 2005. Pheromone reception. *Topics In Current Chemistry*, *240*: 1– 36.

Liu, Z., Vidal, D. M., Syed, Z., Ishida, Y., & Leal, W. S. 2010. Pheromone binding to general odorant-binding proteins from the navel orangeworm. *Journal of Chemical Ecology*, *36*(7), 787–794

Maida, R., Krieger, J., Gebauer, T., Lange, U., & Ziegelberger, G. 2000. Three pheromone-binding proteins in olfactory sensilla of the two silkmoth species *Antheraea polyphemus* and *Antheraea pernyi*. *European Journal of Biochemistry 267*(10): 2899–2908.

Maida, R., Ziegelberger, G., & Kaissling, K.-E. 2003. Ligand binding to six recombinant pheromone-binding proteins of *Antheraea polyphemus* and *Antheraea pernyi*. *Journal of Comparative Physiology. B, Biochemical, Systemic, and Environmental Physiology*, *173*(7): 565–573.

Matamala, J. 1976. *Biología, niveles de infestación, daño y combate químico de Hylastinus obscurus (Marsham) (Coleoptera: Scolytidae)*. Tesis Ingeniero Agrónomo. Universidad Austral de Chile. 51pp.

Nagnan-Le Meillour, P., François, M.-C., & Jacquin-Joly, E. 2004. Identification and molecular cloning of putative odorant-binding proteins from the American palm weevil, Rhynchophorus palmarum L. *Journal of Chemical Ecology*, *30*(6): 1213–1223.

Nikonov, A. A., Peng, G., Tsurupa, G., & Leal, W.S. 2002. Unisex pheromone detectors and pheromone-binding proteins in scarab beetles. *Chemical senses*, *27*(6): 495–504.

Pelosi, P., Zhou, J.-J., Ban, L. P., & Calvello, M. 2006. Soluble proteins in insect chemical communication. *Cellular and Molecular Life Sciences*, *63*(14): 1658–1676.

Picimbon, J.-F. 2003. Biochemistry and evolution of OBP and CSP proteins. In Gary J Blomquist & R. Vogt (Eds.), *Insect Pheromone Biochemistry and Molecular Biology* (pp. 539–566). London: Elsevier Academic Press.

Robertson, H. M., Martos, R., Sears, C. R., Todres, E. Z., Walden, K. K., & Nardi, J. B. 1999. Diversity of odourant binding proteins revealed by an expressed sequence tag

project on male *Manduca sexta* moth antennae. *Insect Molecular Biology*, *8*(4): 501–518.

Rudinsky, J. A. 1962. Ecology of Scolytidae. *Annual Review of Entomology*, *7*(1): 327–348.

Ruebenbauer, A. 2006. Olfactory coding-from molecule to the brain. Introductory Paper N°181. *Department of Ecology. Lund University, Sweden.* 41pp.

Rützler, M., & Zwiebel, L. J. 2005. Molecular biology of insect olfaction: recent progress and conceptual models. *Journal of Comparative Physiology. A, Neuroethology, Sensory, Neural, and Behavioral Physiology*, *191*(9): 777–790.

Tegoni, M., Campanacci, V., & Cambillau, C. 2004. Structural aspects of sexual attraction and chemical communication in insects. *Trends in Biochemical Sciences*, *29*(5): 257–264.

Vogt, R G, Callahan, F. E., Rogers, M. E., & Dickens, J. C. 1999. Odorant binding protein diversity and distribution among the insect orders, as indicated by LAP, an OBP-related protein of the true bug *Lygus lineolaris* (Hemiptera, Heteroptera). *Chemical senses*, *24*(5): 481–495.

Vogt, R G, Prestwich, G. D., & Lerner, M. R. 1991. Odorant-binding-protein subfamilies associate with distinct classes of olfactory receptor neurons in insects. *Journal of Neurobiology*, *22*(1): 74–84.

Vogt, R G, Riddiford, L. M., & Prestwich, G. D. 1985. Kinetic properties of a sex pheromone-degrading enzyme: the sensillar esterase of *Antheraea polyphemus*. *Proceedings of the National Academy of Sciences of the United States of America*, *82*(24): 8827–8831.

Vogt, R G, Rybczynski, R., & Lerner, M. R. 1991. Molecular cloning and sequencing of general odorant-binding proteins GOBP1 and GOBP2 from the tobacco hawk moth *Manduca sexta*: comparisons with other insect OBPs and their signal peptides. *The Journal of Neuroscience*, *11*(10): 2972–2984.

Vogt, R.G., & Riddiford, L. M. 1981. Pheromone binding and inactivation by moth antennae. *Nature*, *293*(5827): 161–163.

Vogt, R.G. 2003. Biochemical diversity of odor detection: OBPs , ODEs and SNMPs. In G.J. Blomquist & R.G. Vogt (Eds.), *Insect Pheromone Biochemistry and Molecular Biology* (pp. 391–446). London: Elsevier Academic Press.

Vosshall, L. B. 2000. Olfaction in *Drosophila*. *Current Opinion in Neurobiology*, *10*(4): 498–503.

Wojtasek, H. 2002. *Molecular Mechanisms of Insect Chemical Communication: From Pheromone Biosynthesis to Perception and Degradation*. Studia I M., Poland: Uniwersytet Opolski. 108pp.

Wojtasek, H., Hansson, B. S., & Leal, W.S. 1998. Attracted or repelled?--a matter of two neurons, one pheromone binding protein, and a chiral center. *Biochemical and biophysical research communications*, *250*(2), 217–22.

Wojtasek, H., & Leal, W.S. 1999. Conformational change in the pheromone-binding protein from Bombyx mori induced by pH and by interaction with membranes. *The Journal of Biological Chemistry*, *274*(43), 30950–30956.

Zhu, J.-Y., Zhao, N., & Yang, B. 2012. Global Transcriptional Analysis of Olfactory Genes in the Head of Pine Shoot Beetle, *Tomicus yunnanensis*. *Comparative and Functional Genomics*, *2012*, 491748.

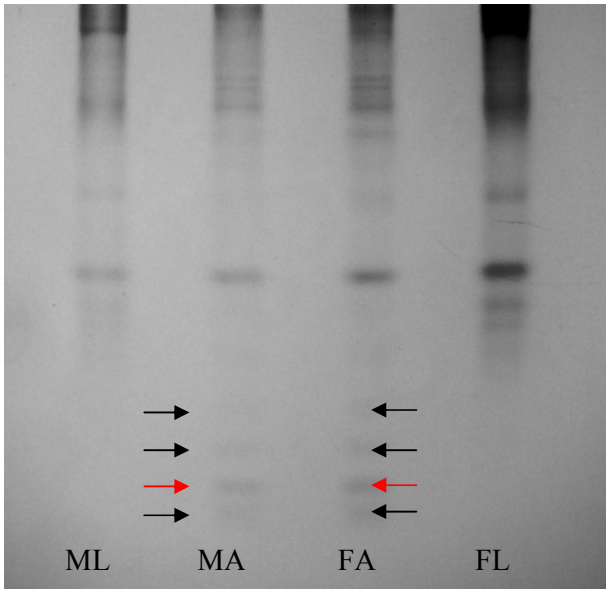

Figure 1. Separation of proteins in 15% Native-PAGE from 180 hindlegs and 500 antennaes of *H. obscurus*. Arrows indicate antenno-specific bands, red arrow indicates band sequenced by Edman-degradation technique. ML, male legs; MA, male antennaes; FA, female antennaes; FL, female legs.

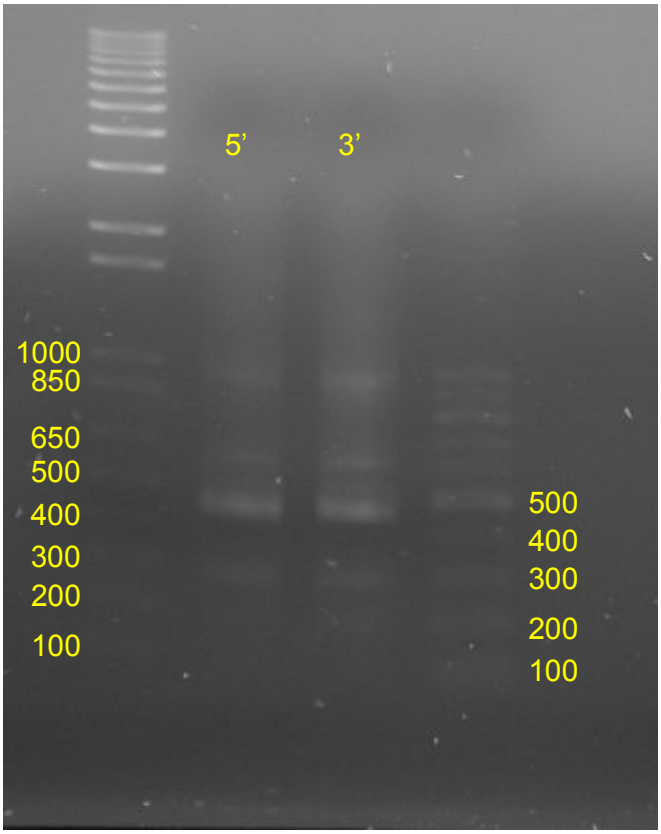

Figure 2. cDNA fragments amplified by RT-PCR with gene specific primers to actin and templates of 5’ and 3’ ends from antennae of adult *H. obscurus*. Numbers indicate weight in bp.

Chapter 6
General Discussion and Conclusions

Discussion

The microscopic study of antenna showed similar shape and types of sensilla occuping the appendices in males and females. As the antenna in other Scolytidae species (Borg & Norris, 1971; Dickens & Payne, 1978; Whitehead, 1981), these are formed by a strong scape, an articulated funicle and a compacted club which concentrate most of sensilla. While the sensilla in scapus and funicle are eminently mechanoreceptive, the club is covered by a mix of mechano- and chemosensitive sensilla forming three sensory bands. The great abundance of chaetica in the club may be related to the habits of this species, due to scolytids spend their whole life inside their host (Knizek & Beaver, 2004).

Although the chemoreceptive ability of chaetica may be proposed but not confirmed, the olfactory functions of basiconica and trichodea appear as the most probable because the TEM images coincide with those describing olfactory sensilla. This is thick walls interrupted by pores which communicate to the sensillar lumen where several dendrites are found (Klowden, 2005).

Is well known that insect antenna hold the most olfactory sensilla, however the high abundance of no olfactory sensilla in the antennal club of *H. obscurus* may be helpful to explain the difficulties to get notorious responses in the electroantennographic studies. Due to more than 40% of sensilla covering the club are designed to respond to mechanical stumuli like air movement (Keil, 1997) resulting in a high proportion of individuals which respond to all the odor stimuli (Saïd et al., 2003), and making difficult to get a larger number of reliable readings. Thus, the using of single sensillum recording (SSR) gives the possibility of isolate individual sensillum to be stimulated, and is proposed as an alternative method to avoid some artifacts that may appear in electroantennographic technique (Wibe, 2004).

The use of Solid Phase Microextraction (SPME) to collect biologically interesting volatile compounds from complex matrices has been noted as a fast (Cornu et al., 2001) and a solvent-free technique (Rochat et al., 2000), which offers cleaner chromatograms with much fewer coelution than the commonly used headspace extraction (Cornu et al., 2001). Thus, the analysis of samples by Gas Chromatographic coupled to Mass Spectrometry (GC-MS) showed the presence of six compounds in roots of red clover, and three in volatiles collected from borers. Both ethanol and hexanal are new records to the chemical relationship between red clover and clover root borer, while β-pinene was a distinguishable compound that appeared in borer volatiles but it was not found in red clover volatiles.

All the compounds found were electroantennographically active for males and females, but not all of them had the same effect in the behavior of insects in the olfactometric assays.

In the current work, ethanol had remarkable activity in electroantennographic and behavioral assays. This compound has been reported as an active kairomone to other Scolytidae species (Roling, 1975; Montgomery & Wargo, 1983; Kelsey, 2001) and their presence is reported as a cue of diseased or weak host (Miller & Rabaglia, 2009). Other compounds like limonene is mostly reported as defensive compounds elicited by biotic or abiotic stress (Thoss & Byers, 2006), which explain the repellency obtained in the bioassays. Meanwhile, The green leaves volatiles hexanal and *E*-2-hexenal (Huber et al., 2001) resulted to have opposite effects in *H. obscurus* females. The attraction observed to *E*-2-hexenal was reported by Tapia et al. (2007), but now the study with separate sexes showed that females were attracted with lower doses than males. The repellent effect of hexanal on females is opposed to those reports which notes this compound is one of the most important volatiles in conipherous bark (Vrkocová et al., 2000) and the adding of it in lures increases the catching number in a scolytid pest (Dickens et al. 1990).

In the intraspecific volatiles, the presence of β-pinene marked a difference respect to the compounds found in the red clover roots, and behaviorally was active just in males marking a difference respect to the response elicited by the remaining compounds, but the both isomers tested were active in the highest assayed doses resulting in opposite behaviors. Electrophysiological assays showed dose-dependent response with higher but mostly not significant response from males than females. The literature noted that β-pinene is involved in the chemical ecology of other scolytid pest species and is used in their monitoring as an attractant (Joseph et al., 2001; Brockerhoff et al., 2006). However, in the current study the mixes which included β-pinene were not able to produce attraction in the bioassays.

All in all, females responded to a larger number of compounds than males, due to they have the responsibility to find a suitable host and their decision has a critical consequence for the life history of the offspring (Anfora et al., 2009).

The repetitive attempts for finding evidence of antenno-specific proteins which could be related to the odors perception were not successful. Poliacrylamide gel electrophoresis showed the presence of proteins with similar migration pattern than odor-binding proteins reported to other insects. The use of degenerate primers designed from an electroblotted and sequenced protein as well as other reported for *Rhyncophorus palmarum* (Curculionidae) did not amplify any PCR product. The small sizes of antenna and the

scarce number of genes that is possible to isolate by conventional methods (Zhu et al., 2012), are named as reasons to the limited genomic resources avalaible in this group of insects, restricting the use of genomic-based information to the management of the scolytid pests (Keeling et al., 2012).

Conclusions

-The presence of pores in the wall of basiconica and trichodea sensilla of *H. obscurus* agrees with the olfactory purpose of those described in other Scolytidae species. However the high proportion of mechanoreceptive sensilla explains the difficulty to get a larger number of reliable replications in the electrophysiological study. The use of an alternative technique like single sensillum recording is proposed to improve the efficiency in this kind of studies.

- The use of SPME to collect volatiles was helpful to find volatiles possibly implicated in the chemical ecology of *H. obscurus*, such as ethanol, hexanal and β-pinene, not reported before. Their electroantennographical and behavioral effects make them interesting subject of further studies.

- All the studied compounds resulted to be active in the electroantennographical assays, but most of them produced response in females only. These responses may be related to the active role of females to locate a suitable host to their offspring.

-The scarce evidences of antenno-specific proteins in the antenna of *H. obscurus* do not allow to state the species has this kind of proteins that should participate in the odor recognition process. The limited genomic information available about the Scolytidae may be one of the main reasons to explain these results. However, the recent reports may be very useful to increase the molecular knowledge of these insects in near future.

Perspectives

Further studies should be focused in testing the new reported compounds being behaviorally active, alone in different doses or in blends with those reported before. In that way, the new findings about molecular evidence of OBPs in other Scolytidae species may be useful to speed up this research and develop an efficient alternative to the control of *Hylastinus obscurus* in the red clover crops.

References

Anfora, G., Tasin, M., De Cristofaro, A., Ioriatti, C., & Lucchi, A. 2009. Synthetic grape volatiles attract mated *Lobesia botrana* females in laboratory and field bioassays. *Journal of Chemical Ecology*, *35*(9): 1054–1062.

Borg, T. K., & Norris, D. M. 1971. Ultrastructure of sensory receptors on the antennae of *Scolytus multistriatus* (Marsh.). *Cell and Tissue Research*, *113*(1): 13–28.

Brockerhoff, E. G., Jones, D. C., Kimberley, M. O., Suckling, D. M., & Donaldson, T. 2006. Nationwide survey for invasive wood-boring and bark beetles (Coleoptera) using traps baited with pheromones and kairomones. *Forest Ecology and Management*, *228*(1-3): 234–240.

Cornu, A., Carnat, A. P., Martin, B., Coulon, J. B., Lamaison, J. L., & Berdagué, J. L. 2001. Solid-phase microextraction of volatile components from natural grassland plants. *Journal of Agricultural and Food Chemistry*, *49*(1): 203–209.

Dickens, J., Jang, E., Light, D., & Alford, A. 1990. Enhancement of insect pheromone responses by green leaf volatiles. *Naturwissenschaften*, *77*(1): 29–31.

Dickens, J., & Payne, T. 1978. Structure and function of the sensilla on the antennal club of the southern pine beetle, *Dendroctonus frontalis* (Zimmerman)(Coleoptera: Scolytidae). *International Journal of Insect Morphology and Embryology*, *7*(3): 251–265.

Huber, D. P. W., Borden, J. H., & Stastny, M. 2001. Response of the pine engraver, *Ips pini* (Say) (Coleoptera: Scolytidae), to conophthorin and other angiosperm bark volatiles in the avoidance of non-hosts. *Agricultural and Forest Entomology*, *3*(3): 225–232.

Joseph, G., Kelsey, R. G., Peck, R. W., & Niwa, C. G. 2001. Response of some scolytids and their predators to ethanol and 4-allylanisole in pine forests of central Oregon. *Journal of Chemical Ecology*, *27*(4): 697–715.

Keeling, C. I., Henderson, H., Li, M., Yuen, M., Clark, E. L., Fraser, J. D., Huber, D. P. W., Liao, N., Docking, T.R., Birol, I., Chan, S.K., Taylor, G.A., Palmquist, D., Jones, S., & Bohlmann, J. 2012. Transcriptome and full-length cDNA resources for the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, a major insect pest of pine forests. *Insect Biochemistry and Molecular Biology*, *42*(8): 525–36.

Keil, T. 1997. Functional morphology of insect mechanoreceptors. *Microscopy Research and Technique*, *39*(6): 506–531.

Kelsey, R. 2001. Attraction of *Scolytus unispinosus* bark beetles to ethanol in water-stressed Douglas-fir branches. *Forest Ecology and Management*, *144*(1-3): 229–238.

Klowden, M. J. 2005. *Physiological Systems in Insects*. Second Edition. Academic Press. 688pp.

Knizek, M., & Beaver, R. 2004. Taxonomy and systematics of bark and ambrosia beetles. In F. Lieutier, K. Day, A. Battisti, J.-C. Grégoire, & H. Evans (Eds.), *Bark and wood boring insects in living trees in Europe, a synthesis* (pp. 41–54). Dordrecht: Springer.

Miller, D. R., & Rabaglia, R. J. 2009. Ethanol and (-)-alpha-pinene: attractant kairomones for bark and ambrosia beetles in the southeastern US. *Journal of Chemical Ecology*, *35*(4): 435–48.

Montgomery, M. E., & Wargo, P. M. 1983. Ethanol and other host-derived volatiles as attractants to beetles that bore into hardwoods. *Journal of Chemical Ecology*, *9*(2): 181–190.

Rochat, D., Ramirez-Lucas, P., Malosse, C., Aldana, R., Kakul, T., & Morin, J. P. 2000. Role of solid-phase microextraction in the identification of highly volatile pheromones of two *Rhinoceros beetles, Scapanes australis* and *Strategus aloeus* (Coleoptera, Scarabaeidae, Dynastinae). *Journal of Chromatography A*, *885*(1-2): 433–444.

Roling, M. 1975. Scolytidae Attracted to Ethanol in an Oak-Hickory Forest in Missouri. *Journal of the Kansas Entomological Society*, *48*(4): 504.

Saïd, I., Tauban, D., Renou, M., Mori, K., & Rochat, D. 2003. Structure and function of the antennal sensilla of the palm weevil *Rhynchophorus palmarum* (Coleoptera, Curculionidae). *Journal of Insect Physiology*, *49*(9): 857–72.

Tapia, T., Perich, F., Pardo, F., Palma, G., & Quiroz, A. 2007. Identification of volatiles from differently aged red clover (*Trifolium pratense*) root extracts and behavioural responses of clover root borer (*Hylastinus obscurus*) (Marsham) (Coleoptera: Scolytidae) to them. *Biochemical Systematics and Ecology*, *35*(2): 61–67.

Thoss, V., & Byers, J. A. 2006. Monoterpene chemodiversity of ponderosa pine in relation to herbivory and bark beetle colonization. *Chemoecology*, *16*(1): 51–58.

Vrkocová, P., Valterová, I., Vrkoc, J., & Koutek, B. 2000. Volatiles released from oak, a host tree for the bark beetle *Scolytus intricatus*. *Biochemical systematics and ecology*, *28*(10): 933–947.

Whitehead, A. T. 1981. Ultrastructure of sensilla of the female mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera: Scolytidae). *International Journal of Insect Morphology and Embryology*, *10*(1): 19–28.

Wibe, A. 2004. How the choice of method influence on the results in electrophysiological studies of insect olfaction. *Journal of Insect Physiology*, *50*(6): 497–503.

Zhu, J.-Y., Zhao, N., & Yang, B. 2012. Global Transcriptional Analysis of Olfactory Genes in the Head of Pine Shoot Beetle, *Tomicus yunnanensis*. *Comparative and Functional Genomics*, *2012*, 491748.