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**"Biology, ecology and management of *Monochamus galloprovincialis* (Olivier),
vector of the pine wood nematode"**

**"Biología, ecología y manejo de *Monochamus galloprovincialis* (Olivier), vector
del nematodo de la madera del pino"**

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With the recent introduction of pine-wood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Bührer) Nickle (Nematoda: Aphelenchoididae), in Europe, measures to contain the disease are urgent. Human activity has proven to be the primary mechanism of spread of the disease over long distances, but knowledge of the dispersion to local scale is just as important. Knowledge of the vector population dynamics therefore plays a key role here. Knowing where the insects are going to disperse, which hosts will it colonize, how it will disperse and how far will be able to reach even when the beetles are without food, all together with a good capture device, it will enable the development of biological control techniques such as mass trapping or even the early detection of disease.

Transmission of the causing agent of the pine wilt disease, the PWN, between *Pinus pinaster* Aiton trees in Portugal is known to occur either during pine sawyer, *Monochamus galloprovincialis* (Olivier; Col.: Cerambycidae), adult feeding on twigs of healthy trees or during female oviposition on branches and trunks of dying or dead trees. Still, the disease does not affect other potentially susceptible local pine species such as *Pinus pinea* L. Several experiments evaluated the suitability of *P. pinea* as a host for *M. galloprovincialis* feeding, oviposition and progeny development. Feeding responses were first studied in two-choice experiments pairing *P. pinea* twigs with *P. pinaster*, *P. halepensis* Miller, *P. sylvestris* L., *P. uncinata* Miller, and *P. nigra* Arnold. Another test evaluated the effect on feeding of increased dosages of limonene applied to *P. pinaster* twigs. Oviposition preferences by *M. galloprovincialis* females between *P. pinaster*, with either intact or manipulated bark thickness, *P. pinea* and *P. sylvestris* were also studied in two-choice tests. Finally, suitability of *P. pinea* as a substrate for the development of *M. galloprovincialis* offspring was tested. Results showed that *P. pinea* could be an acceptable host for the pine sawyer feeding under lab conditions.

Long-distance transportation of goods containing nematode-infested beetles to distant locations constitutes the main pathway for the expansion of the disease. Management actions involving early detection and eradication under this scenario are critical to stop pathogen's spread. Although the dispersal of mature *M. galloprovincialis* have been successfully tracked down using commercial baits and traps, dispersal behavior and potential of immatures is poorly understood. Several physiological traits related to

dispersal and maturation were studied for newly emerged *M. galloprovincialis* during several shoot-feeding spans. Similarly, field survival and dispersal capabilities of immature insects were studied by mark-release-recapture studies in which the individuals were released from an area lacking hosts and recaptured in a small-sized pine stand. Results showed that insects reached sexual maturity after feeding for 12-13 days, marked by changes in their gonads. *Monochamus galloprovincialis* emerged with enough lipid and muscular fractions for sustaining dispersal, regardless of size, age or sex. Micro-CT images made it possible to create cross-sections of insects at different developmental stages, showing the wing muscular structures. Unfed adults survived 12 days on average in the lab (6-20 days). Based on the fitted negative-exponential dispersal model, immature insects were expected to disperse up to 3109 m from their release point in the middle of a crop field to a nearby pine stand. Overall, our results show that newly emerged *M. galloprovincialis* are able to sustain long flights in the field without requiring hosts for feeding. Furthermore, our results complement previous findings and can be integrated in the design and implementation of management measures in high risk areas or where PWD has been recently reported.

The spread of the PWN, is greatly constrained to the dispersal of its vectors, long-horned beetles of the *Monochamus* genus. Disease spread at global and regional scales has been mainly caused by human-mediated transport, yet at a local scale, the short- and long-distance dispersal behaviour of the beetles determine colonization dynamics. Three mark-release recapture experiments using commercial traps and lures allowed the parametrization of the dispersal kernel under two landscape fragmentation scenarios for the only known European PWN vector, *M. galloprovincialis*. The respective release of 171 and 353 lab-reared beetles in continuous pine stands in 2009 and 2010, resulted in 36 and 28% recapture rates, yet, at a fragmented landscape in 2011, only 2% of the released 473 individuals could be recaptured. Recaptures occurred as soon as 7-14 days after their release, in agreement with the requirement of sexual maturation to respond to the pheromone-kairomone attractants. Data from the first two experiments was fitted to one mechanical and two empirical dispersal models, from which the distance dispersal kernels could be computed. Derived estimated radii enclosing 50% and 99% of dispersing *M. galloprovincialis* under continuous pine stands ranged between 250-532m and 2344-3495m depending on the replicate and choice of model. Forecasted recaptures in 2011 resulted in a moderate underestimation of long-distance dispersal, probably influenced by the high degree of habitat fragmentation. In addition, trapping parameters such as the

effective sampling area (0.57-0.76ha) or the seasonal sampling range (426-645m) could be derived. Observed results, derived dispersal kernels and trapping parameters provide valuable information for the integrated pest management of PWD. Furthermore, estimated dispersal distances indicate that ongoing clear-cut measures for eradication in the European Union are likely ineffective in stopping the vectors dispersal.

Understanding dispersal behaviour of these beetles is key to a sound disease management. LiDAR techniques were used to describe habitat features that may affect distribution of sexually mature and immature individuals. Predictive models for seven dasometric variables were developed and calibrated using reference field data collected in 20m-fixed radius circular plots. A mark-release-recapture assay was carried out by placing sixty-four traps in the centers of 4 ha square cells in a regular grid and releasing 223 insects (111 immature and 112 mature) in the center of the plot. Seven stand variables at 200 x 200m scale were fitted to a GLM for analyzing beetle habitat selection. Data from recaptures was fitted to 2Dt and negative exponential empirical dispersal models, from which the distance dispersal kernels could be computed. The % of forest canopy cover (FCC) had a significant positive effect on the amount of recaptured native insects, whereas distance and mean height of the regrowth was negatively correlated to the distribution of lab-released insects. A significant edge effect for the native captures of native insects was found in the edge traps. There were not differences in habitat selection between mature and immature insects neither were in the dispersal pattern for both kind of individuals. Estimated radii enclosing 50% and 99% of immatures was 347-365m and 1445-2013m respectively and 317-324 and 1282-1561m for 50% and 99% of matures respectively. Longest tail, by the 2Dt model, estimated that 0.001% of the immature *M. galloprovincialis* would fly over 5000 m distance. Our results contribute to understanding the influence of forest structure on *M.galloprovincialis* abundance at patch-scale, which would help to develop strategies for disease control through forest management.

Traps and attractants have been optimized for the capture of *M. galloprovincialis*, increasing the possibility of developing methods of lowering its population in PWD affected areas with the aim of either eradicating the disease or containing the spread of it. In order to evaluate the effectiveness of such mass trapping campaigns, two sets of experiments were carried out in 2010 and 2013. The release of 353 lab-reared beetles in the experimental area of 2010 facilitated the evaluation of mark-release-recapture (MRR) procedures in the calculation of population abundance estimates using the POPAN formulation of the Jolly-Seber model, a pre-requisite for the assessment of mass-trapping.

Abundance estimates derived from best fitting parameters fell within one standard error of the real figures, proving the method appropriate. In 2013 four trap densities were tested in six 36 ha plots. In order to evaluate the removed proportions, the local beetle population was estimated in a contiguous 260 ha study area. A superpopulation of 21319 individuals could be calculated from the MRR data, corresponding to a rough density of 82 individuals per hectare. Evaluated trapping densities removed 4.66, 20.50, 33.33, and 59.80% of *M. galloprovincialis* population at 0.02, 0.11, 0.25 and 0.44 traps ha⁻¹ respectively, thus the estimated 95% removal would occur at 0.82 traps ha⁻¹. These results suggest that substantial reduction of *M. galloprovincialis* abundances might be achieved via mass trapping, and that this represents a very promising management method for the containment or eventual eradication of *B. xylophilus* at the areas affected by the PWD.

Con la reciente introducción del “nematodo del pino” en Europa, la necesidad de medidas para la contención de la enfermedad son de carácter obligatorio. La actividad humana ha resultado ser el primer mecanismo de dispersión de la enfermedad a larga distancia, pero el conocimiento de la dispersión de forma local es igual de importante a la hora de ver qué medidas tomar. El conocimiento de las dinámicas de la población del vector por tanto aquí juega un papel clave. Conocer cuál es su preferencia de hospedantes, cómo y cuánto se va a dispersar y a qué distancia sería capaz de llegar aun cuando se encuentre sin alimento, junto con un buen dispositivo de captura, van a posibilitar el desarrollo de técnicas de control biológico como captura masiva o una detección temprana de la enfermedad.

La transmisión del agente causante de la enfermedad del marchitamiento del pino, *Bursaphelenchus xylophilus* (Steiner y Bührer) Nickle (Nematoda: Aphelenchoididae), entre árboles de *Pinus pinaster* Aiton en Portugal se sabe que es provocado ya sea durante la alimentación de *Monochamus galloprovincialis* (Olivier; Col.: Cerambycidae) adultos en las ramas de los árboles sanos o durante la oviposición de las hembras en las ramas y troncos de los árboles moribundos o muertos. Sin embargo, la enfermedad no afecta a otras especies de pino locales potencialmente susceptibles tales como *Pinus pinea* L. Varios experimentos han evaluado la idoneidad de *P. pinea* como especie aceptable para la alimentación, oviposición y desarrollo de la progenie de *M. galloprovincialis*. Las respuestas de alimentación del vector fueron estudiadas por primera vez en dos experimentos de doble elección emparejando ramillos de *P. pinea* con *P. pinaster*, *P. halepensis* Miller, *P. sylvestris* L., *P. uncinata* Miller, y *P. nigra* Arnold. Otro ensayo evaluó el efecto sobre la alimentación de altas dosis de limoneno aplicadas a ramillos de *P. pinaster*. Las preferencias de oviposición de las hembras de *M. galloprovincialis* entre *P. pinea*, *P. sylvestris* y *P. pinaster*, este último con el grosor de la corteza intacta o reducida, se estudiaron también en un ensayo en el que se sometía al insecto a elegir entre los hospedantes emparejados dos a dos. Por último, fue probado la idoneidad de *P. pinea* como posible sustrato para el desarrollo de la descendencia de *M. galloprovincialis*. Los resultados mostraron que *P. pinea* podría ser un anfitrión aceptable para el perforador del pino al menos bajo condiciones de laboratorio.

El transporte a larga distancia de mercancías que contengan material infestado de *Monochamus* con nematodo constituye la principal vía de expansión de la enfermedad. Acciones de manejo que contemplen la temprana detección y erradicación bajo este escenario son críticas para poder detener la difusión del patógeno. Aunque la dispersión de insectos vectores maduros puede ya ser exitosamente seguida mediante el uso del cebo comercial y trampas eficaces, el comportamiento de dispersión de los insectos inmaduros todavía es desconocido. Se estudiaron varios rasgos fisiológicos relacionados con la dispersión de los recién emergidos *Monochamus* durante varios intervalos de alimentación. De esa misma manera se estudió la supervivencia y capacidad de dispersión en campo de los inmaduros mediante técnicas de marcado-liberación-recaptura. De esta manera se liberaron en un área desprovista de posibles hospedantes y se recapturaron en una pequeña masa de *Pinus pinaster* mediante trampas cebadas multiembudos. Los resultados de este estudio mostraron que los insectos alcanzan su madurez sexual después de alimentarse durante 12-13 días, lo cual se puede apreciar por los cambios en sus gónadas. *Monochamus galloprovincialis* emergieron con suficiente cantidad de lípidos y fracción muscular para mantener el vuelo de dispersión, sin afectarles el tamaño, edad o sexo.

Imágenes con Micro-CT hicieron posible la creación de secciones del insecto en diferentes estadios de desarrollo, pudiéndose observar las estructuras de musculatura alar. Los insectos sin alimentar sobrevivieron hasta los 12 días de media en el laboratorio (6-20 días).

Basándonos en el ajuste de un modelo negativo exponencial, los inmaduros pudieron dispersarse hasta los 3109m desde su punto de liberación en el medio de un campo de cultivo cercano a una pequeña masa de pinar. En conclusión, nuestros resultados muestran que los insectos recién emergidos son capaces de volar largas distancias en campo sin requerir ningún hospedante para alimentarse. Además, nuestros resultados complementan estudios previos de dispersión y pueden ser integrados en el diseño e implementación de medidas de manejo en áreas con alto riesgo o donde la enfermedad haya sido recientemente introducida.

La propagación del nematodo de la madera del pino (NMP), *Bursaphelenchus xylophilus* (Nematoda; Aphelenchoididae), se ve limitada en gran medida a la dispersión de sus vectores, los cerambícidos del género *Monochamus*. La enfermedad del nematodo del pino se ha extendido a escala mundial y regional principalmente por el accidental transporte humano. Sin embargo, a escala local, el comportamiento de dispersión de los escarabajos va a determinar la dinámica de la colonización. Tres experimentos con técnicas de marcado-captura-recaptura, utilizando trampas comerciales y señuelos permitieron la parametrización del núcleo de dispersión bajo dos escenarios de fragmentación del paisaje

para el único vector europeo conocido, *M. galloprovincialis*. La respectiva liberación en 2009 y 2010 de 171 y 353 escarabajos criados en laboratorio y liberados en un área de rodales continuos de pino, se tradujo en 36 y 28% de tasas de recaptura. Sin embargo, en 2011 en un paisaje fragmentado, de los 473 insectos liberados sólo el 2% de los individuos pudo ser recapturado. Las recapturas se produjeron como pronto a los 7-14 días después de su liberación, lo que puede ser debido a su requisito de maduración sexual para responder a los atrayentes de feromonas-kairomona. Los datos de los dos primeros experimentos se ajustaron a un modelo mecánico y a dos modelos empíricos de dispersión, de los cuales la distancia de los núcleos de dispersión pudo ser calculada. Los radios de distancia derivados de las estimaciones que engloban el 50% - 99% de la dispersión de *M. galloprovincialis* bajo rodales continuos de pino oscilaron entre 250-532m y 2344-3495m dependiendo de la réplica y la elección del modelo. Las recapturas obtenidas en 2011 dieron lugar a una moderada subestimación de dispersión a larga distancia, probablemente influenciado por el alto grado de fragmentación de los hábitats. Además, los parámetros de captura, tales como el área efectiva de muestreo (0.57-0.76ha) o el intervalo de muestreo estacional (426-645m) pudieron también ser obtenidos. Los resultados observados, ya sean los kernels de dispersión como los parámetros de captura proporcionaron información valiosa para el manejo integrado de la enfermedad del nematodo del pino. Por otra parte, las distancias de dispersión obtenidas indican que las medidas vigentes de la Unión Europea consistentes en la realización de klareos probablemente sean una medida ineficaz para detener la dispersión de sus vectores.

Trampas y atrayentes se han optimizado para la captura de *M. galloprovincialis*, aumentando la posibilidad de desarrollar métodos de reducción de su población en las zonas afectadas PWD con el objetivo de erradicar la enfermedad o contener la propagación de la misma. Con el fin de evaluar la eficacia de este tipo de campañas de trampeo masivo, dos series de experimentos se llevaron a cabo en 2010 y 2013. La liberación de 353 insectos criados en laboratorio en la zona experimental de 2010 facilitó la evaluación de la metodología de marcado-captura-recaptura (MCR) para el cálculo de las estimaciones de abundancia de la población mediante la formulación Popan del modelo Jolly-Seber, un requisito previo para la evaluación de la captura masiva. Las estimaciones de abundancia derivados de los mejores parámetros de ajuste estaban dentro del intervalo de las cifras reales, lo que demuestra que el método es adecuado. En 2013 cuatro densidades de trampas se pusieron a prueba en seis parcelas de 36 hectáreas cada una. Con el fin de evaluar las proporciones de insectos eliminados, la población de *Monochamus* locales se estimó en un área de estudio contigua de 260 hectáreas. Se calculó haber una superpoblación de 21319

individuos a partir de los datos de MCR, que corresponde a una densidad aproximada de 82 individuos por hectárea. Las densidades de trapeo evaluadas extrajeron el 4.66, 20.50, 33.33, y 59.80% de la población de *M. galloprovincialis* en 0.02, 0.11, 0.25 y 0.44 trampas ha-1, respectivamente, por lo que la eliminación de aproximadamente el 95% de captura se produciría en 0.82 trampas ha-1. Estos resultados sugieren que una reducción sustancial de la abundancia de *M. galloprovincialis* podría lograrse a través de la captura masiva, y que esto representa un método de gestión muy prometedor para la contención o erradicación futura de *B. xylophilus* en las zonas afectadas por la enfermedad del nematodo del pino.

Pine wilt disease (PWD) is caused by the pine wood nematode (PWN), *Bursaphelenchus xylophilus* Nematoda: Aphelenchoididae (Steiner et Buhrer) Nickle, and it is considered as one of the biggest threats to *Pinus* spp. forests worldwide (Vicente et al. 2012; Futai 2013). Considered indigenous to North America, *B. xylophilus* follows a saprophytic lifestyle colonizing dead or dying coniferous trees. However, its introduction to new regions worldwide (i. e. Japan, Korea, China and Taiwan in the Far East, and Portugal and Spain in Europe) has resulted in significant economical and environmental damage (Vicente et al. 2012). The first incidence of PWD was reported in Japan in 1905 (Yano 1913), but *B. xylophilus* was not identified as the causal agent of the disease until 1971, when Kiyohara and Togushige demonstrated the pathogenicity of the nematode by inoculating 25-year-old *Pinus densiflora* (Siebold & Zucc) with it, and observed the characteristic wilt symptoms (Kiyohara and Tokushige 1971). Cerambycid beetles of the genus *Monochamus* (Coleoptera: Cerambycidae) have been reported as the sole vectors of the PWN, although other members of *Bursaphelenchus* are found associated with beetles belonging to Scolytinae, Curculionidae, Cerambycidae and Buprestidae (Futai, 2015). So far, the list of PWN vectors, include *M. alternatus* in East Asia (Mamiya and Enda 1972) *M. saltuarius* in Japan (Sato et al. 1987), *M. caroliensis* in North America (Linit et al. 1983), and *M. galloprovincialis* in Portugal (Sousa et al. 2001)

In 1984, the PWN was detected in a shipment of wood from North America upon its arrival in Finland. Because of this, and in order to avoid the spread of the disease along Europe, European authorities obliged to carry out more rigorous inspections at sea ports, in particular, wood products coming from East Asia. In 1985 Finland restricted the importation of conifer chips shipped from the USA and Canada. Other Nordic countries acted similarly, and the EPPO recommended the prohibition of the import of softwood products from countries with PWN presence to Europe. Both, *B. xylophilus* and its vectors, were classified as a quarantine organism of class “A-1” by the European and Mediterranean Plant Protection Organization (OEPP/EPPO 1986). Despite the dedicated and concerted actions of government agencies, the PWD continues to spread (Zamora et al. 2015).

In 1999 PWD was observed for the first time in Europe on *Pinus pinaster* Aiton trees at two sites approximately 3 km from each other, Marateca/Pegoes and Vale de Landeira

located in the area of Setubal (Portugal;Mota et al. 1999),. The work carried out by Sousa et al. (2001) in PWN infested areas in Portugal, resulted in the capture of 69 *M. galloprovincialis*; 54% of them *B. xylophilus*; between a few hundred and a few thousand per insect. As soon as the PWN was detected, containment measures were immediately applied through the National Eradication Programme for the Pinewood Nematode Control (PROLUNG). Portuguese authorities were initially aiming at stopping the spread of the disease within an area of 30 km in radius from Setúbal. During the first years, the disease was succesfully contained within this demarcated area. Yet, the actions carried out to eradicate the PWN did not succeed. The current situation remains worrying: the whole of Portuguese territory is considered a demarcated zone since 2008, due to the absence of guarantee of the existence of free zones inside

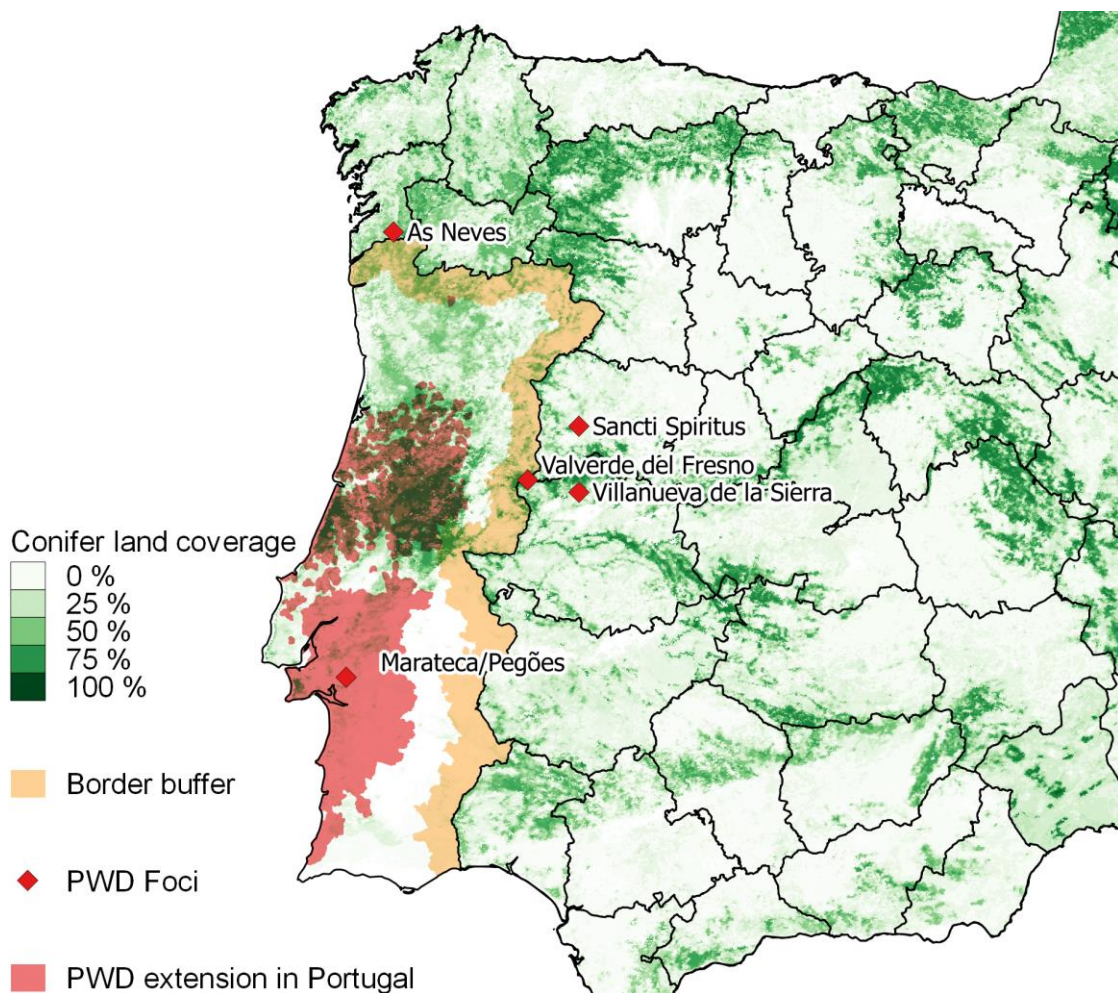


Figure 1. Distribution of PWN in the Iberian Peninsula. Infested regions within Portugal highlighted in red, and the intensive sampling border-buffer in orange. Background images represent raster images of conifer land coverage

An official contingency plan against the PWD, headed by the Spanish Ministry of Environmental Affairs in agreement with the European Union launched preventive measures in 2008. As a consequence of systematic sampling along the Portuguese border and within the national territory, *B. xylophilus* has been detected in four instances in Spain. In all cases, a small number of trees were found to hold the PWN, and eradication measures have been taken in each of the foci. The first outbreak reported was in Villanueva de la Sierra (Cáceres, Extremadura) in 2008 (OEPP/EPPO 2010a), and was recently considered eradicated. A second positive tree was found in As Neves (Pontevedra, Galicia) in 2010 (OEPP/EPPO 2010b); then in 2012, a declining *P. pinaster* tree was found at Valverde del Fresno (Cáceres, Extremadura) (OEPP/EPPO 2012a), while the last one was reported in Sancti-Spíritus (Salamanca, Castilla y León) on an isolated multi-species stand (OEPP/EPPO 2014), yet, the nematode was retrieved from samples taken on a *Pinus radiata* D. Don (Zamora et al. 2015). All four foci were located close to the Portuguese border (Figure 1). There is a clear risk of spread of the PWN into Spanish territory from Portugal, especially in the northern half of the Portuguese border, where many pine stands expand towards both sides of the frontier. The expansion of the PWN's range may come both as a result of natural spread and as a consequence of human mediated dispersal (Robinet et al. 2011). A very important timber trade occurs between the two countries, and not surprisingly, two of the PWD foci in Spain have been found in the vicinities of wood mills. Yet, although PWN might be present in the wood, *M. galloprovincialis* plays a fundamental role in the establishment of the invasive species. There is an urgent need to halt the spread of PWD in Spain, as the country serves as a preventing further spread to other European countries, where the climatic conditions, the presence of *M. galloprovincialis*, and of several highly susceptible pine species might generate catastrophic damages. Soliman et al. (2012) have recently estimated a conservative 11.9 billion € loss caused by PWD in Spain during the period 2008-2030. Successful integrated management methods of *M. galloprovincialis* may help reducing such losses.

- **Disease symptoms**

Pine wilt is a dramatic disease since it usually kills affected trees within a few weeks to a few months after infection. One of the current hypothesis on how such death may occur suggests that as nematodes multiply within the host pine trees, abnormal substances accumulate heavily around the pit membranes of tracheid cells. The blockage of the water conduction results in "tracheid cavitation", i. e. the formation of air pockets in the water transport system (Futai 2013). As the infection proceeds, the cavitations increase and ultimately lead to the destructioun of the water-conducting pathway. During this process, transpiration decreases, ultimately causing yellowing and wilting of the needles; the most visual signs of the disease. In susceptible trees, the nematodes develop into adults and translocate throughout the tree, feeding on parenchymatic cells, while they reproduce. If conditions are optimal for nematode development, internal host responses can be seen as soon as one to three days after infection (Melakeberhan and Webster 1990).

Wilting progresses downwards from the top of the tree, one of the characteristics which allow to visually distinguish PWD from other needle diseases. Needle discoloration is usually the first symptom (Figure 2). Needles first turn to grayish green and then to a tanned brown color. An additional symptom, yet difficult to detect in situ, is the amount of oleoresin exudate, as the reduction and cessation of it matches with the onset of the disease. Yet, it has been shown that the development of the disease varies environmental factors, which also modify hosts' susceptibility to PWN; high temperatures and water deficit in the growing season favors the intensity and spread of the disease (Pérez et al. 2008).



Figure 2. Trees affected by the PWD in Portugal

- **The Pine Wood Nematode: *Bursaphelenchus xylophilus***

The genus *Bursaphelenchus* comprises mycophagous nematodes, mainly distributed in the northern hemisphere (Braasch et al. 1998). *Bursaphelenchus xylophilus* (EPPO A1 list: No. 158) was first described in the USA as *Aphelenchoides xylophilus* (Steiner and Buhner 1934). Later it was described again as *Bursaphelenchus lignicolus*, when it was recognized as the causal agent of PWD in Japan (Mamiya and Kiyohara 1972). *Bursaphelenchus xylophilus* is found mainly on *Pinus* spp. and can apparently use the dead wood of most pine species as a substrate for its development. However, only a limited number of host species are susceptible to be attacked as living trees. So far, the Asian *P. bungeana*, Zucc. ex Endl., *P. densiflora* Siebold & Zucc., *P. luchuensis* Mayr, *P. massoniana* Lamb. and *P. thunbergii* Parl (in their native habitats), and the European *P. nigra* Arnold (Inácio et al. 2015) *P. sylvestris* L., *P. pinaster*, (Evans et al. 1996) or even the introduced *P. radiata* Don (Zamora et al. 2015) in Spain, are the only known species to be affected by PWD as mature trees in the field. Many other species have been found to be damaged or killed by the nematode but only under experimental conditions (mainly as seedlings in greenhouses). Other conifers can also act as hosts (primarily *Larix*, *Abies* and *Picea*) but reports of damage are rare. In addition, a few isolated cases of affected *Picea* and *Pseudotsuga* trees have been reported in the USA (Malek and Appleby 1984). Within the EPPO region, *P. sylvestris* would be the species of *Pinus* most at risk in northern and central.



Figure 3. Nematode *Bursaphelenchus xylophilus* causative of the PWD

<http://plpnemweb.ucdavis.edu/nemaplex/Taxadata/G145S1.HTM>

- Infection cycle of the pine wilt disease

One of the main characteristics of the PWD cycle is the close relationship between the developmental stages of the PWN and those of its vector beetles. A full knowledge of the relationships between these two organisms is necessary to understand the epidemic mechanism of the PWD. The nematode is transported between host trees almost exclusively by cerambycid beetles in the genus *Monochamus* (Linit; et al. 1983; Akbulut & Stamps, 2012). Other families of beetles besides *Monochamus* have been shown to carry PWN, but none has been shown to transmit it (Linit et al. 1983; Kobayashi et al. 1984; Robertson et al. 2008).

Two main phases are distinguished during the life cycle of *B. xylophilus*: the so-called propagative or mycophage phase, and the phytophagous or dispersive phase. These phases are mainly deriving from the type of transmission to the hosts: either by vector oviposition in stems of dead or dying trees during the propagative phase, or by the infection of host twigs while the vector beetles feed during the dispersion phase. The propagative phase can be held without the vectors' presence and does not serve for the purpose of dissemination. The dispersal cycle starts with the formation of third and fourth-stage *dauer* larva (DL3 and DL4), which are morphologically different from L3 and L4 stages at the propagative phase. These are considered survival stages, with high starvation resistance, whereas DL4 larvae are only able to tolerate moderate dryness. When stimulated by the presence of the vector beetle, the DL3 molts to DL4, in preparation to board the vector. As the adult beetle emerges, the nematodes migrate and settle beneath the elytra or within the trachea of the beetles, which then transports them to another host (Kikuchi et al. 2011). Distribution of *B. xylophilus* on the body of *M. galloprovincialis* was studied by Naves et al. (2006b). They found nematodes in 11% of the larvae, 17% of pupae and 91% of callow adults; the highest number of nematodes was found in the thoracic region of the beetles.

As mentioned, both dispersal stages are closely related to the development of the cerambycid vector (Mamiya 1976). When the beetles feed on branches of healthy trees, the nematodes emerge from the beetle's respiratory system and enter the trees through the feeding-wounds created by the beetles (Naves et al. 2007). On the other hand, once mature the insects will look for a dead or dying tree or trunk where to lay eggs, facilitating the establishment of the propagative phase on new hosts (Naves et al. 2007). Next, the beetle larvae feed several weeks in the cambial wood and then bore into the sapwood to overwinter. As the time of beetle emergence get closer, the nematodes aggregate around

the pupal chambers of their vectors, and when the beetle is preparing to emerge, the nematodes migrate to the beetle, closing the cycle.

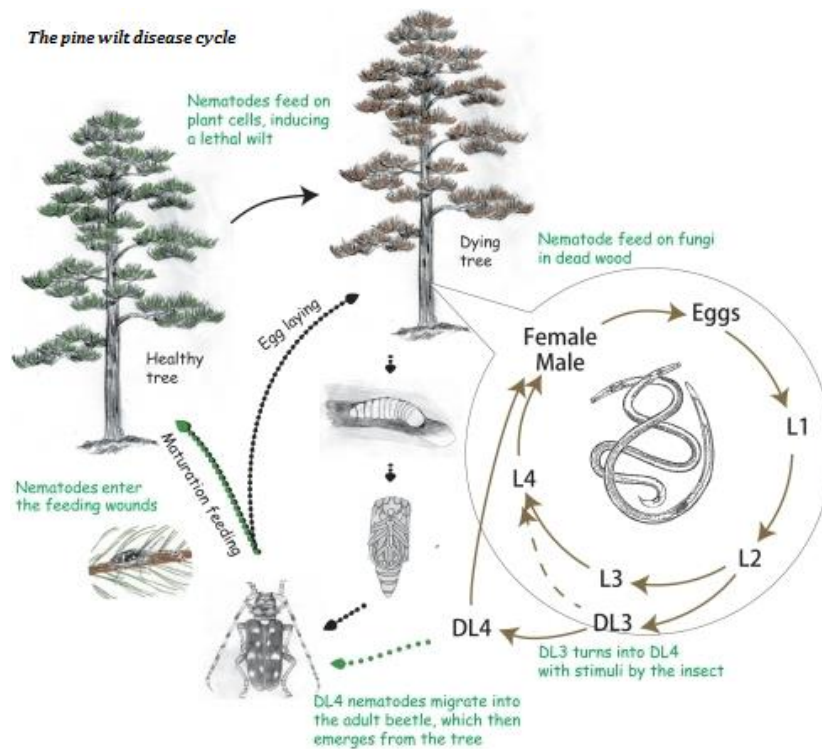


Figure 4. Interaction of the pine Wood nematode with sawyer beetles to cause pine wilt. (Kikuchi et al. 2011).

These routes of transfer demonstrate the significance of the vectors in the life cycle of PWN as well as their central role in PWD epidemics. Beetles can be loaded with high numbers of PWN. On average 15 000 and up to 289 000 were found on *M. alternatus* (Mamiya and Enda 1972). Similarly, *M. galloprovincialis* was able to transfer up to 324 000 nematodes to *P. pinaster* twigs, although the average transfer was of 25 813 (Naves et al. 2007). Total nematode load is apparently correlated with vector weight (Linit et al. 1983; Wingfield and Blanchette 1983). A study conducted with *M. alternatus* and *M. saltarius*, reported that PWN left their bodies for approximately 35 and 24 days of feeding respectively, reaching the peak scape during the second week after the emergence (Dong Soo Kim et al. 2009). Similarly, in the case of the European vector, *M. galloprovincialis*, the transmission occurs during the first 8 weeks after emergence, with transmission peaks recorded during the second and the sixth week (Naves et al. 2007). Accordingly, the knowledge of the whereabouts of the behavior of the vector beetles during this period of time plays a key role in the control of the disease.

- *Monochamus galloprovincialis*

Monochamus beetles inhabiting conifers constitute the principal confirmed and potential vectors of *B. xylophilus* (Akbulut and Stamps 2012). The genus *Monochamus* belongs to the subfamily Lamiinae within the coleopteran family Cerambycidae (Bense 1995). Of the more than 130 described species in the world, five are present in Europe, while two of them, *Monochamus galloprovincialis* (Olivier, 1795) and *Monochamus sutor* (Linnaeus, 1758), are in Spain (Figure 3). Elsewhere, *M. alternatus* Hope is the main vector in Japan, whereas *M. carolinensis* Olivier and *M. scutellatus* Say are known vectors in North America. In Europe however, *M. galloprovincialis* is to date the only vector of the disease (Sousa et al. 2001). Yet, its distribution in Europe overlaps with other *Monochamus* beetles, especially *M. sutor*, which is considered as one of the potential vector candidates if the PWD spreads northwards (Pajares et al., 2015). *Monochamus sutor* inhabit only in montane pine and fir, and in Spain was found only in the Pyrenees.

Monochamus beetles are secondary insects that can attack only weakened or dying trees (Cesari et al. 2005). In fact, under normal circumstances, they provide very important ecosystem services, especially in forest biomass, where they act as primary decomposers and initiators of wood breakdown (Vives 2000; Saint Germain et al., 2004). However, many of the *Monochamus* spp. have been recorded as having non-pathogenic *Bursaphelenchus* spp. as associates. It is supposed, therefore, that most, if not all, species would also be capable of transmission of *B. xylophilus* to a greater or lesser extent. For example, European *M. sutor* have been shown to transmit the related *B. mucronatus* (Schroeder and Magnusson 1992; Abelleira et al. 2015) and could potentially transmit *B. xylophilus* as *M. galloprovincialis* in Portugal or Spain, or as *M. alternatus* in Asia.

Monochamus galloprovincialis is distributed through the coniferous forests of Europe, North Africa, Siberia, Caucasus and Mongolia (Vives 2000). At the Iberian Peninsula and the Balearic Islands, *M. galloprovincialis* can be found in stands of most of the pine species. Notably, its presence is rare in pure *P. pinea* L. stands, while it is altitudinally excluded from *P. uncinata* Miller natural stands, where it is substituted by *M. sutor*. Despite the wide distribution, until its role as the vector of PWN was discovered, very few studies had been conducted on its bio-ecology, as it was considered a secondary insect, involved in the decomposition of wood. However, the recent events have created the demand for deepening the knowledge on the PWN vector, and hence numerous scientists dedicate to its study.

This way, the life cycle of *M. galloprovincialis* has been widely studied. *Monochamus galloprovincialis* follows an univoltine life cycle in southern Europe while it might be semivoltine in the North, adults taking up to two years to emerge (Koutroumpa et al. 2009; Akbulut and Stamps 2012). Naves et al. (2008) reported that *M. galloprovincialis* beetles started to emerge in May in Portugal, peaking during July, while the last beetles emerged in September. In Spain the adults emerge from late May to mid August (Mas i Gisbert 2015). As they emerge, beetles fly towards fresh pine branches, where they feed on twigs for about 10 to 20 days, before they reach sexual maturity (Naves et al. 2006a, 2008; Koutroumpa et al. 2008).

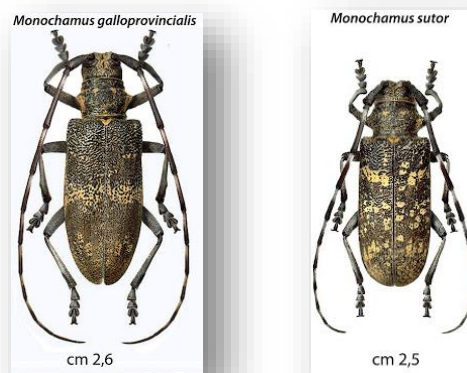


Figure 5. *Monochamus* spp. present in the Iberian Peninsula

So far immature attraction to feeding trees has not been linked to chemical attraction mediated by host terpenes (Álvarez et al. 2015b). Yet, the same study showed that immature *M. galloprovincialis* are able to physiologically respond to host cues. Similarly, lab-bioassays failed to show cross attraction between immature and mature *M. galloprovincialis* (Ibeas et al. 2008). On the other hand, chemical cues, such as host and bark beetle kairomones are used by mature beetles looking for mates or breeding grounds (Ibeas et al. 2007; Pajares et al. 2004). In addition, at least one male produced pheromonal compound mediates aggregation in *M. galloprovincialis* and other *Monochamus* beetles (Pajares et al. 2010; Pajares et al. 2013). These advances in the chemical ecology of PWN vector have helped improving the tools available for research and management of their populations.

Female *M. galloprovincialis* oviposits in bark crevices or in niches chewed through the bark, forming slit-like scars on recently dead or severely stressed trees (Naves et al., 2006b).

After hatching (Figure 6a), larvae bore under the bark, feeding on phloem. At the end of the season, they mine into the sapwood sealing the entrance with a plug of frass, while they remain within a characteristic “L” shaped gallery (Figure 6b). They spend the winter in the form pupa or imago. Diapause apparently occurs during the fourth larval instar, after which pupation occurs, ultimately leading to emergence (Koutroumpa et al. 2008; Naves and Sousa 2008).



Figure 6. (a) *M. galloprovincialis* larva recent eclosionated; (b) "L"-shaped gallery

Research on the bioecology of *M. galloprovincialis* has provided much data such as the nematode enters the vector and spread it again (Naves et al. 2007), PWN dynamics inside the vector, (Naves et al. 2006a), or reproduction of the beetle (Naves et al. 2006a, 2008; Ibeas et al. 2008; Koutroumpa et al. 2008). As new technologies develop, new possibilities to study the insects' physiology arise. X-ray computed microtomography (micro-CT) is a non-destructive powerful tool that allows visualizing details of the inner structures of beetles (Figure 7). Recently, the micro-CT with a high spatial resolution was considered as a potential tool for the morphological classification of insects (Alba-Tercedor and Caparrós 2012; Alba-tercedor 2014) and therefore is a great tool to study insect morphology (Zhang et al. 2010; Li et al. 2011). This technique allows imaging of a small specimen with spatial resolution in the range 1–50 μm , values that fit well within the dimensions of the beetle. Such potential has allowed the anatomical study the developmet of organs directly involed with the ecology and behavior of *M. galloprovincialis* in the field, such as the reproduction system (Hubweber and Schmitt 2009), flight muscles and their evolution (Bozkurt et al. 2007), energetic reservoirs in the shape of fat content or even the location of the inner organs (Zhang et al. 2010).

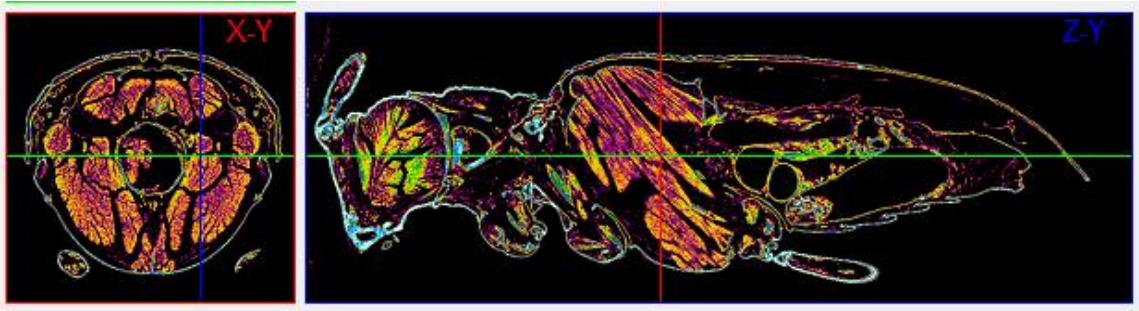


Figure 7. Three dimensional reconstruction of *Monochamus galloprovincialis* using X-ray computed microtomography.

- **Vector-host-pathogen relationship**

Monochamus beetles are secondary insects that only attack weakened or dying trees (Cesari et al. 2005). However, with the recent introduction of the PWN in Europe and Asia, the study of longicorn is considered to be helpful in clarifying the outbreaks and PWN epidemics and therefore, the knowledge of the relationship vector-host-nematode is of vital importance.

Insects host preferences show a strong heritable component and are thought to represent the suitability of hosts for larval survival (Thompson 1998). Suitability can depend upon a number of factors such as nutritional quality, host plant defense chemicals, prevalence of natural enemies or microenvironment (Thompson and Pellmyr 1991). Considering its role in the dispersion of the nematode, defining host spectrum and preference of *M. galloprovincialis* is of primary importance for forest management. *Monochamus* species are mainly *Pinus* feeders, but some may utilize *Picea* and *Abies* species as well. In Portugal, only *P. pinaster* has been found to be infested by the nematode, although other *Pinus* species (*P. pinea* and *P. halepensis* Miller) grow in the infested zone (Mota et al. 1999; Naves et al. 2006b). High resistance to PWN was reported earlier for *P. pinea* (Evans et al. 1996), although recent results have reported only moderate resistance (Santos et al. 2012). Several studies focused on the study of host preferences, reported that *P. sylvestris* L. is the most frequent host of *M. galloprovincialis* in Northern and Central Europe while its Southern and Mediterranean populations are more frequently collected on *P. pinaster* and *P. halepensis* (Francardi 2000; Naves et al. 2006b; Koutroumpa et al. 2009).

Several environmental stimuli or events will affect the behavior of the insect, such as fires, availability of host material or even forest characteristics. Landscape structure and

dynamics influence ecological processes (e.g., population dynamics, spatial distribution) of the organisms living in the landscape (Turner 1989; Wiens et al. 1993). However, mechanisms leading to habitat location by *Monochamus sp.* at larger scale are still largely unknown (Saint-Germain et al. 2004). The explosive technological advances and development in recent years enormously facilitates the detection and monitoring of pests (Work et al. 2011; Mueller et al. 2014). The importance of landscape structure and how organisms interact with spatial and temporal landscape heterogeneity has come to the forefront of ecology, and this perspective is making inroads into pest management as well. The recent incorporation of Light Detection and Ranging (LiDAR) techniques in forestry, may provide appropriate tools for the achievement of this goal. The information obtained through the LiDAR, can be related to the main variables of woodland, such as the number of trees, basal area, standing volume, the mean square diameter, dominant height, foliar biomass or branch biomass, by relating them with LiDAR readings via statistical models. The availability of such LiDAR data repositories in Spain, justifies the implementation of these new forest resource mapping techniques. This way, the habitat of *M. galloprovincialis* can be better characterized and used to improve pest management strategies.

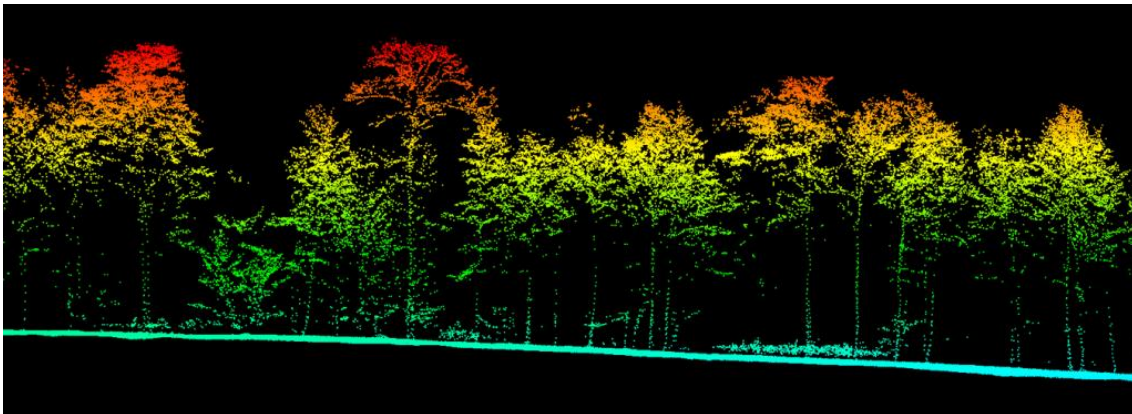


Figure 8 .Point cloud image from LiDAR

Pine Wilt Disease Management

- History and management of the PWD

Once introduced into a susceptible tree the control of *B. xylophilus* is very hard considering the options available so far. In the United States, PWD management is mainly achieved by the removal of symptomatic trees. In East-Asia, where the disease is much more devastating (Yoshimura et al. 1999), insecticide and nematicide treatments, biological control, and induced resistance using less virulent strains of PWN have been investigated. After the disease was found in Asia, the governments of the affected countries strengthened quarantine measures at ports of entry and established quarantine stations within the countries to prevent the movement of infected logs, lumber and wood products from infected to uninfected areas. In addition, clear cutting and methyl bromide fumigation of pine trees killed by the PWN were implemented too (Ikeda 1984). Traps with attractant lures for monitoring the vector were developed and insecticide sprays or trunk injections have resulted in some success. However, such insecticide have resulted in harmful side effects to the environment. In addition, nematicide and insecticide treatment of trees are regarded as impractical, very expensive and ineffective due to the extensive areas of affected forests areas (Dwinell 1997), furthermore, many of these treatments have been removed due to their non-selective nature.

A national regulatory control system is recommended to all EPPO countries for the detection, containment and eradication of *B. xylophilus*, yet allowing sufficient guarantees to export host commodities within and outside the region. The recommendations to prevent the introduction of *B. xylophilus* and its vectors cover plants and wood of all conifers, apart from *Thuja plicata*, from countries where the nematode occurs (OEPP/EPPO 2012b). It is also recommended that coniferous plants should be prohibited but that countries may choose whether to prohibit wood. If it is not prohibited, wood must have been heat treated to a core temperature of 56°C for 30 minutes. In the case of packing wood (crates, dunnage etc.), kiln drying could be accepted instead, whereas for particle wood, the alternative of fumigation is also acceptable.

Since the detection of PWN in Europe, Portugal implemented an eradication program in the demarcated areas of that country. Yet, in 2008, the PWN was found at 65 of the 2,443 surveyed Portuguese locations (Ministry of Agriculture, Portugal, unpublished report). An outbreak in 2008 in Spain led the European Union to intensify control measures,

especially in Portugal, where a demarcated area consisting of the sum of the infested area with the presence of widespread damage, was created over a belt 20 km around the border with Spain (Figure 2). Timber from Portugal must be subject to the conditions laid down in the Community Decision 2012/535 / EU.

Due to the provisions of the Implementing Decision of the Commission 2012/535 / EU, Spain has undertaken the development of a national contingency plan to prevent the introduction and spread of PWN. Adopted measures include establishing protocols for the inspection of sawmills and wood industries, commercial shipments. In each of the declared PWD foci, all coniferous hosts were eliminated within a 3 km wide clear cut belt, in order to prevent further spread. In addition, traps were installed along the outer limit of the affected zone to capture the vector during its flight period. Periodic surveys, eradication and insect vector control actions are being conducted on the whole affected and buffer zone too (known as the demarcated area).

The nematodes can move actively within the wood tissues, and thus, it can leave one piece of wood to move into an adjoining piece (Wingfield and Blanchette 1983). However, without their vectors they are incapable of moving from one host tree to another. Infested wood is the most probable way of international transport of *B. xylophilus*, and the species has been intercepted in a number of occasions on sawn wood, round wood and wood chips imported into the EPPO region from the USA and Canada (Rautaapa 1986). The most likely pathway of introduction of *B. xylophilus* is when it is imported together with the insect vectors, which may then carry the nematodes to coniferous trees in the vicinity upon emergence (Robinet et al. 2009).

In order to obtain practical information to enable the Standing Committee on Plant Health Union develop European scientific and health policy on the problem of PWN and submit them to the Commission for be incorporated as binding decisions. The spread of the disease, whether locally or worldwide, requires the study of biotic and abiotic factors that influence the disease agents (nematode and insect vector) are dispersed between stands.

- **Monitoring the vector**

Direct attempts to control the pathogen resulted impractical in large areas so the management of the insect vectors seems a better strategy for dealing with the disease. Even though the human transport of infested wood is responsible for long-distance dispersal of the disease (Robinet et al. 2009, 2011), the beetle's flight activity and dispersal is also important when considering the spreading of PWD at a local scale. With the development of effective trapping devices such as modified multiple-funnel (Lindgren 1983; Álvarez et al. 2015a), or cross-vane traps that are well suited for the catch of *M. galloprovincialis* and the development of a very attractive lure for *Monochamus* spp. (Pajares et al. 2004, 2010b; Álvarez et al. 2015b) the monitoring has now converted in an efficient managing tool. Traps provide now the possibility to be used not only for monitoring the insect vector but also for direct control of its population. The incorporation of the Teflon film seemed to be an important factor to gain highest beetle amounts while that for successful *Monochamus* spp. trapping, escape-proof dry collection cups either with insecticide or enlarged depth and Teflon film or water containing collection cups are required (Álvarez et al. 2015a).



Figure 9. Modified multiple funnel with Teflon and enlarged cup



Figure 10. Galloprotect 2D plus (α -pinene, pheromone and kairomone)

Previously, short- and long distance dispersals by PWN vectoring beetles have been studied. Yet, without an effective trapping system, used methods were quite imprecise,

resulting in few or poor results (Shibata 1986; Togashi 1990a; Fujioka 1993; Yoshimura et al. 1999; Robinet et al. 2009). A further advance in monitoring of *Monochamus* sp. occurred when the commercial *M. galloprovincialis* lure kit (Gallopsect 2D®, SEDQ S. L., Barcelona, Spain) was developed (Pajares et al. 2010a). This lure contains two bark beetle kairomones (ipsenol and 2-methyl-3-buten-2-ol) and the pheromone (2-undecyloxy-1-ethanol), and results highly attractive for both sexes of *M. galloprovincialis* (Pajares et al., 2010). These developments have in turn allowed carrying out demanding techniques such as mark-release-recapture of *M. galloprovincialis* (Gallego et al. 2012; Mas i Gisbert et al. 2013; Torres-Vila et al. 2014), which represent a very useful methodology for the estimation of the population density, the study of the vectors' dispersal behavior, or can potentially be used to provide key information of the nematode load carried by the vector, allowing early detection of infections. It might also provide a more proactive management strategy for the eradication or containment of the PWD using mass-trapping techniques or even opening up opportunities for integrating entomopathogenic fungi into PWD management (Alvarez-Baz et al. 2015).



Figure 11. Numbered *M. galloprovincialis* in a mark-capture-recapture assay

Even so, while the research has been directed towards the development of highly attractive commercial lure for mature pine sawyers, none of the tested blends were successful in attracting immature pine sawyer adults (Álvarez et al. 2015b). This represents a serious handicap when the population dynamics of immature insects need to be studied.

According to Naves et al. (2007b) the nematode transmission in *M. galloprovincialis* is more frequent during the first 6 weeks after emergence. So, this is a crucial period to be considered when the spread of the disease is analyzed, since the dispersal behaviour of immature *Monochamus* will play a key role in the dynamics of disease expansion. However, in the case of immature *M. alternatus*, these disperse by flying randomly (Togashi 1990a), whereas mature adults are strongly attracted to volatiles emitted from dying or newly killed trees (Ikeda et al. 1980). These adults move by walking or short-range flights in the pine canopy concentrating in those dying trees where they mate and oviposit (Togashi 1990a, 1990b), while that in the Iberian Peninsula a study with mature *M. galloprovincialis* seems that even if most of recaptures occurred close to the origin, some beetles could disperse at 14 km from the release point (Gallego et al. 2012; Mas i Gisbert et al. 2013). Long-distance dispersal of PWN by beetle flight and the human transportation of pine logs infested with PWN and the insect vector both accelerate the spread of the PWD (Robinet et al. 2009). Local spread of the disease from infested pine stands to surrounding, un-infested pine stands is likely caused by long-distance dispersal by beetles. Accurate estimates of the flight capacity of this insect vector are required if we are to understand and predict the spread of PWD in Europe, and for managing the spread of the PWN in European forests developing new or improved tools to track vector activity and dispersal.

On the other hand, the development of aforementioned new mapping techniques such as LiDAR, creates the possibility of producing high-resolution maps which could eventually lead to an earlier detection of pests (Mei et al. 2011). By combining LiDAR data with other information about an area's vegetation, rainfall patterns and temperature, researchers can get a detailed picture about the characteristics of the micro-habitat preferences that could influence in the dispersion of the insects (Work et al. 2011). LiDAR techniques may be an interesting possibility to study the behavior and migration of insects, of relevance to ecology, agriculture and evolution (Brydegaard et al. 2009). Therefore, LiDAR techniques could lead to obtain the level of detail needed to model the activity of pests on a landscape-scale and so, it can be relevant for planning and decision making.

Main questions

Shoot feeding and oviposition preferences (Chapter 1)

- What is the host preference in the Iberian Peninsula?
- Is *Pinus pinea* an acceptable host?
- Can limonene act as a repellent for *M. galloprovincialis*?
- Can *M. galloprovincialis* develop a progeny in *Pinus pinea*?

Physiology and dispersal of immature Monochamus galloprovincialis (Chapter 2)

- What is the *M. galloprovincialis* sex maturation period?
- Do immature beetles have enough fat content and wing muscles to undertake sustained dispersal flight?
- How long can recently emerged insects survive without eating?
- How far can a newly emerged *M. galloprovincialis* disperse in an area deprived of hosts?

Monochamus galloprovincialis dispersal under continuous and fragmented pine stands (Chapter 3)

- Which is the dispersal behaviour of *M. galloprovincialis* in a continuous stand? Which dispersal kernels describe best this behaviour?
- How far can *M. galloprovincialis* disperse? Which is the probability for long distance dispersal events?
- How is the dispersal pattern in a fragmented habitat?
- Are there differences between the dispersal behaviour of *M. galloprovincialis* between sex and size of the individuals?
- Can we parametrize the trapping effort? What is the effective sampling area of commercial traps and lures for *M. galloprovincialis*? And their seasonal sampling range?

Monochamus galloprovincialis abundance and dispersal in relation to stand characteristics (Chapter 4)

- What is the influence of stand characteristics on tracking local populations of *M. galloprovincialis*? How do densities of the PWN vector vary within a continuous stand?
- Which stand variables influence the distribution of released *M. galloprovincialis* in the pinewood?
- Do mature and immature insects have different dispersion patterns?

Monochamus galloprovincialis population reduction for PWD management (Chapter 5)

- Is mark-released-recapture a valid method to estimate absolute population densities of *Monochamus galloprovincialis*? How precise are the estimates?
- Do populations of *M. galloprovincialis* behave as open or as close? How does this affect the estimates?
- Is mass trapping an effective control method for the insect vector? What is the optimal trap density?

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Chapter 1: *Shoot feeding, oviposition, and development of Monochamus galloprovincialis on Pinus pinea relative to other pines species*

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Introduction

Pine wilt disease was detected for the first time in Nagasaki (Japan) in 1905 (Yano, 1913), but has spread since to most of Japan, east of China, Korea and Taiwan (Zhao et al., 2008). Despite the widespread pine mortality caused, it took almost seven decades for the pine wood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner et Buhner) Nickle (Nematoda: Aphelenchoididae), to be shown as the causal agent of the disease (Kiyohara T., 1971). This nematode is originally associated with dead and dying conifers of North America where it lives as a saprophyte, but can cause a fatal wilting disease in susceptible host species, especially out of its natural range, and is now considered one of the most dangerous diseases that threaten conifers worldwide. PWN necessarily requires of an insect vector for being introduced into new hosts. Species of the genus *Monochamus* Dejean (Col.: Cerambycidae), a group of woodboring beetles that colonize dead, dying, or severely stressed woody plants, are the only demonstrated vectors of the PWN (Linit, 1988).

In 1999, PWN was declared the agent causing death of *Pinus pinaster* Aiton trees in Setubal Peninsula (Portugal), some 20 Km south of Lisbon (Mota M., 1999). Studies soon demonstrated that *Monochamus galloprovincialis* (Olivier), a species widely distributed throughout Europe, and particularly in the Mediterranean region (Vives, 2000), was vectoring the disease (Sousa et al., 2001). The discovery and subsequent spread of PWN through Portugal, and its recent occurrence in Spain affecting *P. pinaster* trees close to the Portuguese border (Abelleira et al., 2011), has created great concern in Europe.

Life cycles of the nematode and its vector are closely linked (Linit, 1988; Togashi, 2008). Eggs are usually deposited in bark crevices or in egg niches chewed by female *M. galloprovincialis* through the bark and forming slit-like scars on recently dead or severely stressed trees (Naves et al., 2006b). Larvae bore under the bark, feed on phloem, and mine

into the sapwood at the end of the season. Their development is completed when they finally pupate in characteristic pupal cells located at the end of a U-form gallery within the sapwood (Bense, 1995). Juveniles of PWN aggregate in the xylem tissue surrounding these pupal cells and, after moulting to dauer larvae, climb onto newly formed callow beetles (Linit, 1988). After emergence during the following summer, imagines feed on young pine shoots in the crown of healthy trees, initially for sexual maturation, but they keep doing so for nutrition through all their life. Primary transmission of nematodes into healthy trees occurs during this shoot feeding, but transmission can also occur during oviposition into dying or dead trees, in the so called secondary transmission.

Interestingly, nematode infection and tree mortality in Portugal has been restricted to *P. pinaster*, whereas *Pinus pinea* L., which commonly occurs in the infested zone, has shown no symptoms so far (Naves et al., 2006a). High resistance to PWN had been reported earlier for *P. pinea* (Evans et al., 1996), although recent testing points towards just moderate resistance (Daub, 2008; Santos et al., 2012). Other pine species susceptible to PWN occur in the Iberian Peninsula (Naves et al., 2006b), that aided by the predominant Mediterranean climate could provide the required temperature range for disease development (Perez et al., 2008; Rutherford & Webster, 1987). Among possible reasons for trees escaping nematode infection, host rejection or avoidance by vector beetles during feeding or oviposition, or impaired or reduced progeny development may play a key role. Very few studies have been addressed to study host preferences by *M. galloprovincialis* among European pines. Naves *et al.* (2006b) found *Pinus sylvestris* L. to be significantly preferred by adult males in multiple choice feeding tests. Preference for this pine species over *P. pinaster* had been also found by Koutroumpa *et al.* (2009). Feeding on *P. pinea*, on the other hand, was reported to not differ from *P. pinaster*, *P. radiata* D. Don or *P. halepensis* Miller (Naves et al., 2006b). The same authors reported that *M. galloprovincialis* females laid significantly more eggs on *P. sylvestris* than in other tested species; on the other hand, very few eggs were laid on *P. pinea* and none of the larvae successfully developed to adult beetles. These facts suggest that even though *P. pinea* is accepted by *M. galloprovincialis* adults for feeding, it may be rejected by reproductive females. Limonene, the most abundant terpene of *P. pinea* (Santos et al., 2006), is a known feeding deterrent for other conifer insects such as *Thaumetopoea pityocampa* (Petraakis et al., 2005), *Hylobius abietis* or *H. pinastri* (Nordlander, 1990; Nordlander, 1991), and can also influence hosts choice by ovipositing *T. pityocampa* females (Tiberi et al., 1999). In relation to *M. galloprovincialis*, it has been suggested as one of the chemical compounds influencing host selection (Naves et al., 2006b), as is the case for *M. alternatus* (Fan & Sun, 2006).

In this paper we report the results of six laboratory experiments aimed to determine: (i) the preferences of *M. galloprovincialis* for *P. pinea* as a feeding substrate compared to other Iberian pines; (ii) if limonene is a feeding deterrent to *M. galloprovincialis* (iii) the relative host preferences by ovipositing *M. galloprovincialis* females; and, (iv) the suitability of *P. pinea* for the development of the *M. galloprovincialis* progeny. Gathered results are discussed and considered within the frame of pine wilt disease etiology in Mediterranean pinelands.

Materials and methods

Insects, host material and experimental conditions

Tested *M. galloprovincialis* adults were obtained from *P. pinaster* bolts of naturally infested trees in 2009 and 2010 after forests fires in the vicinity of Arenas de San Pedro (Avila, Spain) and from *P. sylvestris* bolts of Tabuyo del Monte (Leon, Spain) in 2011. Upon emergence in the lab, insects were sexed and the length of their right elytrum was measured. Insects used for feeding bioassays were kept unfed for 1-3 days before used, whereas those for oviposition bioassays were fed with *P. halepensis* twigs for 2 weeks to allow for sexual maturation. In all cases, insects were kept in individual containers. All experiments were carried out under laboratory conditions (25 °C; 15:09 L: D photoperiod).

Pine twigs of *P. pinaster*, *P. uncinata*, *P. sylvestris* and *P. nigra* trees used in the feeding experiments were collected at the clonal bank of the Spanish Ministry of Agriculture and Environment in Valsaín (Segovia; Spain). *Pinus pinea* twigs were obtained from trees at the seed orchard of the Autonomous Government of Castile and Leon in Tordesillas, (Valladolid; Spain). *Pinus halepensis* samples were collected from trees of the Meseta Norte provenance planted at Megeces (Valladolid, Spain). In all cases, used twigs were similar in age (2-3 years old), diameter (1 cm) and had been pruned from trees of similar age and characteristics. Upon collection, twigs were carried to the laboratory in plastic bags, ends were sealed with paraffin to prevent desiccation, kept at 5 °C and used within 24h of their collection. Similarly, logs for the oviposition and developmental studies were harvested from nearby localities. These were 50 cm long and between 5 and 15 cm in diameter. *Pinus pinea*, *P. pinaster* and *P. sylvestris* stands in which felling was done, were located in Valladolid, Tabuyo del Monte and Saldaña (Palencia; Spain) municipalities respectively.

Bioassay 1: Feeding preference tests between P. pinea and Pinus spp.

Two-choice tests were carried out to study *M. galloprovincialis* adult feeding preference between *P. pinea* and *P. halepensis*, *P. pinaster*, *P. uncinata*, *P. sylvestris* and *P. nigra*. Each individual was presented a pair of test twigs, 15-20 cm long and one cm in diameter, arranged vertically 10 cm apart between each other, held upright on a Styrofoam platform and within a two-litre glass jar. Pairings were set using *P. pinea* as the control twig, and one of the other species as the test twig. For each host species pairing, ten replicates were set (five males and five females). Adults were allowed to feed for 60 hours and then each twig was wrapped with plastic film and feeding wounds were outlined with a permanent marker. As two types of feeding wounds could be identified on the bark of the twigs (regular feeding, i. e. wounds that regularly reached to the phloem and irregular feeding, i.e. wounds that reached the phloem patchily), and this could have been linked with different feeding preferences, each wound type was measured and analyzed independently. After unrolling the film, outlined areas were scanned and measured using the digital image analysis software ImageTool 3.0 (University of Texas Health Science Centre, S. Antonio, U.S.A.). The amount of fed bark (mm²) was used as the response variable for the analysis of host preference.

Bioassay 2: Feeding preference test between P. pinea and P. pinaster

To test for a possible positional bias in Bioassay 1 due to pair arrangement, a false 10 cm long twig was built joining two 5 cm long twigs halves, one from *P. pinea* and another from *P. pinaster*. A metallic filament inserted into the pith connected both halves, and the central junction area was covered by parafilm. Size and bark thickness of both twig parts were as similar as possible. These false twigs were then laid horizontally on the bottom of the jars. Twenty-six replicates were bioassayed (13 with each sex). Sampling and experimental procedures were as in Bioassay 1.

Bioassay 3: The effect of limonene on feeding

To test for a possible feeding deterrent effect, three different concentrations of limonene were tested on 10 cm long *P. pinaster* twigs and 6-10 mm in diameter. The (+) isomer of limonene (97%, Sigma, St. Louis, MO), naturally occurring in the phloem of *P. pinea* (Santos et al., 2006), was dissolved in ether to give low (1 mg/g), medium (2 mg/g) and high (3 mg/g) dosages based on the dry bark weight. The low dosage corresponded to the natural concentration of limonene found in the phloem of *P. pinea* (Macchioni et al., 2003). Different solutions were topically applied to one half of the twig, whereas the other only received the solvent. As in the previous assay, the central area between both parts was

covered with parafilm. For each test dosage, twenty-six replicates were evaluated. Sampling and experimental procedures were as before.

Bioassay 4: Oviposition preferences between *P. pinaster*, *P. pinea* and *P. sylvestris*.

Two-choice bioassays were carried out to evaluate oviposition preferences of *M. galloprovincialis*. Two 60 cm long logs were paired inside plastic boxes (ca. 70×40×30 cm). The three combinations between *P. pinaster*, *P. pinea* and *P. sylvestris* initially had 15 replicates each (Pairings I to III), although by the end of the experimental period some of the replicates had to be removed as no activity could be detected on them. To account for the influence of physical features of bark on the egg-lying preference by females, paired logs were selected to be as similar as possible in diameter and total bark thickness. Although no significant differences in total diameter could be found, bark thicknesses differed significantly, even if *P. pinaster* logs had their bark previously filed down in an attempt of evening bark thicknesses (Table 1). Two extra pairings (IV and V) were set comparing *P. pinaster* intact logs with *P. pinea* and *P. sylvestris*. These extra pairings had 20 replicates initially although, as before, some were removed from the analysis due to lack of activity (Table 1). Sexually-mature unmated *M. galloprovincialis* adults, one female and two males, were released into the centre of each box, and were allowed to move freely, mate and lay eggs during 4-7 days. Fresh *P. halepensis* shoots were also placed inside the boxes for adult feeding. After adults were removed, oviposition slits were detected and bark around was excised with a scalpel so number of eggs laid, or recently hatched larvae, and slits without eggs could be tallied.

	<i>n</i>	Mean log diameter (mm) ± SEM			<i>P</i>	Mean log bark thickness (mm) ± SEM			<i>P</i>
		<i>P. pinaster</i>	<i>P. pinea</i>	<i>P. sylvestris</i>		<i>P. pinaster</i>	<i>P. pinea</i>	<i>P. sylvestris</i>	
Pairing I	14	83.07±3.34	77.07±3.01		0.19	4.14±0.45	2.61±0.29		<0.01
Pairing II	13		91.46±2.42	85.77±2.72	0.13		3.38±0.25	1.54±0.17	<0.01
Pairing III	15	65.93±6.38		67.33±6.06	0.87	3.33±0.57		1.7±0.32	0.02
Pairing IV	17	96.06±4.96	99.06±3.87		0.64	6.47±0.97	8.18±0.92		0.21
Pairing V	18	108.83±6.99		108.22±5.96	0.95	8.39±1.26		4±0.76	<0.01

Table 1. Mean and SEMs of diameter and bark thicknesses of logs used in the Bioassay 4. *P*, probability values of paired Welch's two sample t-tests.

Bioassay 5: Development of *M. galloprovincialis* progeny in *P. pinea*

In order to check whether the *M. galloprovincialis* offspring could complete its development from egg to adult in *P. pinea*, 12 logs, 50-60 cm long and ca. 10 cm in diameter, were placed in pairs within plastic boxes as above. One pair of mature *M. galloprovincialis* adults was released into each box, and was left to interact for one week. After this period, adults were removed, oviposition slits were recorded and laid eggs were left to hatch under laboratory conditions. Larval activity at each oviposition wound (i. e. frass occurrence) was tracked during the first two weeks after egg laying. Then, larval activity was recorded for the entire log, as it was not possible to assign to any particular slit. Six months after oviposition, logs were transferred to a cool chamber (5°C) for three weeks to induce diapause (Koutroumpa et al., 2008). Subsequently, development was left to resume in the laboratory to adult emergence. Upon emergence, fifteen months after oviposition, logs were debarked to check for larval entries into the xylem to pupate. Finally, logs were cut in pieces and larvae still alive within the wood were recorded.

Bioassay 6: Offspring performance in *P. pinea* vs. *P. sylvestris*

Overall performance of *M. galloprovincialis* on *P. pinea* and *P. sylvestris* logs was compared. Eight 60 cm long logs of each species, similar in diameter (11 ± 0.9 SEM and 10.5 ± 1.2 SEM cm for *P. pinea* and *P. sylvestris* respectively; $t=-0.394$, $p=0.700$), were arranged in pairs inside plastic boxes (ca. 70×40×30 cm). A couple of sexually mature unmated *M. galloprovincialis* adults was released into each box and was allowed to mate and oviposit. Egg laying slits were tracked daily and adults were removed when at least 15 slits on each log were recorded, thus no differences in the number of slits were detected between species ($F_{1,14} 1.17$, $P (>F)=0.298$). Progeny was left to develop until adult emergence, and logs were debarked and larval entries into the xylem recorded. Collected adults were measured and weighted upon emergence.

Statistical analysis

As a Welch's t-test on the feeding responses of male and female *M. galloprovincialis*, both for figures at each evaluated pine species pair and for total figures, showed no significant differences ($P > 0.05$), data was pooled for sexes. For each experimental pairing in Bioassays 1 and 2, and each treatment level in Bioassay 3, univariate differences in regular and irregular feeding were estimated using Welch's t-test for paired samples, whereas Hotelling's T^2 test (Lockwood, 1998) was used to analyze the registered response

in a multivariate fashion. In addition, a new variable vector of differences, resulting from the differences in the response between control twigs (*P. pinea*) and each *Pinus* species in Bioassay 1, was used to fit a generalized linear model (GLM) against tested species, followed by an analysis of variance. If significant effects were detected, means were separated using Tukey's HSD test, applying a Bonferroni correction to the value of α for the confidence intervals. In addition, both regular and irregular feeding responses in Bioassay 3 were fitted in a linear regression model against tested limonene dosages.

The number of egg laying wounds with or without eggs, standardized to the log bark area, as well as the percentage of laying wounds containing eggs, were used as the response variables for fitting GLMs against tested species within each pairing. Log diameters and bark thicknesses were taken into account as covariates.

Weight and elytral length of emerged *M. galloprovincialis* individuals were compared through the analysis of variance of GLMs fitting the responses against tested species for each sex and in conjunction. Similarly, the number of egg laying wounds, xylem entries and emergences tallied for *P. sylvestris* and *P. pinea* were compared also by an analysis of variance on GLMs fitted against tested species, and accounting for the Poisson error distribution. All statistical tests and calculations were performed using R statistical analysis software (The R Development Core Team, 2012).

Results

Bioassays 1 and 2: Feeding preference tests

Monochamus galloprovincialis adults did not reject *P. pinea* twigs when feeding. Yet, regularly fed areas were generally lower for *P. pinea*, although significant differences could only be shown for *P. sylvestris* and *P. pinaster* (Table 2). In the case of the irregularly fed area, except for a significant difference with *P. sylvestris*, there was no evident difference with evaluated pine species.

		Mean consumed area \pm SEM						
		Regular feeding			Irregular feeding			Hotelling's T^2
Species (n)	Tested Species	<i>P. pinea</i>	<i>P</i>	Tested Species	<i>P. pinea</i>	<i>P</i>	<i>P</i>	
Bioas. 1	<i>P. halepensis</i> (10)	64.55 \pm 20.24	36.95 \pm 17.48	0.42	44.36 \pm 16.76	38.65 \pm 14.32	0.83	0.53
	<i>P. nigra</i> (10)	63.31 \pm 36.54	53.79 \pm 25.90	0.85	55.67 \pm 16.00	85.07 \pm 49.41	0.62	0.82
	<i>P. pinaster</i> (9)	100.71 \pm 20.11	61.53 \pm 32.45	0.38	16.95 \pm 5.10	28.90 \pm 11.98	0.34	0.34
	<i>P. sylvestris</i> (8)	108.58 \pm 25.94	14.94 \pm 11.84	<0.01	301.59 \pm 99.38	1.36 \pm 1.36	0.02	<0.001
	<i>P. uncinata</i> (10)	75.07 \pm 26.04	84.08 \pm 49.02	0.88	288.03 \pm 89.09	151.24 \pm 35.86	0.23	0.34
Bioas. 2	<i>P. pinaster</i> (9)	168.92 \pm 31.86	77.54 \pm 22.28	<0.01	10.04 \pm 3.52	19.15 \pm 8.69	0.25	0.03

Table 2. Mean area consumed on twigs of tested species in feeding bioassays 1&2 \pm SEM. *P*, probability values of paired Welch's two sample t-test, and probability value for Hotelling's T^2 test for two sample multivariate test considering regular and irregular feeding.

No significant differences were found in the vector of differences of regularly consumed areas among the tested species ($F_{4, 42}$ 0.715, $P = 0.59$). On the other hand, this variable was found to differ significantly among species for irregularly consumed areas ($F_{4, 40}$ 3.99, $P = 0.008$; Figure 1).

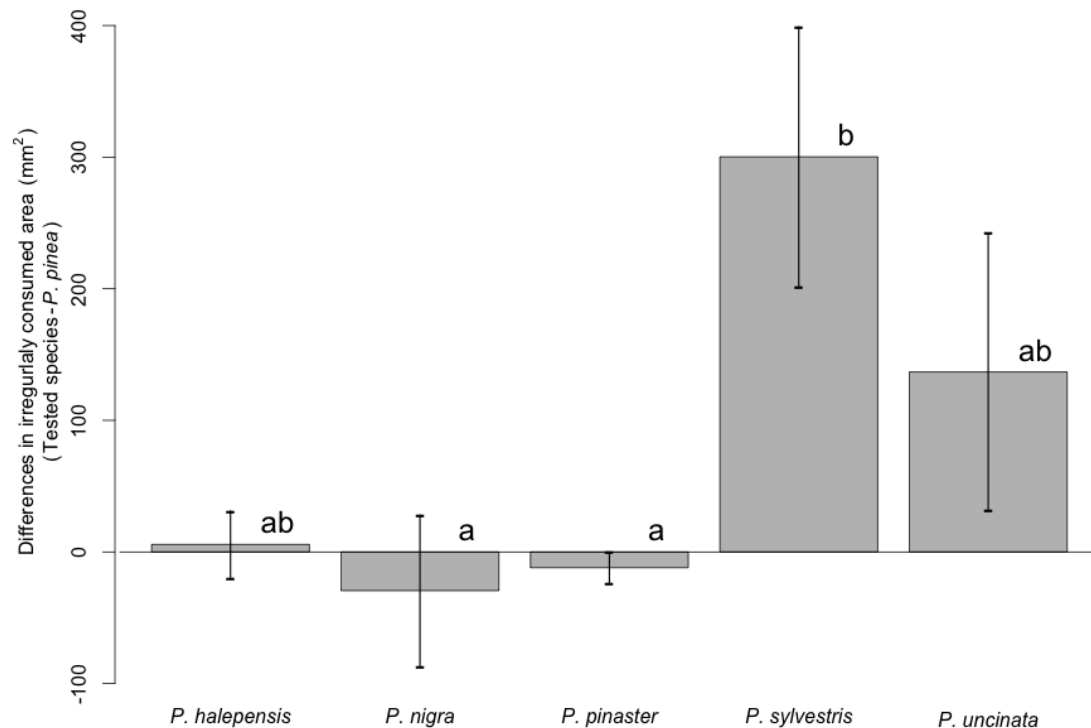


Figure 1. Pairwise differences in regularly consumed areas (mm^2) between tested pine species and *P. pinea* in Bioassay 1. Bars sharing the same letter are not significantly different (Tukey's HSD test, Bonferroni's adjustment, $P < 0.05$).

Bioassay 3: The effect of limonene on feeding

No differences were found in both types of feeding between control and limonene treated twigs, regardless of any tested dose (Table 3).

Limonene Dose (n)	Mean consumed area ± SEM						Hotelling's T^2 P
	Regular feeding			Irregular feeding			
	Test Twig	Control twig	P	Test Twig	Control twig	P	
1 mg/g DW (26)	165.66±20.27	175.46±29.11	0.69	41.72±11.09	43.24±7.89	0.92	0.95
2 mg/g DW (25)	158.76±25.38	119.28±23.30	0.24	37.22±9.05	42.78±14.27	0.72	0.48
3 mg/g DW (26)	84.18±17.01	116.54±26.90	0.21	33.52±13.06	20.75±10.64	0.46	0.35

Table 3. Mean area consumed on tested twigs in Bioassays 3 ± SEM. P, probability values of paired Welch two sample t-test. and probability value for Hotelling's T^2 test for two sample multivariate test considering regular and irregular feeding

Fed area however, decreased as the dose of limonene increased, and a significant treatment effect could be shown for limonene treated twigs ($P = 0.008$), still with a very low fit ($r^2 = 0.079$). The same trend was registered on control twigs, but the linear modeling resulted in no treatment significance and a low fit ($r^2 = 0.02$, $P = 0.11$).

Bioassay 4: Oviposition preferences between *P. pinaster*, *P. pinea* and *P. sylvestris*

The results of Bioassay 4 showed that *M. galloprovincialis* produced more oviposition wounds and laid more eggs on *P. pinea* when it was offered together with *P. pinaster* or with *P. sylvestris* (Pairings I, II and IV; Figure 2), although the difference in laid eggs could not be significantly proven when the bark of *P. pinaster* was artificially smoothed (Pairing I; Figure 2). Significantly higher number of oviposition wounds, both with and without eggs, were detected on *P. sylvestris* than on intact *P. pinaster*, but the number of eggless wounds was significantly lower on *P. sylvestris* when the bark of *P. pinaster* was manipulated (Pairings III and V; Figure 2).

	n	% Egg containing laying wounds ± SEM			F	df	P
		<i>P. pinaster</i>	<i>P. pinea</i>	<i>P. sylvestris</i>			
Pairing I	14	33.64±0.09	23.32±0.06		1.06	1, 9	0.329
Pairing II	13		55.51±0.06	65.66±0.05	2.31	1, 10	0.159
Pairing III	15	50.49±0.09		70.33±0.07	4.68	1, 11	0.053
Pairing IV	17	85.42±0.06	33.53±0.09		16.73	1, 6	0.006
Pairing V	18	68.71±0.07		53.20±0.07	24.48	1, 14	<0.001

Table 4. Percentage of egg containing laying wounds ± SEM on tested species in Bioassay 5. F, df and P; F statistic, degrees of freedom and probability values respectively, of the species factor on the analysis of co-variance of registered responses.

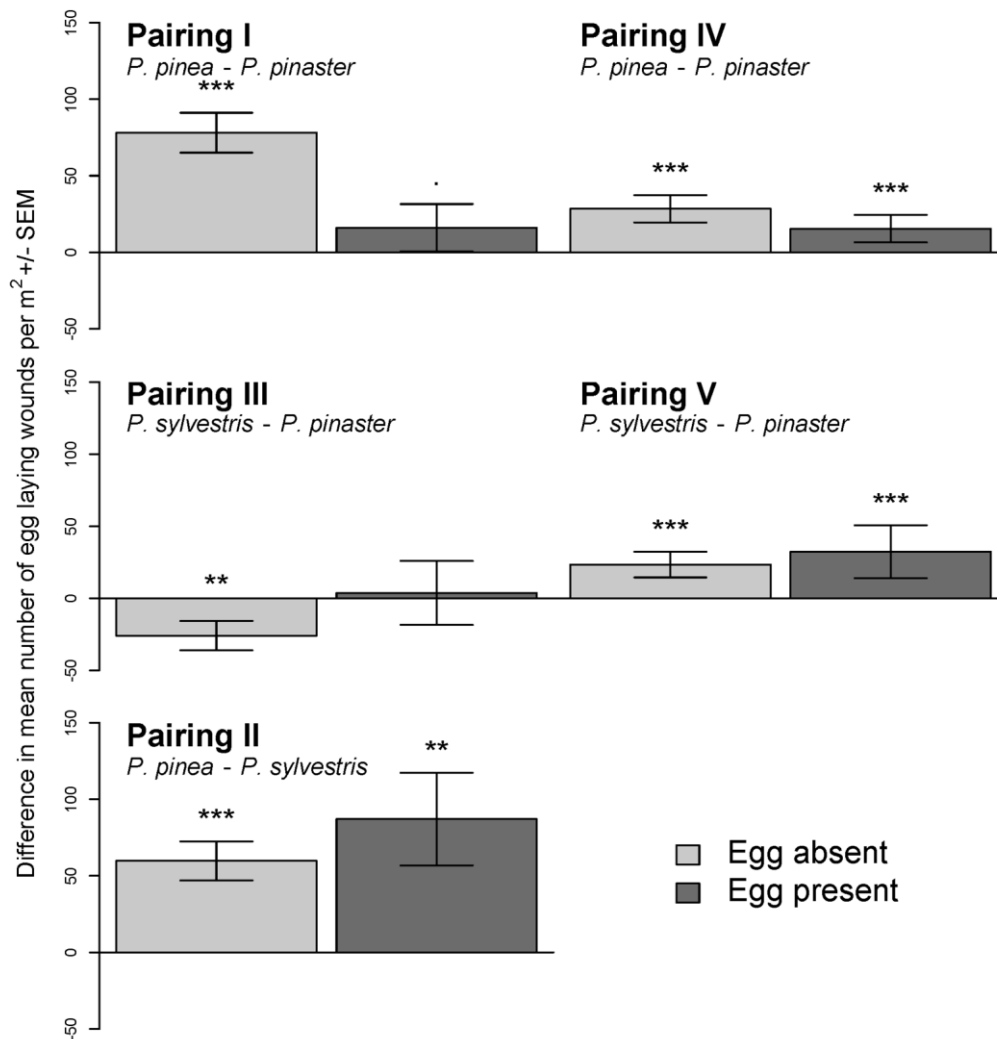


Figure 2. Pairwise differences in the mean number of egg laying wounds per m² with eggs present or absent in Bioassay 4. Asterisks over bars denote significance level for the species factor in the analysis of co-variance: . $P < 0.1$; * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$.

However, when the proportion of eggs on oviposition wounds was analyzed, *P. pinea* resulted in a less suitable host (Table 5). A significantly lower percentage of eggs per wound was detected when *P. pinea* was paired to *P. pinaster* with intact bark (Pairing IV), a difference that could not be proven in the case of manipulated bark thickness (Pairing I; Table 5). On the other hand, no significant differences were found between *P. pinea* and *P. sylvestris* (Pairing II; Table 5). Significant difference was found favouring *P. pinaster* to *P. sylvestris* on Pairing V, whereas the opposite was almost true when *P. pinaster* bark was manipulated (Pairing III; Table 5).

	<i>Pinus pinea</i>	<i>Pinus sylvestris</i>	<i>F</i>	<i>d. f.</i>	<i>P</i>
Weight (mg)	481.36±16.46	312.05±11.93	65.73	1, 84	<0.001
Female	479.85±26.20	321.48±16.60	27.18	1, 38	<0.001
Male	482.67±21.56	303.84±17.14	37.47	1, 44	<0.001
Elytral length (mm)	15.68±0.21	13.89±0.2	30.08	1, 94	<0.001
Female	16.33±0.27	14.48±0.26	19.74	1, 44	<0.001
Male	15.06±0.23	13.35±0.28	15.46	1, 48	<0.001

Table 5. Mean weight (mg) and elytral length (mm) ± SEM of *M. galloprovincialis* adults emerged from *P. pinea* and *P. sylvestris*. *F*, *df* and *P*; *F* statistic, degrees of freedom and probability values respectively, of the species factor on the analysis of variance of registered responses.

Bioassay 5: Development of *M. galloprovincialis* progeny in Stone pine

A total of 269 egg laying wounds made by *M. galloprovincialis* females were tallied on the experimental *P. pinea* logs. After one week, a mean of 25% of these wounds per log showed activity of newly hatched larvae (69 in total; Figure 3). This figure further decreased to a 23% of the initial wounds one week later. Four months later, 57 larvae (84% of hatched larvae) were found to have entered the xylem to pupate, but only 14 (23% of hatched larvae) came out to emerge as adults ten months after the onset of the bioassay. Furthermore, 7 larvae (11% of hatched larvae) were found alive within the xylem 14 months after eggs were laid.

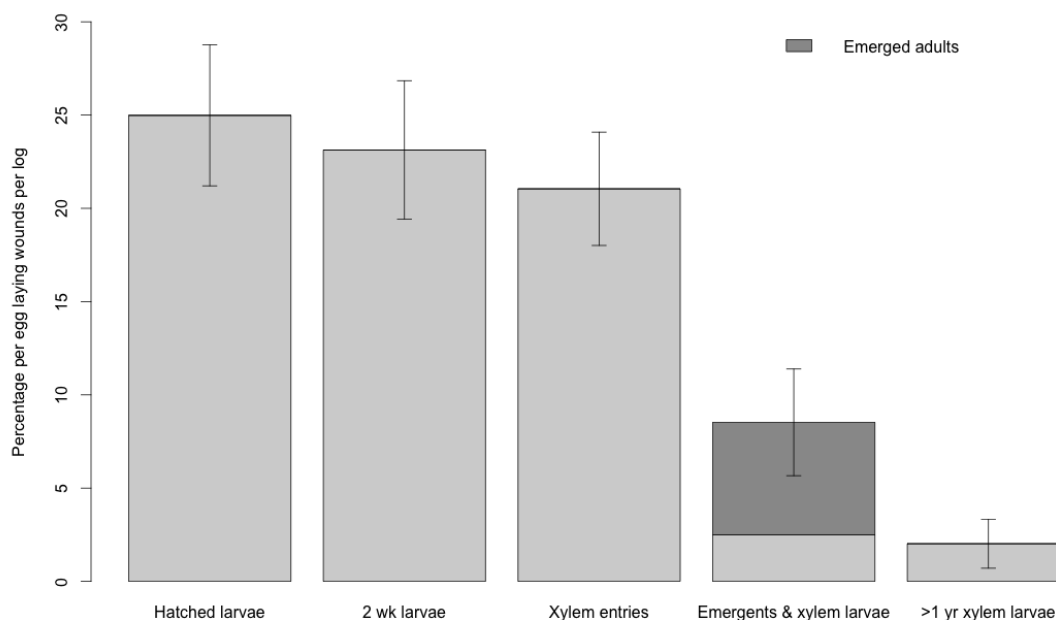


Figure 3. Mean percentages ± SEM of *M. galloprovincialis* developmental stages in relation to the number of egg laying wounds per *P. pinea* log (Bioassay 5).

Bioassay 6: Offspring performance in *P. pinea* Vs. *P. sylvestris*

The comparison of *M. galloprovincialis* offspring performance resulted on poorer figures in *P. pinea* than in *P. sylvestris* (Figure 4, Table 5). The mean number of oviposition wounds was similar in both species ($F_{1,14} 1.17, P = 0.30$), but the proportion of larvae that had entered the xylem to pupate was almost three times lower in *P. pinea* ($F_{1,14} 10.53, P = 0.006$) and, similarly, the number of emerging adults was found to be significantly higher in *P. sylvestris* ($F_{1,14} 4.87, P = 0.046$; Figure 4). Overall, only 14% of egg laying wounds resulted in the emergence of new adults in *P. pinea*, whereas 36.5% did so in *P. sylvestris*. Mortality of the progeny once within the xylem, however, was similar for both pine species, as the proportion of emergences per larval entries was not different (ca. 75%). On the other hand, adults emerging from *P. pinea* were significantly larger and heavier (Table 5) than those reared on *P. sylvestris*, regardless of their sex.

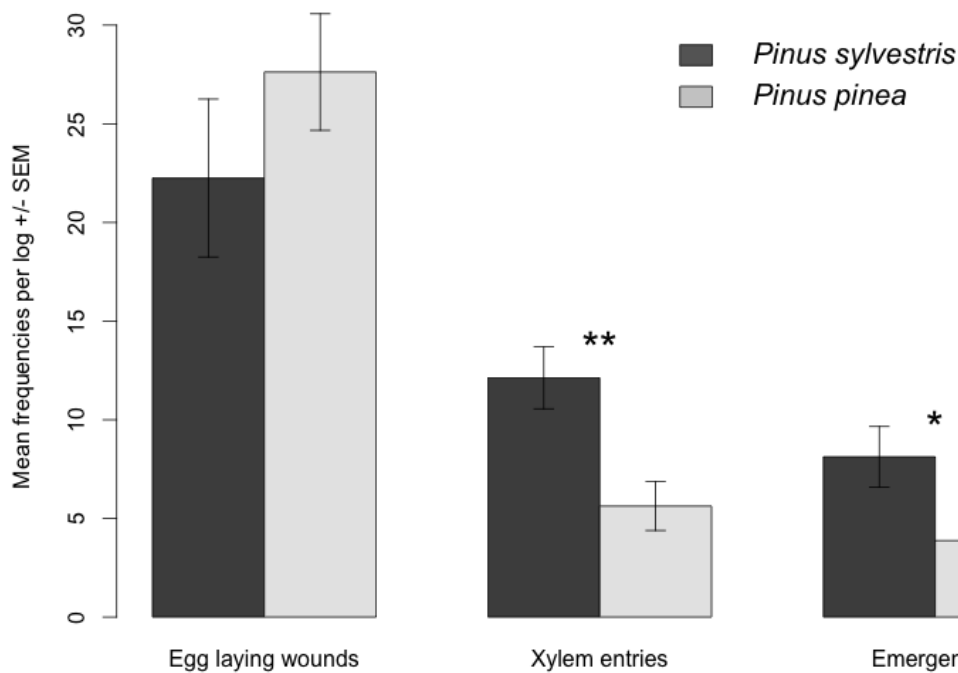


Figure 4. Mean number \pm SEM of egg laying wounds, xylem entries and emergences per *P. pinea* and *P. sylvestris* log in Bioassay 6. Asterisks over bars denote significance level for the species factor in the analysis of variance: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

Discussion

On the basis of the results presented above, although differences in magnitude could be found, it seems that *M. galloprovincialis* is able to feed, lay eggs and develop on *P. pinea*, at least under lab conditions. The analysis of the outcome of feeding bioassays showed that two patterns of feeding behavior could be recognized, as the phloem of test twigs was found to be eaten in a regular or irregular way by evaluated insects. These patterns were evaluated separately, but the analysis showed similar preferences for species, so it did not apparently represent a different *M. galloprovincialis* behaviour (i. e. irregular feeding was not linked to rejection or no-preference). Insects consumed a larger area on *P. sylvestris* twigs, as previously reported either for multiple-choice experiments (Naves et al., 2006b) or when confronted just with *P. pinaster* (Koutroumpa et al., 2009). Thus, it seems that *P. sylvestris* might be the preferred host for twig feeding by *M. galloprovincialis*. *Pinus pinaster* could be ranked as a second candidate, also shown by Naves et al., (2006b). As for the rest of evaluated species, no significant differences could be found between them and *P. pinea*. Due to its biogeographical importance, it is noteworthy to mention that no differences were found with *P. uncinata*, the main host for *Monochamus sutor* in the Pyrenees (Vives Noguera, 2001), and hence host preference can not be considered a biological barrier for the spread of PWN by *M. galloprovincialis* through the Pyrenees.

It is known that beetle preferences are mediated by chemical cues associated with hosts and non-hosts (Allison et al., 2004). Limonene, the most abundant terpene on *P. pinea*, (Santos et al., 2006), is a known oviposition deterrent for several pine-infesting insects (Nordlander, 1990), and a feeding inhibitor of *M. alternatus* (Fan & Sun, 2006). It is also known to reduce up to 40% the *M. galloprovincialis* catch in pheromone-kairomone baited traps (Pajares et al., unpub.). Even if no differences were detected between treated and control twigs, a progressive reduction in feeding was detected as the limonene dosage increased. This could indicate a plausible deterrent effect of limonene in the diet of adults, which could have also manifested in the feeding of control twigs, as the atmosphere of the experimental arena was polluted by limonene. Still, the lack of an unbiased control precludes to conclude whether if the application of a low limonene dosage, i. e. the equivalent to what has been reported for the contents of *P. pinea* (Macchioni et al., 2003), could be sufficient to deter the feeding response on *P. pinaster* and achieve a similar result to that on Bioassay 2.

M. galloprovincialis females laid eggs on all pine species tested and the results in oviposition tests did not show a clear preference towards any particular host. Still, figures showed that females of *M. galloprovincialis* did not negatively discriminate *P. pinea* as a host for reproduction over other pines. In fact, Stone pine logs received more egg laying wounds and more eggs than the other species. However, when the proportion of eggs at egg laying wounds was considered, lower values were consistently registered for *P. pinea* than for other pine species, although differences were only significant to *P. pinaster*. This fact could indicate a lack of preference as a substrate for egg laying towards *P. pinea*. (Akbulut et al., 2008) have reported between 1 to 4 eggs in *M. galloprovincialis* oviposition scars, with 8% of these containing multiple eggs. In our bioassays, percent of egg containing wounds ranked from 23,3 in *P. pinea* to 85,4 in *P. pinaster*, and not a single wound was found with multiple oviposition. On the other hand, preference between *P. pinaster* and *P. sylvestris* remains unclear, as the proportion of egg containing scars was higher on Maritime pine when its bark was not manipulated, but resulted lower when the bark was smoothed. In any case, *P. sylvestris* logs always received a higher number of eggs, significant if they were confronted to non manipulated *P. pinaster* logs. Other published studies have similarly reported *P. sylvestris* to be the most oviposited host by *M. galloprovincialis* (Koutroumpa et al., 2009; Naves et al., 2006b). Thus, it seems likely that bark thickness is a main host feature involved in *M. galloprovincialis* oviposition preferences. Interestingly, Nakamura (1995) had reported that the optimum bark thickness for *M. alternatus* oviposition was between 1-2 mm and Francardi (1996) had shown a negative correlation between bark thickness and the density of *M. galloprovincialis*. Also Naves (2006b) had suggested that *M. galloprovincialis* preference for *P. sylvestris* over other native Portuguese pines was due to its thinner bark. In our case, number of eggs were similar in thin-barked *P. pinaster* than in *P. sylvestris* (mean of 59 and 61 respectively), but much lower if thick-barked *P. pinaster* logs were presented (mean of 17,6 and 49,9 respectively).

Several laboratory studies have reported low values of within-log generation survivorship of *Monochamus* species. For example, the survival of *M. carolinensis* (Olivier) in *P. sylvestris* ranged between 6 and 15% (Akbulut & Linit, 1999; Akbulut et al., 2004). Akbulut et al., (2008) showed that between 12 and 15 % of the initial cohort completed development and emerged as *M. galloprovincialis* adults on *P. sylvestris* and *P. nigra*. The results presented above showed that the proportion of emergences per egg laying slit in *P. sylvestris* was 36.5% and 14% in *P. pinea*. A closer look to the data, revealed that peak mortality occurred between oviposition and larval entry to the xylem, i. e. during the early larval instars. The fact that almost a quarter of the hatched larvae became adults would come to demonstrate that *P. pinea* is not lethal to *M. galloprovincialis* under lab conditions,

which contradicts previous results by Naves et al., (2006b). Still a lower survivorship than in *P. sylvestris* could mean that *P. pinea* is not as good a host as the latter. In addition, even if initial colonization densities were not significantly different on *P. pinea* and *P. sylvestris*, emerged adults were bigger and heavier on *P. pinea*. These parameters have been repeatedly linked to breeding success and fitness (e. g. Anderbrant & Schlyter, 1989), and hence it seems that emerging *M. galloprovincialis* had better fitness parameters in *P. pinea* than in *P. sylvestris*, far from what could be expected. However, we should take in consideration that this may be the result of reduced larval competition in *P. pinea* due to a higher mortality of young larvae. Furthermore, even if it is generally accepted that *M. galloprovincialis* has one generation per year (Francardi, 1996), the appearance of alive larvae in *P. pinea* logs after one year indicates the possibility of cycles of variable duration, as has been shown on other pine species (Koutroumpa et al., 2008; Naves, 2008). Thus it seems possible, that most of *M. galloprovincialis* follow a univoltine life cycle, while for a small fraction of the population a two-year development might be required, also on *P. pinea* logs.

Taken together, the experiments presented above showed that all pines species tested were accepted by *M. galloprovincialis* as host for feeding, and *P. sylvestris*, *P. pinaster* and *P. pinea* as hosts for breeding, albeit at different preference levels. Remarkably, *M. galloprovincialis* fed and reproduced on *P. pinea* and, although it was not the preferred host, no particularly detrimental effects were observed. Still, this beetle has not been found in natural, pure stands of *P. pinea* after intensive trapping surveys conducted in the Northwest of Spain (Sánchez et al., unpub. results). As mentioned, *P. pinea* and *P. pinaster* co-occur in areas affect by PWN in Portugal (Naves et al., 2006a), but somehow *P. pinea* does not get infected, even if this pine is moderately susceptible to the pathogen and *M. galloprovincialis* populations are certainly present. The possibility that conducting experiments in confinement may have influenced the results of the study, since the beetles tended to attack plant species normally ignored in the field (Morewood et al., 2004), seems insufficient to explain the contradiction between field and laboratory reports regarding *M. galloprovincialis* biology on *P. pinea*. Further research should focus on the unravelling of the traits of *P. pinea* that could explain this elusive question and provide managers with information that could be exploited both in breeding and silvicultural management programs aimed to increase resistance to the PWN.

Acknowledgments

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Chapter 2: Physiological development and dispersal ability of newly emerged *Monochamus galloprovincialis* (Coleoptera: Cerambycidae).

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Introduction

The pine sawyer *Monochamus galloprovincialis* (Olivier, Coleoptera, Cerambycidae) is the only known European vector of *Bursaphelenchus xylophilus* Steiner & Buhrer (Nickle, 1970). This pathogenic nematode, known as the pine wood nematode (PWN), induces the Pine Wilt Disease (PWD), a major cause of conifer mortality in Eurasia (Mota et al., 1999; Sousa et al., 2001; Futai, 2013). Prior to the introduction of the disease into Europe, *M. galloprovincialis*, was regarded as a secondary forest pest that merited little attention. However, the obligate phoretic association of the PWN during its dispersal has transformed it into a priority species. Recent spread of the disease throughout Portugal and the detection of four foci in the bordering regions of Spain (Zamora et al., 2015), has increased the demand to understand the vector's ecology.

Healthy hosts are infected with the PWN when *Monochamus* spp. feed on the shoots of susceptible trees. Although adult beetles feed on shoots throughout their life span, initially for sexual maturation after emergence and later for maintenance, most of successful PWN transmissions occur during the first 49 days after beetles have emerged (Naves et al., 2007). Vector dispersal is thus a key feature for nematode spread and thus for PWD management. As is the case in many invasive species, active or passive dispersal mechanisms of dispersal can be considered for the vector. Recent findings indicate that *M.galloprovincialis* adults usually disperse actively over rather short distances within pine stands, though some individuals may disperse over much longer distances, particularly in fragmented habitats (Etxebeste et al., 2015). On the other hand, passive dispersal of *Monochamus* larvae in nematode-infested wood may result in the establishment of the pathogen in geographically distant areas. This way accidental transport by humans has carried the beetles and the nematodes over very long distances (Robinet et al., 2009, 2011). In fact, human activities involving the movement of wood products are regarded as the single-most important factor in spreading PWN (Robinet et al., 2009). Once in destination

ports, warehouses or timber processing facilities, the emerged nematode-infested immatures will disperse in search of hosts and potentially spread the disease to new areas.

Dispersal and reproduction depends mainly on the ability of *M. galloprovincialis* individuals to explore and colonize new suitable resources. Shoot feeding is regarded as a requirement for sexual maturation, manifested with the development of gonads and mature eggs. Even though the genital structure of *M. galloprovincialis* has already been described (Tomminen & Leppanen, 1991), the gonad development sequence is not fully understood. Similarly, the time required to reach sexual maturity is assumed to range from 15 days (Álvarez et al., 2015; Sanchez-Husillos et al., 2015) to 3 weeks (Koutroumpa et al., 2008; David et al., 2013, 2015), but has not been studied in detail. Additional shoot feeding by adult *M. galloprovincialis* may contribute to further development or recovery of wing muscles as well as for the accumulation of lipids for further dispersal events (Zera & Denno, 1997). Furthermore, an energetic trade-off between dispersal and reproductive capacities has been described for *M. galloprovincialis* and other species (Zera & Denno, 1997; Ronce, 2007; David et al., 2015). Gonad development and other physiological changes during the initial days of feeding of model insects might affect their dispersal abilities (Wang et al., 2014). David et al. (2015) recently reported that newly emerged *M. galloprovincialis* were capable of flying in a flight mill, and that the reproductive maturity of females had no effect on the distance flown. Thus, detailed observation of the physiological development of these beetles as they shoot feed and mature might help understanding the dispersal capabilities of immature pine sawyer beetles.

The dispersal range of mature *M. galloprovincialis* has recently been studied in laboratory and field experiments. Flight mill records have shown that mature beetles are capable of flying accumulated distances of about 16 km (David et al., 2013), whereas field studies involving mark-capture-recapture (MCR) techniques have reported maximum flight distances between 8.3 and 13.6 km in fragmented landscapes (Gallego et al., 2012; Mas i Gisbert et al., 2013; Etxebeste et al., 2015). However, the dispersal ability of newly emerged adults has been only studied by means of 10 min flights in a flight mill (David et al., 2015). Classical field experiments based on MCR techniques could not be used, as immature beetles failed to respond to any of the known attractants for mature beetles. Several studies have shown that freshly emerged adults released in a pine stand did not respond to the standard pheromone-kairomone blend until at least 2 weeks after release (Etxebeste et al., 2015; Sanchez-Husillos et al., 2015). This span was therefore interpreted as the time required for the beetles to mature and respond to the attractants, which include a male produced pheromonal component (Pajares et al., 2010). Though antennal response of immature

beetles to several host terpenes was recorded, field trials in a pine stand or an area deprived of competitive hosts failed to attract these adults to terpene-baited traps (Álvarez et al., 2015).

Further accumulation of knowledge regarding the survival, physiological development and dispersal behaviour of immature *M. galloprovincialis* will be very relevant for developing PWD management strategies. In order to address these issues, several experiments were carried out to quantify (1) physiological changes related to (a) gonad development, (b) lipid content, (c) wing muscle content and (d) survival in immature *M. galloprovincialis* adults during shoot feeding after emergence; and (2) the dispersal behaviour of newly emerged unfed immature beetles over areas deprived of hosts in comparison to mature beetles that had been feeding for at least 15 days.

Materials and methods

Insects, feeding material and experimental conditions

Monochamus galloprovincialis individuals were obtained from *Pinus sylvestris* Ait. bolts field colonized by wild-parents during early summer in 2011, 2012 and 2013 in Tabuyo del Monte (42°15'N, 6°25'W; Castile and Leon, Spain) attracted to the logs by commercial *M. galloprovincialis* lures (Gallopprotect 2D®, SEDQ SL, Barcelona, Spain). Colonized logs were transferred to an outdoor cage at the end of the summer, and stacked in racks until the following season. The cage was checked daily for emergences between May and August. Upon collection, *M. galloprovincialis* individuals were transferred to the lab, sexed, weighted and length of the right elytrum was measured; after, each beetle was kept in an individual container. In the case that planned experiments required insects to be fed, *Pinus pinaster* Aiton twigs were provided to each individual. Insects fed ad libitum. Indoor experiments were carried out under laboratory conditions (25 °C; 15:09 L: D photoperiod).

Gonad development

After 0, 4, 8, 10, 12, 14 and 18 days of feeding, a total of 140 insects (10 females and males at each sampling occasion) were dissected out. For each individual, the presence of oocytes and eggs in the ovarioles and the ovaries respectively in the case of females, and the colour of testes in the case of male individuals were recorded under a binocular microscope (10X-23X).

Lipid content

Lipid contents were determined following the protocol presented by Anderbrant (1988). *Monochamus galloprovincialis* individuals were sampled after 0, 4, 8, 14 and 18 days of feeding (n=117 females and 94 males). Beetles were handled individually, killed by freezing, dried at 60° C for 24 h and weighed dry. Lipid extraction consisted of macerating the insect in a petroleum ether (60-71°) solution for 24 hours at 38° C. After the extraction, beetles were dried for additional 24h at 60° C, and weighed again for the fat-free value. Mean lipid content was calculated as the percentage of the ratio between fat (dry weight minus fat-free weight) and the initial dry weight.

Wing muscle content

X-ray computed microtomography (micro-CT) was used for observing wing muscles. This recently proposed imaging method for studying muscular structures is entirely non-destructive and does not require slicing the target insect (Zhang et al., 2010; Li et al., 2011; Alba-tercedor, 2014). Micro-CT imaging was applied to describe wing muscle development for individuals (one each) based on a cellular length scale after 0, 4, 8 and 18 days of feeding. Micro-CT images were obtained with a Skyscan 1172 Micro-CT high resolution scanner (Bruker MicroCT, Kontich, Belgium).

Lipid-extracted insects were used to determine wing muscle content (n=211). The pterothorax fraction of the insect was excised from the body using micro-scissors. Guided by the 3D images obtained from the micro-CT, a binocular microscope (10X-23X) was used to carefully remove the main muscles of the pterothorax (Pringle, 2010): the direct (dorso-longitudinal) and indirect flight muscles (dorso-ventral) and the pleural inserted in the wing esclerites. Leg, pleurocoxal and trochanter muscles were not removed. The pterothorax fraction was weighed and macerated in a 10% KOH solution at 40°C until only the sclerotized parts remained. Pterothorax dry weight was measured again after extraction and wing muscle content was determined as % of pterothorax dry weight.

Beetle survival

Twenty-nine newly emerged adults (12 males, 17 females) were placed in 2l glass jars and individually fed with *P. pinaster* twigs until their weight was stabilized (24 days). At the same time, 10 newly emerged insects (5 males, 5 females) were left unfed until death. All beetles were checked daily. All beetles were weighed regularly (once every 1-3 days) and the age in days at beetle death was recorded.

Dispersal of newly emerged immature beetles

In order to test dispersal capacities of recently emerged *M. galloprovincialis*, two similar experiments were conducted in Cervatos de La Cueva (42° 18' 10'' N, 04° 45' 58'' W, 849 m. a. s. l., Castile and Leon, Spain) during two consecutive years (July 25th to September 30th 2011, and from June 18th to October 9th 2012). The experiments consisted of releasing unfed, freshly emerged (maximum 2 days before release) *M. galloprovincialis* individuals in a crop area with no host trees, except for two adjacent small (2.56 and 4.51 ha) *P. pinaster* stands. It was hypothesized that immature insects would need to disperse to these pine stands, where they would feed, become sexually mature and eventually, be attracted to baited traps in the stand. Released beetles were individually marked using numbered bee tags (Opalith Plättchen, Christian Graze KG, Germany), glued to their pronotum. A total of 434 immature beetles of similar weight (359.51± 10.28 mg males and 359.45± 10.15 mg females) were released at distances of 100, 250, 500, 750, 1000 and 1500m (in 2012) and 1000, 1500, 2000, 2500 and 3000 m (in 2013) away of the nearest pine stands' edge (Table 1 and Fig 1). At each forest patch, multi-funnel traps (Econex SL, Murcia, Spain) were installed, 12 and 14 at the southern and northern patch respectively. Traps were baited with the standard commercial lure for *M. galloprovincialis* (Galloprotect 2D®, SEDQ S. L., Barcelona, Spain). Lures were replaced every 6 weeks and traps checked every other day. The distance flown by the insects was estimated as the distance from the release point to the nearest pine tree in the trap stands (i. e. stands' edge). As a positive control, 169 mature beetles (two to three weeks old, lab reared beetles) were also released as controls from the same locations.

	Distances (m)	100	250	500	750	1000	1500	2000	2500	3000	Total
Immature (n=434)	F	12	13	13	17	40	37	16	23	20	191
	M	20	17	18	15	41	42	33	27	30	243
Mature (n= 169)	F	7	7	7	8	15	17	3	6	8	78
	M	8	10	9	7	14	15	12	9	7	91

Table 1 Number of immature and mature *M. galloprovincialis* males and females released at different distances from the trap stand

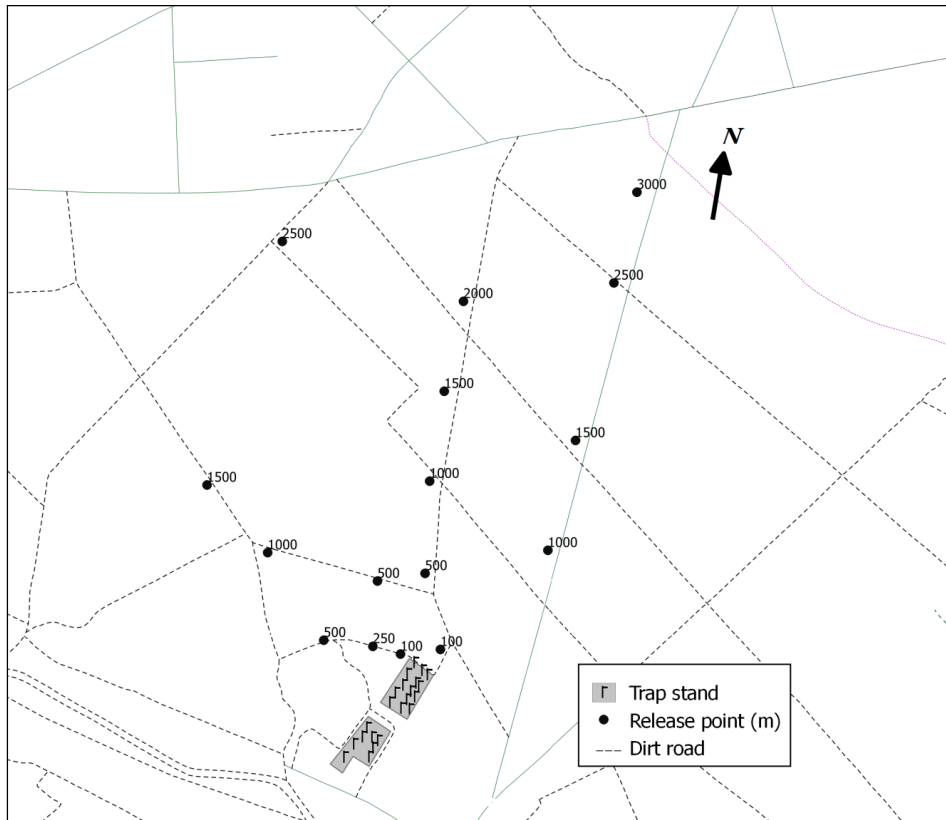


Figure 1 Localization of trap stands and release points in the *M.galloprovincialis* dispersal experiments. Release points are labelled with the distance to the nearest pine tree in the stand

Statistical analyses

One-way ANOVA was used to analyze the size differences between sexes. Lipid and wing muscle contents as % of total or pterothorax dry-weight respectively, were fitted against feeding time (number of days), size and sex factors using a quasi-binomial error distribution in a generalized linear model (GLM; Crawley, 2007). Tukey's HSD test with Bonferroni adjustment to the value of $\alpha= 0.05$ was used for post hoc mean comparisons. A survival analysis was performed using censored data of fed and unfed beetles with time to death data. Welch's t-test was used to analyze differences in survival time between sexes. Data from the two experiments on dispersal of immatures were pooled for analysis. Dispersal of immatures was approached by fitting a negative exponential linear model for the frequency of recapture proportion per released insect for each distance, modified as the logarithm ($x + 1$), and the logarithm of flight distance, pooled for sexes and experimental years. These variables could not be analyzed separately due to low recapture numbers. The intercept of the modeled function with the x-axis was considered an estimator of the maximum distance that a beetle can fly (Östrand & Anderbrant, 2003). In addition, a possible difference in the dispersal behavior of mature and immature beetles was tested by

including the maturation state factor within the same modeling strategy and regressing pooled recapture data for both beetle classes. All statistical analyses were performed using the R 3.2.2 statistical programming environment and language (The R Development Core Team, 2015).

Results

Gonad development

Female *M. galloprovincialis* emerged with 6 pairs of empty elongated ovarioles (Figure 2a). By the 12th day of feeding, the first oocytes had been observed in almost all females and eggs had appeared in 20% of females (Table 2).

Feeding days	Female gonads		Male gonads		
	Oocytes	Eggs	White	Light Yellow	Dark yellow
0	0	0	100		
8	0	0	90	10	
10	0	0	90	10	
12	70	20		40	60
14	100	90			100
18	100	100			100

Table 2 *Monochamus galloprovincialis* female (n=70) and male (n=70) gonad development (%) after different feeding spans

Two days later, almost all females (90%) had eggs in their ovaries (Figure 2c). Newly emerged males on the other hand, had two pairs of white circular testes (Figure 2d). After 8 days of feeding, signs of maturation (yellowing of testes) were apparent in only 10% of males, but four days later, more than half the males appeared to have completed maturation, shown by the dark yellow coloration of testes (Table 2; Figure 2f). After 14 days of feeding, all males presented mature testes. If these characters are taken as proxies of maturation, 50% of the individuals reached maturity after $11.96 \pm 0.001SE$ and $12.78 \pm 0.05SE$ days of feeding for males and females, respectively.

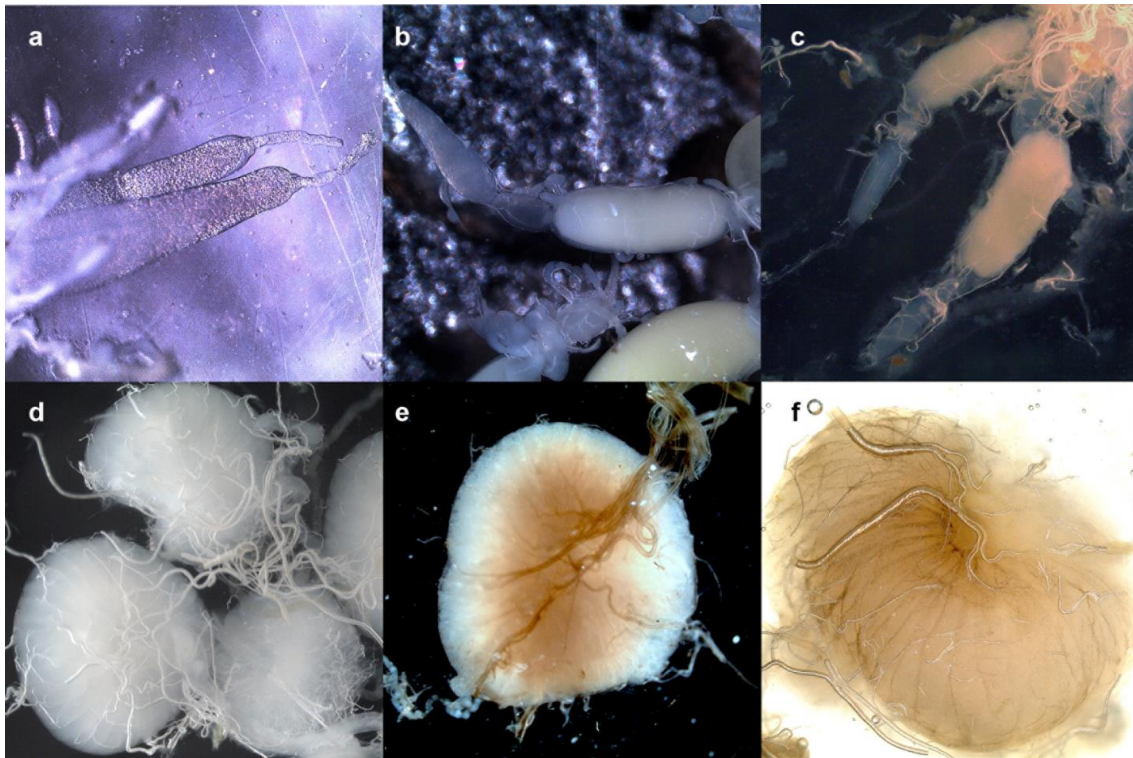


Figure 2 Gonad development in *M. galloprovincialis*. a to c, pictures of ovarioles of female *M. galloprovincialis* taken 0, 8 and 14 days after emergence, showing the presence of oocytes already at day 8. d to f, change in coloration of male *M. galloprovincialis* gonads.

Lipid content

Preliminary analysis showed that adult size was significantly affected by sex ($F_{1,341}=24.35$, $P<0.001$); correspondingly, mean elytral length in females was 15.28 ± 0.3 mm, while males' measured 13.91 ± 0.4 mm on average. The proportion of lipid content was not significantly affected by elytral length ($F_{1,186}= 0.205$, $P=0.651$), yet, it was positively correlated with insect dry weight ($r= 0.69$, $t = 12.97$, $P<0.001$). Mean lipid content was not significantly affected by sex ($F_{1,186}=0.001$, $P= 0.980$), or number of feeding days ($F_{1,188}=0.306$ $P=0.581$; Figure 3a). Lipid content remained fairly constant at around 11% of dry weight in males and 10% in females, though a slight increase (1-3%) was observed after the first days of feeding, particularly in males.

Wing muscle content

Micro-CT scanning provided crisp spatial imaging of the thorax, leg and wing muscles as well as the abdomen with the reproductive system and eggs was clearly visible, as can be seen in Figure 3. The A-A' section shows the cross-section of dorso-longitudinal wing muscles (Figure 4, upper row) whereas the B-B' section shows the cross-section of the dorso-ventral wing muscles (Figure 4, lower row). Lack of replicates precluded statistical

analysis, but observation revealed that unfed, freshly emerged females had a well-developed wing muscle array like that of fed females.

The proportion of pterothorax muscle content presented no significant differences based on sex ($F_{1,135}=0.519$ $P=0.690$) or size ($F_{1,135}= 0.913$ $P= 0.341$). Muscle content averaged $33.37\pm4.9\%$ and $31.21\pm4.6\%$ of pterothorax dry weight in males and females, respectively (Figure 3b). In newly emerged adults, wing muscles accounted for $23.83\pm4.2 \%$ (males) or $29.91\pm6.8\%$ (females) of pterothorax dry weight. These proportions did not change significantly after feeding for females ($F_{4,62}=0.739$, $P=0.569$) or males ($F_{4,65}=1.266$, $P=0.292$), which corroborated the 3D Micro-CT observations.

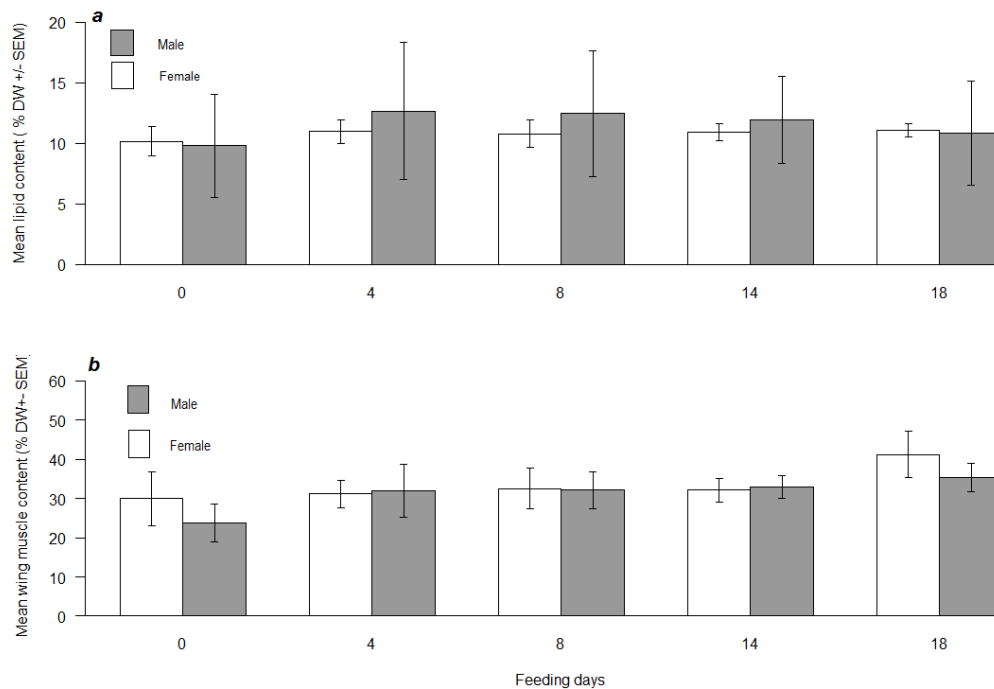


Figure 3 a, Mean fat content % (\pm SEM) and b, Mean wing muscle content % (\pm SEM), of newly emerged *M. galloprovincialis* adults after different feeding spans.

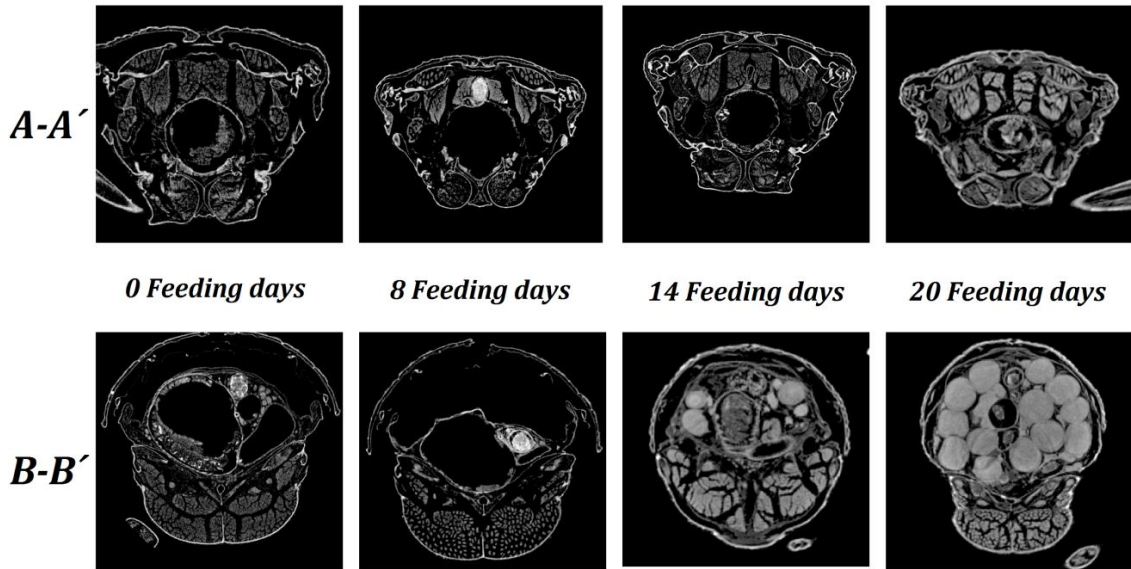
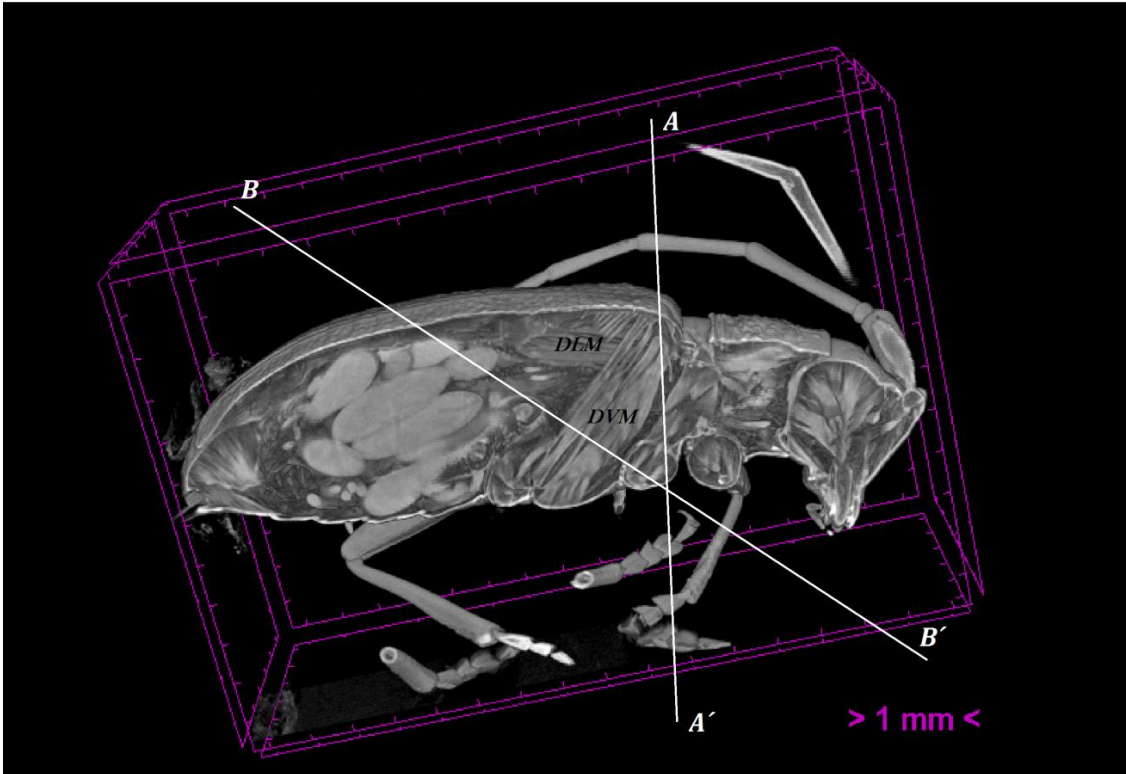


Figure 4 CTvox volume rendering reconstruction of mature *M. galloprovincialis* female, showing the cross-section axis (upper). Cross-section of dorso-longitudinal (Dlm) (A-A' row) and dorso-ventral (Dvm) (B-B' row) wing muscles after different feeding spans (lower).

Beetle Survival

Fed adults steadily gained weight for 14-16 days, after which the weight stabilized at around 30% and 49.3% increase over initial weight for males and females, respectively (Figure 4). Female weight increased by 23.8% during the first 4 days of feeding, then more slowly by 10% more through day 11, then rose sharply by another 24% through day 16, concurrent to egg development in the ovaries (Table 2). Weight increment in fed males was 11.2% after 3 days, and then rose steadily during the first 2 weeks of feeding. Unfed males and females behaved similarly and steadily lost 31.1% and 38.2% of their body weight, respectively, from emergence to death. Most fed insects (83.3% males and 70% females) lived to the end of the experiment, though 18% mortality occurred between the Day 6 and Day 8. Unfed males died 4 to 17 days after emergence, while females did so 11 to 20 days after emergence. Mean survival time for unfed insects was 10.0 ± 2.07 and 14.8 ± 1.74 days for males and females respectively, but this difference was not statistically significant ($t=1.77$, $p=0.115$).

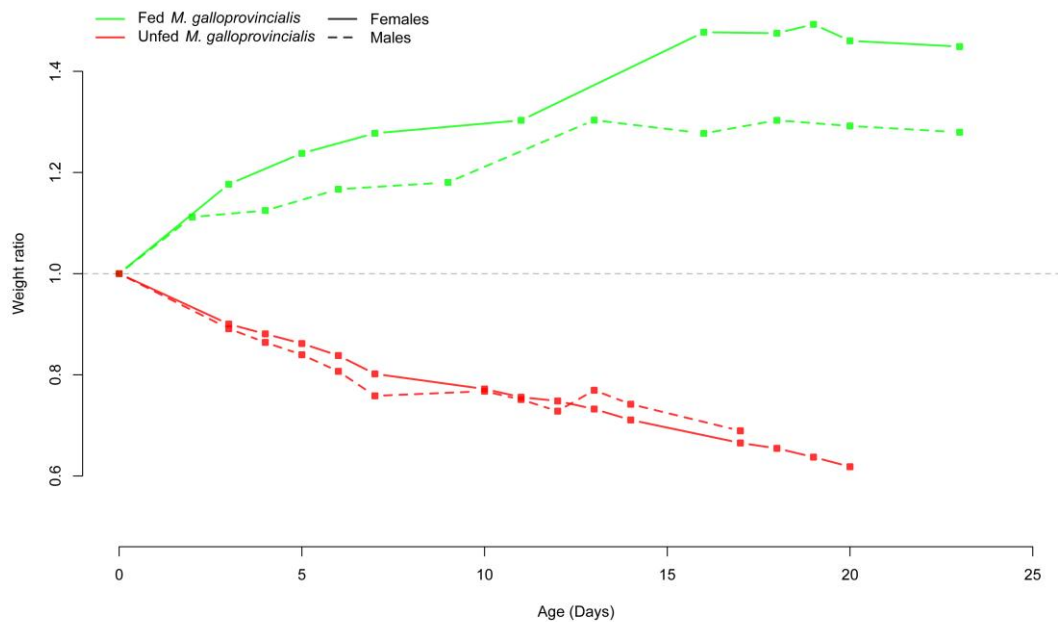


Figure 5 Weight ratio fed and unfed of newly emerged *M. galloprovincialis* adults over time.

Dispersal of newly emerged immature beetles

A total of 57 immature (13.13%) and 24 mature (14.20%) insects were recaptured in the trap stands. Earliest recapture occurred as soon as 4 days after release for mature insects (one male) and after 14 days for immature insects (3 males and 6 females), confirming that these insects must feed to mature for nearly 2 weeks before responding to

the pheromone-kairomone attractants. The last recapture of an immature individual (a male) occurred 98 days after release at the last sampling occasion, whereas the last mature beetle (a female) was recaptured after 53 days. Recaptured immature adults had been originally released over a range of 100 to 2000 m away of the stands, while matures were so from 100 to 1500 m (Figure 6). A high proportion of adults released close to the trap stands were recaptured, indicating that at least 28.1-40% of immature and 60-17% of mature adults released at distances of 100 or 250m from the stands were able to reach them. The numbers of beetles that were recaptured in the stands decreased as the release distance increased. Still, 11 (13.92%) immature adults released at 1500 m and 3 (6.12%) released at 2000 m were recaptured after 14 and 21 days, respectively. The fitted linear model was: $(\text{Recapture rate}+1) = \exp^{3.80 - 0.001*\text{distance}}$. This model estimated 3109 m (1868-5727 m 95% CI; $r^2= 0.843$, $P< 0.001$) as the maximum dispersal distance for unfed immature beetles.

No mature beetles released at 2000 m were recaptured (Figure 6), but this difference with immature beetles could be related to the number of released mature beetles, which were intended to act as positive controls. Accordingly the analysis of the pooled recaptures, i. e. mature and immature beetles, showed that the inclusion of the maturation state in the model did not significantly improve it ($F_{1, 15} 0.95$, $P=0.345$).

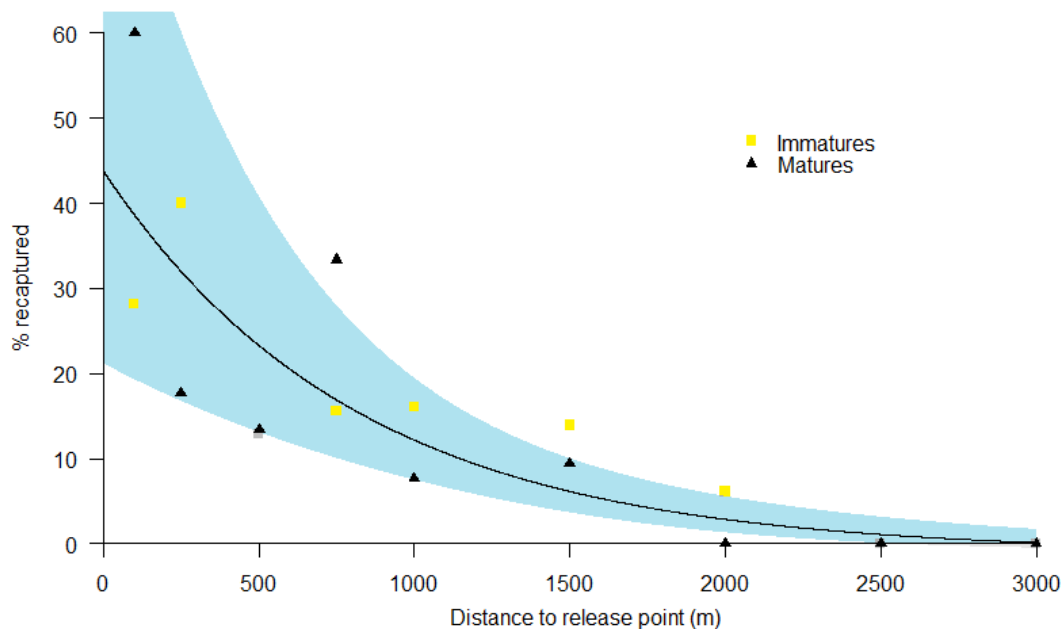


Figure 6 Relative recapture frequencies of mature (yellow rectangles) and immature (black triangles) *M. galloprovincialis* adults released at different distances from the trapping stands. Shown data was pooled from 2102 and 2013 experiments.

Discussion

Physiological development during maturation

Monochamus galloprovincialis females depend on shoot feeding for sexual maturation. They presented immature gonads at emergence and matured to produce eggs after 12-13 days of feeding. Recently, David et al. (2015) reported that most *M. galloprovincialis* females had no eggs until 10 days after emergence, which is in accordance to our findings. Egg production quickly increased after this period, as reflected in the sharp female weight gain observed after Day 11 (Figure 5). Similarly, male testes were completely mature at about 12 feeding days, with 10% showing full maturity after only 8 days. These results indicate that sexual maturation in *M. galloprovincialis* takes longer than the 6 days estimated for *M. alternatus* (Togashi, 1997), but is within the range of 9.8 (at 25° C) and 15.9 days (at 20° C) described for *M. saltuarius* (Togashi 1998).

Our results for the pre-oviposition period align with the 20.4 day span from emergence to the first egg laid reported by Naves et al. (2006) for *M. galloprovincialis*. This would imply that, even if *M. galloprovincialis* females have eggs formed in their ovaries as early as two weeks after emergence, they may require an additional week for copulation and egg fecundation before the onset of oviposition. Females would only respond to the male aggregation pheromone once they are mature, not earlier than about 12-14 days after emergence, as has been shown in behavioural and field assays involving the recapture of released immature beetles (Pajares Alonso et al., 2010). Since males become mature a little earlier than females, one could think that they should also start responding earlier than females to the aggregation pheromone, but such trend could not be established from our results.

Monochamus galloprovincialis females were significantly larger than males, in agreement to what has been reported previously in Portugal (Naves et al., 2007, 2008). Togashi (1997) found a positive correlation between the size of *M. alternatus* females and oviposition rate. David et al (2015), however, found no correlation between emergence weight and the number of eggs present in *M. galloprovincialis* females during the first month. After studying several arthropod taxa, Lease and Wolf (2011) concluded that lipid content increase is proportionate to body size and that females have relatively higher lipid reserves than males. In our study, however, proportion of body lipid content was neither

sex- nor size-dependent. Furthermore, the lipid proportion (about 10% of body dry weight) of freshly emerged males and females remained the same through maturation feeding. Insects can access other compounds for usable energy, however, which are chemically modified from the lipids. These include glycerol, other carbohydrates or glycogen: a glucose polysaccharide that serves as a form of energy storage. The lipid content of emerging insects may affect their capacity for dispersal (Coppedge, 1994). Judging by the results of the dispersal experiments, the lipid contents in recently emerged *M. galloprovincialis* seems high enough to allow for sustained dispersal before they find a new host for feeding.

The Micro-CT technique proved very useful in facilitating non-destructive, 3D observations of *M. galloprovincialis* wing muscle structures. This method, however, is highly dependent on available resources and has only been used to study wing muscles in a few cases (Zhang et al., 2010). Though the small number of insects examined did not allow statistical analysis, 3D imaging of wing muscle development showed that newly emerged insects had well-developed wing muscles that would potentially enable sustained dispersal flights (Figure 4).

Well-developed flight muscles are essential for insect dispersal. Flight muscle size may respond to biotic and abiotic factors, such as food availability or population densities and competition, and may range from as much as 65% of body weight to nearly zero in species that have temporarily or permanently lost the ability to fly. Wing muscles in newly emerged *M. galloprovincialis* adults accounted for 25-30% of pterothorax dry weight and 13% of total body weight, which lies within the range required for an insect to be able to fly (Marden, 2000). However, recent findings have shown that the distance flown by immature *M. galloprovincialis* in a flight mill was positively correlated to the thorax reserves, not to the proportion of wing muscle (David et al., 2015). Similar to the lipid fraction, pterothorax muscle content was neither sex nor size dependent and did not significantly increase after feeding. Even if neither lipids nor pterothorax muscles increased, male and female beetles gained around 30% and 50% of their emergence weight, respectively, during 24 days of shoot feeding. These values are higher than the 28% (both sexes together) reported by David et al (2015). Yet, the increase in body weight could be partially explained by the fact that ether washing does not extract compounds such as undigested food, egg content, abdomen proteins, glycogen and other carbohydrates that accumulate through feeding (Stadler, 1969).

Unfed immature beetles began to die as soon as 4 days after emergence. The longest surviving males and females died at 2.5 and 3 weeks of age respectively, when they had lost

nearly 40% of their body weight. Most nourished adults, on the other hand, survived till the end of the assay. Naves et al. (2006) reported 61.2 days and 64.0 days mean longevity for *M. galloprovincialis* lab-reared males and females, respectively. Mean longevity under field conditions is not yet precisely known, but recaptured beetles released in pine stands immediately after emergence in three different experiments survived in the field for an average of 33.8, 44.1 and 46.8 days; while the oldest was recaptured when it was 105 days-old (Etxebeste et al., 2015). Even if immature adults do not survive long without food, they would live long enough to successfully transmit the pine wood nematode to susceptible hosts. In laboratory experiments, nematode transmission by *M. galloprovincialis* adults to cut twigs occurred from emergence through the 8th week and peaked from weeks 2 to 6 (Naves et al., 2007). Similarly, Togashi (1985) found in Japan that a large proportion of nematodes left *M. alternatus* bodies between 10 and 40 days after emergence, with the peak exit period between days 20 to 35. When nematode escape from *M. alternatus* and *M. saltarius* was studied in Korea, Dong Soo Kim et al., (2009) found these values to average 34.9 ± 12.4 and 23.9 ± 16.2 days after emergence, respectively.

Dispersal of immature beetles

Newly emerged, immature *M. galloprovincialis* adults were able to disperse over an area deprived of food and fly to distant pine stands. Results from the lipid and wing muscle study suggest no apparent sex difference in dispersal, but recaptures were insufficient for proper analysis. Similarly, David et al (2015) reported no sex-biased differences for immature beetles exercised in a flight mill for 10 min. As expected, a higher proportion of the beetles reached the stands when released at a short distance (≤ 250 m) than when freed from larger distances. Even so, 6.12% were able to travel at least 2 km to find the host stand. The fitted model estimated that some emerging immatures would fly up to 3.1 km in an environment deprived of hosts. However, it is important to note that these figures may in fact underestimate the flying capacity of beetles. Firstly, both trap stands were located south of the release points and there were no other stands to trap beetles that may have randomly dispersed in other directions, as has been reported for immature *M. alternatus* (Togashi (1990a;b). Secondly, actual distances traveled by recaptured beetles are unknown. The assay only measured the minimum distance that the beetle had to fly to reach the nearest pine, but it is likely that most beetles did not follow a straight trajectory and thus flew extra distances to find the pine stands. We do not know the time spent by the beetles travelling these distances, since they had to feed for about two weeks before being recaptured. Some of them must have reached the pine stand the same day they were released, such as the three adults released 1500 m away and caught 14 days later. This possibility is supported

by David et al. (2015) who found that newly emerged immature beetles were capable of flying an average of 443m and a maximum of 1000m in a flight mill only in 10 min.

The dispersal ability of mature *M. galloprovincialis* has been studied in flight mills (David et al., 2013) and by mark-release-recapture methods in pine stands (Gallego et al., 2012; Mas et al., 2013; Etxebeste et al. 2015, Torres-Vila et al., 2014). Mature adults may disperse up to 13.6 km in patchy landscapes (Gallego et al., 2012). However, most remained close to the release point under continuous stand conditions and 99% of them did not disperse over estimated 2344-3496 m boundaries (Etxebeste et al. 2015). Our results also showed that lab matured *M. galloprovincialis* were able to cross host-free areas at least during 1500 m. There were no apparent differences in dispersal between the sexes, which agrees with previous studies (Etxebeste et al. 15; Togashi, 1990b; Mas et al., 2013; David et al., 2013), and with results on the physiological traits related to dispersal discussed earlier. Mature beetles did not perform better than immature beetles when dispersing over a food-free area. Humphry & Linit, (1989) reported that immature *M. carolinensis* were more active fliers in a flight mill than mature insects, but David et al (2013, 2015) reported better flight mill performance for mature *M. galloprovincialis*, suggesting that maturation feeding would improve flight capacity. Our results showed no significant improvement in lipid or muscle content throughout the maturation stage. However, our conclusions regarding mature beetle dispersal must be considered with caution since they are derived from a small sample.

To sum up, our results show that recently emerged and immature *M. galloprovincialis* were able to disperse rather long distances through areas deprived of any host. In addition, newly emerged beetles had well-developed thoracic muscles and energetic reserves for sustained such flights. In the absence of food, these adults can survive up to three weeks and would thus be able to transmit PWN to distant susceptible hosts, which constitutes a highly relevant situation for PWD management. Human mediated dispersal through transportation of wood products will reproduce such scenario, and certainly, the dispersal traits of immature *M. galloprovincialis* may facilitate the further scenario of PWD expansion through Europe as has been observed elsewhere (Robinet et al., 2009). Beetles to emerging in new apparently host-less areas, such as ports or warehouses, may spread the threat of infection to susceptible trees as they disperse in search of new hosts, even over non-forested areas. Since semiochemical monitoring of *M. galloprovincialis* is currently only feasible after beetles have matured, it might be asked if providing some host trees in the vicinity of these sites might reduce the risk of beetle dispersal by allowing later on-site trapping. Such management actions would oppose current procedures. However, the

presence of nearby hosts would also increase beetle survival and the subsequent risk of infection, thus making this measure counterproductive. Further studies should account on how to effectively deal with the dynamics of new PWN infestations, taking into account the dispersal capacity of immature *M. galloprovincialis*.

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Chapter 3: *Dispersal of Monochamus galloprovincialis (Col.: Cerambycidae) as recorded by mark-release recapture using pheromone traps.*

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Introduction

Dispersal, defined as the distance travelled by an organism from its site of birth to site or sites of reproduction, is an essential process in the gene flow within and between populations (Clobert et al. 2012), but it is also the mechanism by which species spread towards new environments. Boosted by the increasing human mediated dispersal by trade and global flow (Levine and D'antonio 2003; Robinet et al. 2011), biological invasions are regarded as one of the main causes of biodiversity loss, and cause major economic and environmental damages (Pimentel et al. 2005).

The pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner et Buhner) Nickle, is the causal agent of pine wilt disease (PWD), a worldwide threat to pine forests (Futai 2013). Its introduction in Japan during the early twentieth century has caused massive timber and economic losses (Yoshimura et al. 1999). PWN was reported for the first time in Europe by Mota et al. (1999), and despite intensive management efforts, the disease spread through Portugal, while four foci are under eradication in neighbouring regions in Spain (Abelleira et al. 2011; Zamora et al. 2015). According to a recent economic impact assessment, a 11.9 billion € loss is forecasted for Spain between 2008-2030 (Soliman et al. 2012).

So far, only *Monochamus galloprovincialis* (Olivier) vectors PWN in Europe (Sousa et al. 2001). As other members of the genus, *M. galloprovincialis* reproduce on recently dead or severely stressed trees, where it arrives following chemical cues including host and bark beetle kairomones, and also pheromonal compounds (Pajares et al. 2010; Pajares et al. 2013). New beetles will mostly emerge at the beginning of the summer in Southern Europe (Naves and De Sousa 2009). If present, PWN juveniles aggregate around pupal chambers and enter callow beetles prior to emergence. Newly emerged *Monochamus* beetles feed on the bark of twigs and branches of healthy pines, facilitating the entrance of PWNs into new hosts (primary PWN transmission; Linit 1990). For about 15 days, beetles will keep feeding until they reach sexual maturity while they strengthen flight muscles for further dispersal

(Koutroumpa et al. 2008 and references therein). This phase has been named the maturation feeding, however, shoot feeding takes place throughout adult life, and PWN transmission through primary transmission would occur even after maturity has been reached (Naves et al. 2007). Infection by PWN results in a quick death of most infected trees, which then become attractive to the vector. Secondary transmission of PWN may also occur during oviposition by *Monochamus* females (Futai 2013). Thus, the spread of PWN is greatly constrained to the dispersal capacity of *Monochamus* beetles.

The rearrangement of individuals with distance, the dispersal curve, is often used to numerically characterize dispersal. Its general shape is considered almost universally regular; while the majority of individuals undergo short-distance dispersal (SDD), a few will show a long-distance dispersal (LDD; Nathan et al. 2003). SDD has been thoroughly studied in many organisms, and its characterization allows now for the computation of several population parameters, such as recruitment or small-scale metapopulation dynamics (Clobert et al. 2012; Okubo and Levin 2001; Turchin 1998). On the other end, the scarce LDD events have a disproportionate importance in the evolution of populations, species and communities, and are especially relevant in the case of biological invasions (Clobert et al. 2012; Nathan et al. 2003). Yet, these events are characterised by a high degree of stochasticity, and records often proceed from anecdotal and potentially biased data sets, making LDD characterization very hard (Nathan et al. 2003; Turchin 1998). In addition, human activities such as good transportation or translocation of firewood, contribute significantly to the occurrence LDD events (Pimentel et al. 2005; Taylor et al. 2010; Yoshimura et al. 1999). Correspondingly, long-distance spread of PWN has been primarily linked to human-mediated transportation (Robinet et al. 2011; Takasu et al. 2000; Togashi and Shigesada 2006). The local expansion of the disease on the other hand, is critically affected by the SDD behaviour of *Monochamus* beetles (Robinet et al. 2011; Takasu et al. 2000).

As for most of small animals, insect dispersal is one of the most difficult population parameters to measure. Information gathered using tethered insects at flight mills has provided information about the flight potential, i. e., the dispersal capacity, of studied insects (Ranius 2006). Yet, the lab procedures under which these tests are performed make their field interpretation difficult (Ranius 2006; Taylor et al. 2010). Using this technique, *M. galloprovincialis* has recently been shown to be able to accumulate mean flight distances of about 16 km (David et al. 2014). Telemetry and mark-release recapture (MRR) on the other hand, allow for field observation of the dispersal of studied insects. In addition MRR provides means for population size estimation (Ranius 2006; Sanchez-Husillos et al. 2015).

Mark-recapture has been widely used to study dispersal of wood boring beetles (e. g. Bancroft and Smith 2005; Franklin et al. 2000; Turchin and Thoeny 1993; Turchin and Odendaal 1996), and some figures are also available for *Monochamus* beetles (Bonifacio 2009; Gallego et al. 2012; Mas et al. 2013; Shibata 1986; Togashi 1990; Torres-Vila et al. 2015). Results of MRR studies conducted in Japan registered very low SDD for *M. alternatus*, yet distances above 2 km were recorded at landscape level (Shibata 1986; Togashi 1990). A number of MRR experiments with native and lab-reared *M. galloprovincialis* conducted in fragmented landscapes showed that even if most of recaptures occurred close to the origin, two beetles could be trapped at 8.3 km and 13.6 km from the release point (Gallego et al. 2012; Mas et al. 2013).

Eulerian analysis of the redistribution of populations relies in the recording of large numbers of marked or unmarked individuals at specific points in space (Nathan et al. 2003; Turchin 1998). Two complementary approaches would then result in the computation of the dispersal kernel (Nathan et al. 2012), that is, statistical distribution of dispersal distances of the population under study. While phenomenological or empirical models use dispersal records to fit *ad hoc* functions that ignore the dispersal process, mechanistic models are based in the redistribution process and use data on factors affecting it during modelling, after which the dispersal curve is then predicted independent of the dispersal data (Nathan et al. 2003; Nathan et al. 2012; Turchin and Thoeny 1993; Turchin 1998). One further methodological challenge involves incorporating the estimation of LDD events as these are characterised by their scarcity (Nathan et al. 2003).

The availability of an efficient trapping procedure to conduct MRR studies with *M. galloprovincialis* (Álvarez et al. 2015; Pajares et al. 2010), encouraged us to conduct three field experiments to study the dispersal of lab reared beetles from emergence to a first response to a fake breeding site, i. e., a trap baited with infochemicals. Two main aims were set for these trials: first, the dispersal of *M. galloprovincialis* was studied in order to fit dispersal kernels that could assist in the description of the PWD expansion when vectored by *M. galloprovincialis*, as well as provide information to managers and policy makers in the implementation and application of control measures. The experiments were held in two landscape scenarios, fragmented and continuous pine stands, and gathered dispersal data allowed for the comparison of a mechanical model based on simple diffusion with disappearance (Turchin and Thoeny 1993) with two empirical models, one of which has shown the potential to describe both short- and long-distance dispersal (Clark et al. 1999). Previous reports have generally focused in tracking long distance dispersal events over fragmented scenarios (e. g. Gallego et al. 2012; Mas et al. 2013), or have followed wild-

beetles in a limited spatial scale (Torres-Vila et al. 2015), while no *M. galloprovincialis* dispersal kernel based on MRR results have been published to our knowledge. And second, the same experimental set-ups would help defining the performance of multiple-funnel traps baited with commercial lures that are used in monitoring *Monochamus* beetles (Álvarez et al. 2015), and establishing their effective sampling area as well as their seasonal sampling ranges (Ostrand and Anderbrant 2003; Turchin and Odendaal 1996).

Materials and methods

Insects and host material

Beetles for the mark-recapture experiments in 2009 and 2010 were obtained from *Pinus pinaster* Aiton bolts infested with *M. galloprovincialis*, collected in Arenas de San Pedro (Ávila, Spain; 40°11'02"N, 5°02'22"W). Similarly, *Pinus halepensis* Miller logs were collected in 2011 Callosa d'en Sarrià (Alacant, Spain; 38°38'45"N, 0°08'25"W). Bolts were transported to the lab, placed in outdoor cages or boxes, and monitored regularly for insect emergence (from mid-June to mid-July). Beetles were sexed and the length of their right elytrum was measured upon collection. Numbered and coloured queen bee tags (Opalith Plättchen, Christian Graze KG, Germany) were glued to their pronotum using gum Arabic. Marked beetles were kept individually in glass jars at climatic chambers (15L:9D, 25°C:20°C) until release (≤ 3 days). No food was provided as dispersal records aimed at including any initial displacement providing maturation feeding.

Study sites and experimental design

Lab-reared *M. galloprovincialis* release-recapture experiments were held at three distant sites in the Iberian Peninsula during three consecutive seasons (Fig. S1a). Grids of traps were installed centred on the points of release located at continuous (2009 and 2010; Fig. S1b and S1c) and fragmented pine stands (2011; Fig. S1d). In all experiments, multiple-funnel traps (Econex S.L., Murcia, Spain; Lindgren 1983) baited with the commercial lure for *M. galloprovincialis* (Gallopact Pack®, SEDQ S.L., Barcelona, Spain) were used to track dispersing beetles at week intervals. Traps were hung between pairs of trees, with the collection cup at least 50 cm from the ground. The lures released two bark-beetle kairomones (ipsenol and 2-methyl-3-buten-2-ol, 2.5 and 12 mg/day respectively), a host kairomone (α -pinene, 500 mg/day) and the *M. galloprovincialis* pheromone (2-undecyloxy-1-ethanol; ca. 2 mg/day; Pajares et al. 2010). Baits were renewed every 6 weeks.

The first experiment was carried out between 24 June and 7 October 2009 at a natural ca. 60-year-old *P. pinaster* stand in Tabuyo del Monte (Fig. S1b, Leon, Spain; 42°17'45"N, 6°10'52"W; 961 m a. s. l.). On average, the stand had ca. 900 trees ha⁻¹. Four batches of recently emerged *M. galloprovincialis* were released from the centre of a circular grid consisting of four concentric rings (Fig. S2a).

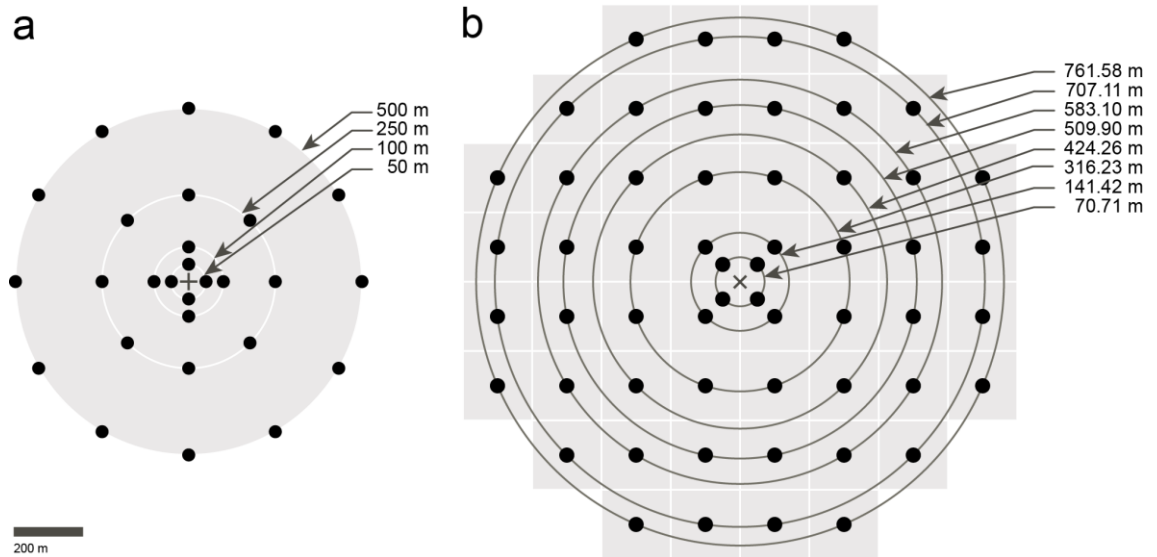


Fig. S2 Detailed trap disposition in experiments held at (a) Tabuyo del Monte (2009) and (b) Villota del Páramo (2010). Filled circles represent trap locations, while crosses represent release sites. Radii lengths of each of the trapping annuli on both sites are shown as well as the underlying 200 m cell grid in 2009 (b).

Four, 4, 8 and 12 traps were placed at regular arc segments respectively at 50m, 100m, 250m and 500m from the release point. In order to prevent escape, traps included a small piece of dimethyl 2, 2-dichlorovinyl phosphate insecticide (Econex S. L., Murcia, Spain). The release platform consisted of dead pine branches staked on the ground. Beetles were placed on the platform between 10:00 and 12:00 at each release occasion, and observed for 20 min for flight initiation. Fifty-four, 55, 40 and 22 lab-reared *M. galloprovincialis* were observed to resume flight on the 24 June, 1, 8 and 15 July respectively (Table 1).

The study site in 2010 was located in Villota del Páramo (Fig. S1c, Palencia, Spain; 42°32'26"N, 4°51'56"W; 1003 m a. s. l.), and was carried out between 18 June and 30 September. The study area consisted of mixed reforested stands of *P. pinaster* (8%), *Pinus nigra salzmannii* Arnold (78%) and *Pinus sylvestris* L. (14%) of varying ages (40 to 60-years old) and densities (150 to 650 trees ha⁻¹; Fig. S1c). Patches of *Quercus pyrenaica* Willd. in the understory could also be found locally. A regular grid of 56 multiple funnel traps were installed centred in the release point (Fig. S1c). Fifty-two of them were placed in the centres

of 4 ha square cells (200×200 m), while the remaining four covered the closest 1 ha cells to the release point. The design intended to provide a homogeneous sampling intensity, while fitting 8 concentric rings around the release point (Fig. S2b).

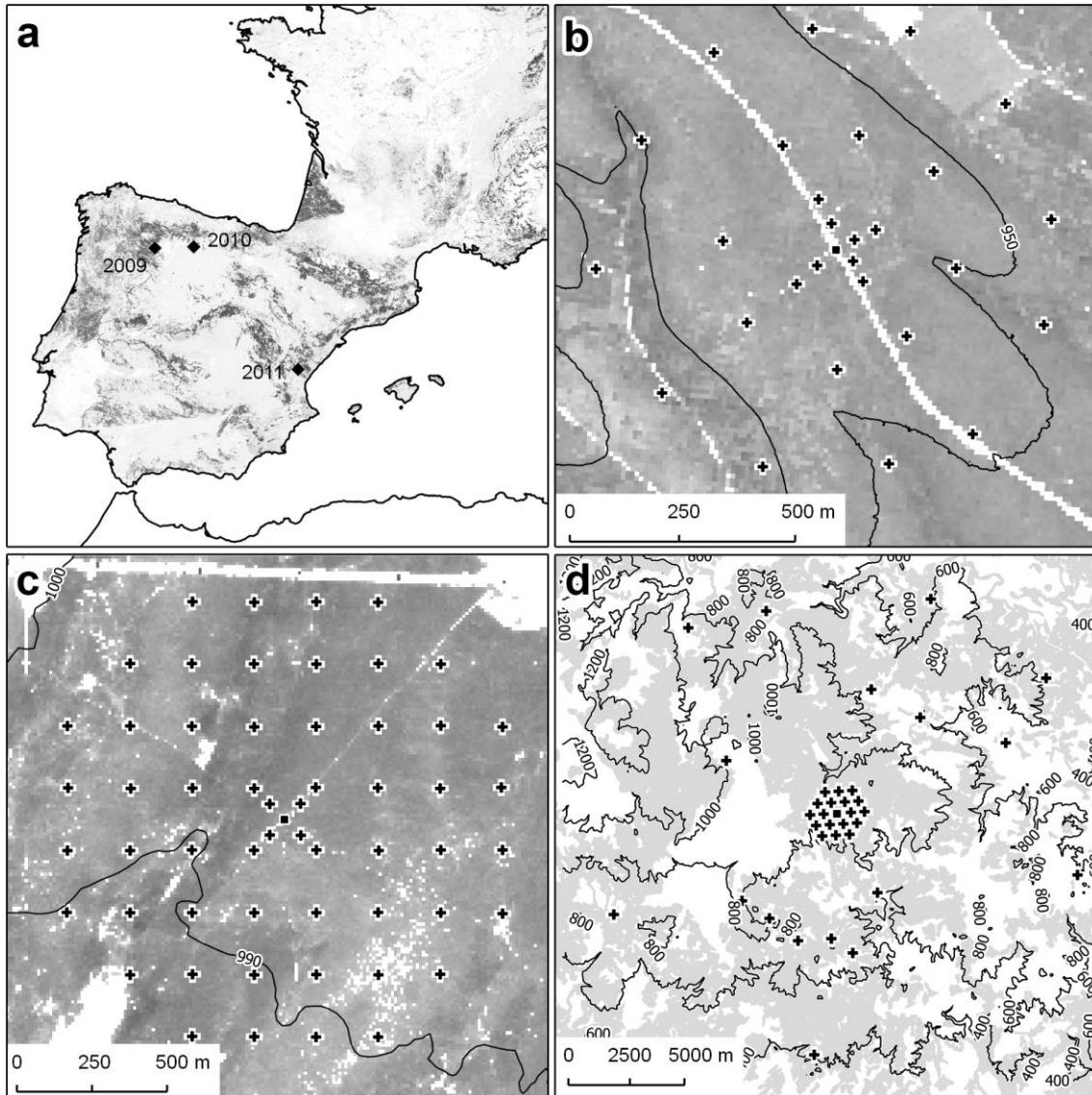


Fig. S1. *Monochamus galloprovincialis* dispersal studies location and design between 2009 and 2011. (a) Geographical location of the experimental sites shown over a raster depicting the distribution of *Pinus* spp. forests in Southern Europe (Tröltzsch et al. 2009). Trap (crosses) and release site locations (filled squares) for (b) Tabuyo del Monte (2009), (c) Villota del Páramo (2010) and (d) Altura (2011). Background images represent raster images depicting canopy height derived from airborne LiDAR data (b and c; PNOA, Instituto Geográfico Nacional, Spain) and forest patches with *P. halepensis* dominance (d; 3rd Spanish National Forest Inventory, Ministry of Agriculture, Food and Environment). See text and Fig. S1 for further design details.

Following the same procedures as in 2010, four batches of 39, 106, 142, and 66 *M. galloprovincialis* were observed to resume flight on the 29 June, 6, 13 and 20 of July respectively (Table 1). Modified multi-funnel traps allowed for live-trapping of *M. galloprovincialis* while minimizing escape (see details in Álvarez et al. 2015). Captured

marked beetles that were recognized as capable of re-initiating flight, i. e., maintained vigour, were re-released 100 m away of the traps position to minimize interference with dispersal. At each sampling occasion, new fresh pine twigs were placed inside the collection cups to allow for insect feeding.

Year	Replicate	Date	No	Recaptures	Distance (m)			Time (days)		
					d _{min}	d _{max}	d _{med}	t _{min}	t _{max}	t _{med}
2009	I	2009-06-24	54	18 (33.3%)	50	500	100	28	105	45.5
		2009-07-01	55 (36♀,19♂)	21 (38.2%)	50	500	50	21	91	52
	III	2009-07-08	40 (19♀,21♂)	11 (27.5%)	50	250	50	7	77	35
		2009-07-15	22 (10♀,12♂)	10 (45.5%)	50	500	75	21	84	35
2010	I	2010-06-29	39 (22♀,17♂)	9 (23.1%)	71	762	141	14	49	14
		2010-07-06	106 (53♀,53♂)	32 (30.2%)	71	762	71	7	84	28
	III	2010-07-13	142 (66♀,76♂)	43 (30.3%)	71	762	141	14	84	28
		2010-07-20	66 (34♀,32♂)	18 (27.3%)	71	762	141	14	77	24.5
2011	I	2011-06-02	128 (61♀,67♂)	1 (0.8%)	0	0	0	29	29	29
		2011-06-10	81 (32♀,67♂)	3 (1.2%)	559	1000	901	52	94	73
	III	2011-06-15	77 (44♀,34♂)	6 (1.3%)	0	2019	0	26	89	75
		2011-06-22	78 (39♀,38♂)	5 (1.3%)	0	559	0	19	78	61
	V	2011-07-04	58 (40♀,18♂)	7 (1.7%)	0	1118	500	35	98	56
		2011-07-11	32 (18♀,14♂)	3 (3.2%)	0	559	559	28	59	49
	VII	2011-07-21	19 (13♀,6♂)	3 (5.3%)	0	5303	5037	14	46	49

Table 1 Summary values of *Monochamus galloprovincialis* release-recapture studies between 2009 and 2011. Initial number of beetles released at the origin (N₀), absolute and relative recaptures, and ranges and median values for distances and times of recapture are provided. Sex distribution for the first replicate in 2009 is not available.

Finally, a third experiment was carried out in a highly heterogeneous landscape during 2011 in Altura (Fig. S1d, Castelló, Spain; 39°49'18"N, 0°38'31"W). Altitudes in the area ranged between 400-1200 m a. s. l., pine stands dominated by *P. halepensis* were present patchily across a wide study area (> 48000 ha). Other pine species could also be found, including *P. nigra*, *P. pinaster*, or *P. sylvestris*. Tree densities in the centre of the setup, a 50-year-old *P. halepensis* stand, averaged 300 to 400 trees ha⁻¹ (Fig. S1d). Thirty-six modified traps were used to track down the dispersal of *M. galloprovincialis* under two trapping regimes. On the one hand, a central set of 19 traps were placed following a staggered trellis on a continuous pine stand of about 600 ha (3-4-5-4-3 disposition; ca. 500

m between traps; Fig. S1d). This set of traps was alternatively removed and installed, following an average 6-days-trapping and 4-days-no trapping cycle, under the assumption that no artificial pheromone source was left at the central stand. On the other hand, 17 additional traps were installed at forest patches with a dominance of *P. halepensis* in two rough concentric rings of 4-6 km and 8-10 km radii. Seven batches of lab-reared *M. galloprovincialis* were released during the time when no traps were present following the procedure described above (Table 1). The release point matched that of the location of the central trap (Fig. S1d). Altogether, 473 *M. galloprovincialis* were released at this third experiment.

Statistical analyses

Due to the low number of recaptures in 2011 (2.1% on average; Table 1), and their poor and sparse distance coverage, modelling was not conducted on this data set. Yet, that information was used to evaluate models fitted with the 2009 and 2010 datasets. Only the distance records for the first time insects were recaptured in 2010 were used for the analysis, so that results would be comparable to those of 2009 when beetles were killed at capture. All calculations and analyses were carried out under the R statistical environment and language (R Core Team 2014).

Rotational symmetry in the effective dispersal of recaptured individuals was tested prior to modelling, in order to check for the assumptions of selected methods (Clark et al. 1999; Turchin and Thoeny 1993). The significance of the deviation of the average displacement from the x and y axes was calculated following the method proposed by Turchin and Thoeny (1993). Relationship between insect size (elytral length) and recapture distance was analysed by simple linear regression.

Mechanistic model – A mechanistic model for the quantitative analysis of the dispersal of a bark beetle, allowing for disappearance, was developed by Turchin and Thoeny (1993). Based on their solution for a time integrated simple-diffusion model (Okubo and Levin 2001; Turchin 1998):

$$C(r) = \frac{\alpha N_0}{2\pi D} K_0 \left(\sqrt{\frac{\delta}{D}} r \right) \quad \text{Eqn. 1}$$

where, $C(r)$ are the captures at distance $r = \sqrt{(x^2 + y^2)}$, α is the recapture efficiency, N_0 is the initial number of beetles released at the origin, D is the diffusion rate, δ is the loss rate and $K_0(z)$ is a modified Bessel function of the second kind. Turchin and Thoeny (1993) proposed the following approximation, written as:

$$C(r) = A r^{-\frac{1}{2}} \exp\left[-\frac{r}{B}\right] \quad \text{Eqn. 2}$$

where, $A \equiv (\alpha N_0)/(\sqrt{8\pi r} \sqrt[4]{D^3 \delta})$ is the scale parameter, proportional to the number of beetles released and the recapture efficiency; and $B \equiv \sqrt{(D/\delta)}$ measures the spatial scale of dispersal. Parameter B can then be used to construct the probability density function, i. e., the distance dispersal kernel (k_D) by numerically solving:

$$k_{D \text{ Diffusion}}(r) = \frac{r^{1/2} \exp[-r/B]}{\int_0^{\infty} r^{1/2} \exp[-r/B] dr} \quad \text{Eqn. 3}$$

Taking natural logarithms of Eqn. 2, and linearizing it, facilitates its fitting by regressing $Y = \ln C(r) + \frac{1}{2} \ln r$ on r (Turchin 1998). Trap catches $[C(r)]$ were averaged by equal distances, thus avoiding problems derived by zero catches at certain traps and distances (Turchin and Thoeny 1993). Response variable Y was then fitted by linear mixed effect models (LME) that allowed to test for possible differences in dispersal patterns between sexes of *M. galloprovincialis*, zonal effects (i. e., spatial heterogeneity between trapping annuli), year of the experiment, replicates, and replicates within years, using a diagonal variance-covariance structure for random effects. Models were fit using the “lme” function of the nlme R package (Pinheiro et al. 2015). Likelihoods of fitted models were compared to a linear model without random effects by means of likelihood-ratio tests (Pinheiro and Bates 2000). A conventional linear model was then fitted for each of the replicates in 2009 and 2010 (Table 1), and k_D and the cumulative probability curves were correspondingly built using fitted B parameters.

Empirical models – Two phenomenological distance location kernels (k_L) were also fitted to the recaptures recorded in 2009 and 2010 via inverse modelling. On the one end, the fat-tailed bivariate Student’s t or “2Dt” model was chosen as was designed to describe both local and long-distance dispersal (Clark et al. 1999; Nathan et al. 2012). The formulation for the 2Dt model is:

$$k_{L2Dt}(r) = \frac{p}{\pi u \left[1 + \frac{r^2}{u} \right]^{p+1}} \quad \text{Eqn. 4}$$

where, p is the shape parameter and u is the scale parameter (m^2). On the other, the negative exponential kernel was chosen as a reference against more fat-tailed diffusion-based and 2Dt kernels (Nathan et al. 2012). Its formulation:

$$k_{L\text{Neg. Exp.}}(r) = \frac{1}{2\pi a^2} \exp\left(-\frac{r}{a}\right) \quad \text{Eqn. 5}$$

where, a is the scale parameter, and the shape parameter c relating the kernel to the gamma function, equals one. The kernel was considered separately to the initial number individuals at each replicate (N_0) and the trapping-area used to detect dispersal (A) so that the expected number of captured individuals would be computed as $C(r) = AN_0 k_L(r)$. The value for the trapping area was taken as the average of the computed effective sampling area for years 2009 and 2010 (6650 m^2 ; see below; Turchin and Odendaal 1996). Results from replicates in 2009 and 2010 were analysed as one single batch accounting for different N_0 . Empirical models were fitted using numerical maximum likelihood estimation assuming a Poisson error distribution for *M. galloprovincialis* recaptures (e. g. Clark et al. 1999). The negative Poisson log-likelihood ($-\ln L$) was numerically minimized using the Stats4 R package. Location and distance kernels in 2D space were related through $k_D(r) = 2\pi r k_L(r)$ (Nathan et al. 2012).

Fitted mechanical and phenomenological kernels were evaluated graphically using the 2011 dataset as the observed recaptures. The A parameter in the diffusion model (Eqn. 2) was re-scaled accounting for the initial number of beetles released on each replicate in 2011. In addition, the mean squared errors of the predictions were computed for each selected kernel. The same set of fitted distance dispersal kernels was also used to produce a table with distances corresponding to the expected 50%, 67%, 95%, and 99% percentiles of dispersal events, as well as the probabilities to disperse over 500, 1000 and 5000 m.

Trapping parameters – Turchin and Odendaal (1996) introduced a computation method, later extended by Ostrand and Anderbrant (2003), that allowed for the estimation of the

effective sampling area (α_{ESA}) and the seasonal sampling range (r_s ; Wall and Perry 1987) of pheromone baited traps under the assumptions that recruitment areas around the traps have circular shapes, and that insect densities (B) remain homogeneous within the area. This way α_{ESA} can be computed as:

$$\alpha_{ESA} = \frac{C}{B} = 2\pi \int_{r=0}^{\text{Pr}(r) \rightarrow 0 \square r_s} r P(r) dr \quad \text{Eqn. 6}$$

where $P(r)$ is the function linking the average proportion of captured insects that started at the distance r of a certain trap and C is the number of captured individuals. The intercept of $P(r)$ with the x -axis provides an estimate of r_s . The ratio between the radius of the α_{ESA} (r_α) and r_s can be used to describe the concentration of the trapped individuals' origin. This way, if both estimates are similar, the catch is very concentrated in space, whilst low catch concentration (CC) values would mean that only a small fraction of the catch comes from the immediate vicinity of the trap (Ostrand and Anderbrant 2003). The influence of sex, year, zonal heterogeneity, and replicates as random effects were tested through a series of LME models. Distance to the origin was \log_{10} transformed for modelling. Values for α_{ESA} , r_α , r_s and CC were numerically computed for fitted models.

Results

Marked lab-reared *M. galloprovincialis* could be consistently recaptured during experiments in 2009 and 2010, yet rates greatly dropped in 2011 (Table 1). Altogether, 60 beetles could be recaptured in 2009, yielding an average 36.1% rate per replicate. Beetles were caught as soon as 7 and as late as 105 days after their release. Although median distances corresponded to those closest to the release point, recaptured beetles were found across all the experimental setup. In 2010, an average recapture rate of 27.7% of released beetles (102 first sightings), showed that trap modifications were avoiding the escape of *M. galloprovincialis* without a significant loss of efficiency, yet 20.6% of the beetles were found dead at their first recapture. Nine and three beetles were sighted on a second and a third occasion (33.3% and 0% mortality respectively). Table 1 shows median, minimum and maximum distance recorded for first sightings, while cumulative track lengths averaged 240 m (mode 71 m, range 71-1090 m); beetles were re-sighted between 1 and 12 weeks

after their release. Out of the 473 *M. galloprovincialis* released in 2011, only an average 2.1 % recapture rate per replicate was obtained (28 first sightings, 64.3% mortality). Three individuals were sighted on a second occasion (66.6% mortality), and one *M. galloprovincialis* could be tracked dead on a third occasion. First recaptures occurred as soon as 14 and as late as 98 days after release. Distances to recaptures ranged from 0 to 5303 m (Table 1). Although cumulative distances ranged between 1000 and 2000 m, the longest records were recorded from two first sightings of beetles that dispersed 5303 m after only 14 days, and 5037 m after 46 days (Fig. S6). No significant influence of beetle size in recapture distance was found at any of the experimental years (r_{adj} -0.04, P 0.70 in 2009; r_{adj} -0.013, P 0.58 in 2010; and r_{adj} -0.019, P 0.47 in 2011).

No significant deviation on the average displacement from the x and y axes was detected in 2009 (5.8 m, t_3 0.65, P 0.56 and -23.68 m, t_3 -0.89, P 0.44 respectively). On the other hand, a significant westward deviation was found in 2010 (-95.21 m, t_3 -5.31 P 0.013), whilst the average displacement from the y axis remained not significant (-87.47 m, t_3 -2.3 P 0.105). Much of the detected drift could be accounted to replicates I and IV (-144.44 and -83.33 m westward drift), which were in turn the replicates with lowest absolute and relative recaptures (Table 1). Their removal from the displacement analysis resulted in a non-significant deviation (t_1 -4.44 P 0.14). Thus, and in spite of this small overall drift, the assumption of rotational symmetry was still acknowledged.

Mix modelling of recaptures pinpointed the significant effects of replicates and experimental years on the diffusion model structure, but the recapture distance was not found to be affected by the sex of dispersing *M. galloprovincialis* (Table S1).

Model	Random Effects	d. f.	AIC	P
LM	-	3	134.71	
LME	Sex	5	138.71	1
	Annulus	5	135.62	0.214
	Replicate	5	131.30	0.025
	Year	5	131.48	0.027
	Replicate into Year	7	132.26	0.200

Table S1 Comparison of diffusion model structures based on likelihood-ratio tests comparing linear mixed effect (LME) models to a linear model (LM) without random effects. Models included recapture data of all replicates in 2009 and 2010. See text for further details. AIC, Akaike Information Criterion.

Nesting replicates within year, did not significantly improve the model either, although the difference in AIC value did not support big differences with un-nested alternative models or with the simple linear regression alternative. Considering these results and the different N_0 values, computation of the parameters for the mechanistic diffusion models were obtained through simple linear regression (Table 2; Figs. S4 and S5).

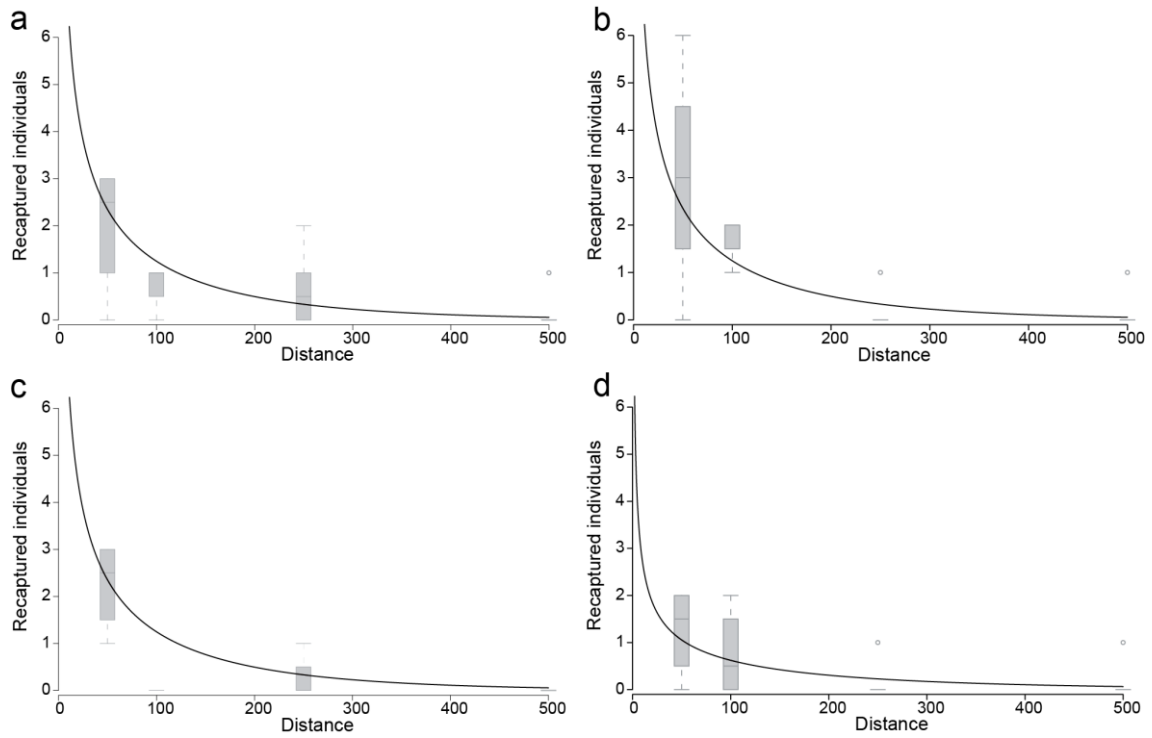


Fig. S4 Mean number of predicted *Monochamus galloprovincialis* recaptures by diffusion models 642 with distance and boxplots of observed recaptures in replicates (a) I, (b) II, (c) III and (d) IV in 2009. 643 Predictions for replicate 2009-III are based on approximate parameters as model could not be fitted.

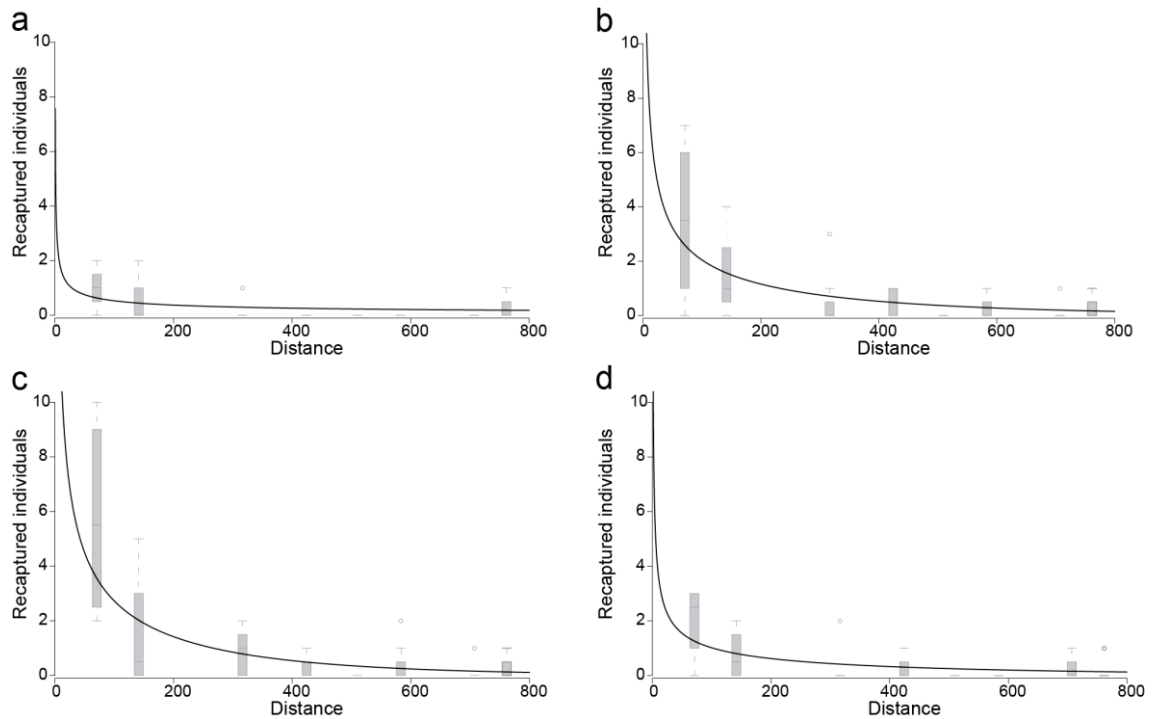


Fig. S5 Mean number of predicted *Monochamus galloprovincialis* recaptures by diffusion models 645 with distance and boxplots of observed recaptures in replicates (a) I, (b) II, (c) III and (d) IV in 2010

Replicate 2009-III only recaptured beetles in two out of four possible trapping distances, making it impossible to follow the same modelling strategy (logs of zeros). In addition, the low and evenly distributed recaptures for replicates 2010-I and 2010-IV resulted in very large B parameters of the diffusion model. Based on lowest AIC values, i.e., highest likelihoods, diffusion models for replicates 2009-I and 2010-II were selected for further analyses and representation purposes (Table 2). Fig. 1 shows the curves corresponding to the fitted models, distance dispersal kernels and cumulative probabilities of dispersal based on those models.

Year	Replicate	Parameter		AIC
		A	B	
2009	I	13.63 ± 0.55	413.26 ± 1984.10	6.08
	II	22.16 ± 1.28	173.97 ± 853.17	12.83
	IV	8.95 ± 0.79	275.27 ± 1390.07	8.93
2010	I	5.36 ± 1.11	18269.99 ± 1454.40	12.00
	II	25.51 ± 0.43	450.09 ± 4474.67	6.13
	III	37.06 ± 0.88	326.08 ± 2169.93	16.27
	IV	11.75 ± 0.64	690.15 ± 2895.42	10.53

Table 2 Diffusion model parameter estimates (\pm SE) after linear modelling of mean *Monochamus galloprovincialis* recaptures per sampling distance and replicate. Model for replicate 2009-III was not fitted because recaptures were only available for two radii (see Results). See Eqn. 2 and text for parameter definition.

In addition, inverse modelling of recaptures allowed the estimation of the parameters of the 2Dt and negative-exponential empirical dispersal location kernels (Table 3).

Dispersal kernel	Parameter	Estimate	95% CI	AIC
2Dt	p	0.804	0.50 to 1.36	375.98
	u	39760.1 m ²	19820 to 82135.8 m ²	
Negative exponential	a	149.14	132.78 to 165.51	368.29

Table 3 *Monochamus galloprovincialis* maximum likelihood estimates for 2Dt and negative exponential empirical dispersal location kernels. Approximated 95% confidence intervals of parameters estimates are provided. See Eqn. 4 and 5 for parameter definition.

The negative exponential provided the best fit, providing the lowest AIC value of both evaluated models. Fig. 2 shows the kernels for location, distances and cumulative probabilities for dispersal computed after the fitted parameters of both models.

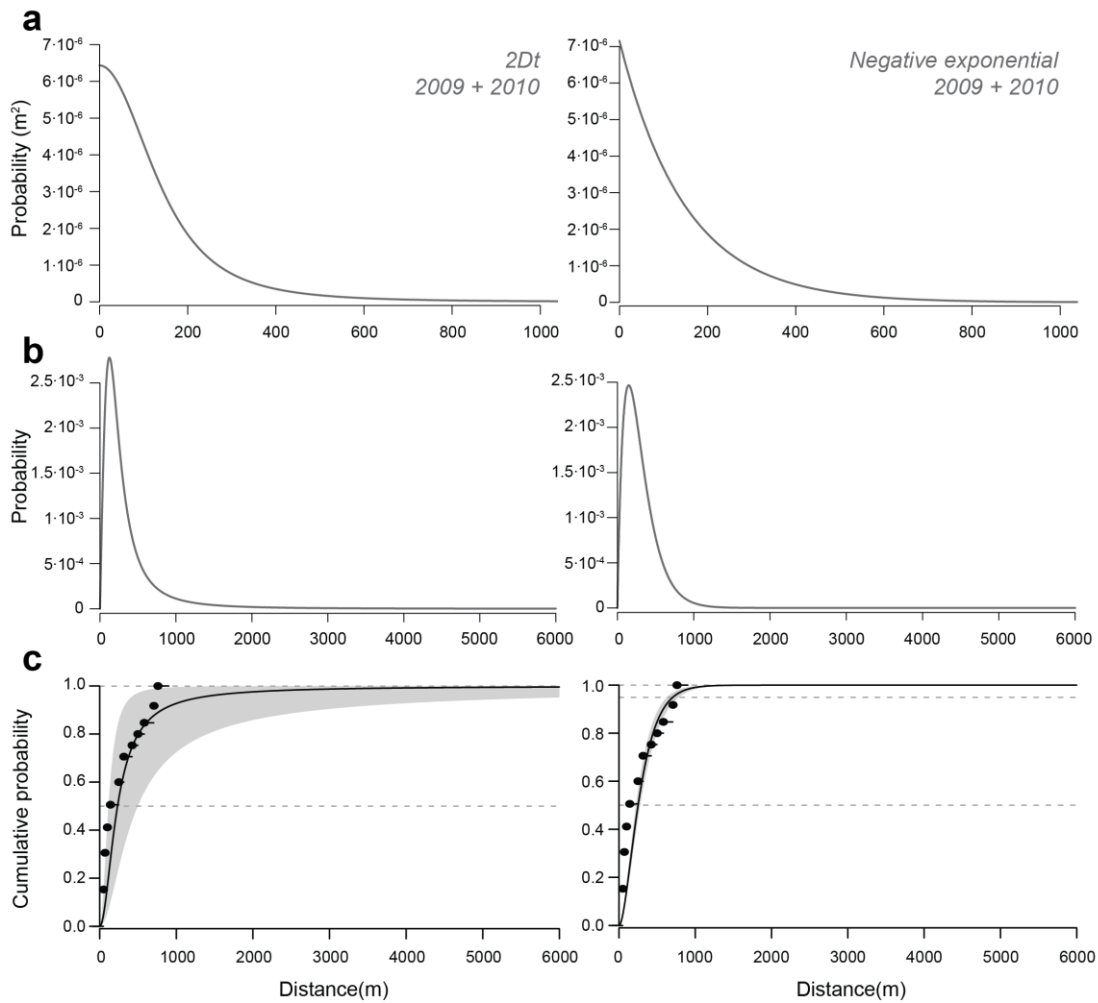


Fig. 2 Analysis of *Monochamus galloprovincialis* dispersal using two empirical models on recaptures of all replicates in 2009 and 2010. (a) Dispersal location kernels, (b) distance dispersal kernels and (c) cumulative probabilities of dispersal over distances fitted with parameters estimated for 2Dt and negative exponential kernels. See text and Table 4 for further details.

Graphical evaluation of fitted kernels is presented in Fig. 3, complemented with the computation of distances to given percentiles of dispersers and probabilities of beetles dispersing to certain distances (Table 4).

Distance	Dispersal kernel		Distance at percentile (m)				Probability over distance		
			r ₅₀	r ₆₇	r ₉₅	r ₉₉	500 m	1000 m	5000 m
Mechanical	2009	I	488.88	708.67	1614.76	2344.19	0.490	0.184	<0.001
		II	205.8	298.33	679.76	986.83	0.125	0.009	<0.001
	2010	IV	325.64	472.04	1075.58	1561.45	0.304	0.064	<0.001
		II	532.45	771.83	1758.67	2553.11	0.528	0.217	<0.001
Empirical	2009+2010	III	385.75	559.17	1274.11	1849.67	0.381	0.105	<0.001
		IV	816.44	1183.49	2696.67	3914.83	0.694	0.408	0.002
	2Dt		233.34	343.87	1270.67	3495.87	0.203	0.073	0.006
		Neg. Exp.	250.32	343.59	707.52	990.07	0.152	0.009	<0.001

Table 4 Estimated radii enclosing 50%, 67%, 95% and 99% of dispersing *Monochamus galloprovincialis* based on fitted distance dispersal kernels. Alternatively, probabilities of dispersal events over 500, 1000 and 5000 m are presented.

Overall, diffusion models for replicates 2009-I and 2010-IV predict that 20% of the beetles will fly over 1000 m, but less than 0.1% would do over 5000 m. On the other hand, 7.3% and 0.9% of the beetles would be found over 1000 m according to the 2Dt and negative-exponential models respectively. The widest tail was forecasted by the 2Dt, predicting that 0.6% of *M. galloprovincialis* would fly over 5000 m. When compared to Fig. 3, observations closest to the release point, i. e., highest observed recaptures, were forecasted in a similar fashion by all models, while above 1000 m, i. e., lowest observed recaptures, diffusion models provided a better guess than empirical models.

Data set	Model parameters and fit					Derived trapping parameters			
	a	95% CI	b	95% CI	AIC	α	r_s	r_α	CC
2009	0.122	0.066 to 0.179	-0.046	-0.101 to 0.008	-555.52	0.57 ha	426 m	43 m	0.10
2010	0.076	0.051 to 0.1	-0.027	-0.039 to -0.015	-1429.39	0.76 ha	645 m	49 m	0.08

Table 5 Trapping parameters in 2009 and 2010. LME model parameters with their 95% CI for each data set, relate the proportion of captured insects and distance to release point considering zonal effects as random. Derived trapping parameters: effective sampling area (α_{ESA}), its radius (r_α), the seasonal sampling range (r_s) and the catch concentration (CC; see Materials and Methods for further explanations).

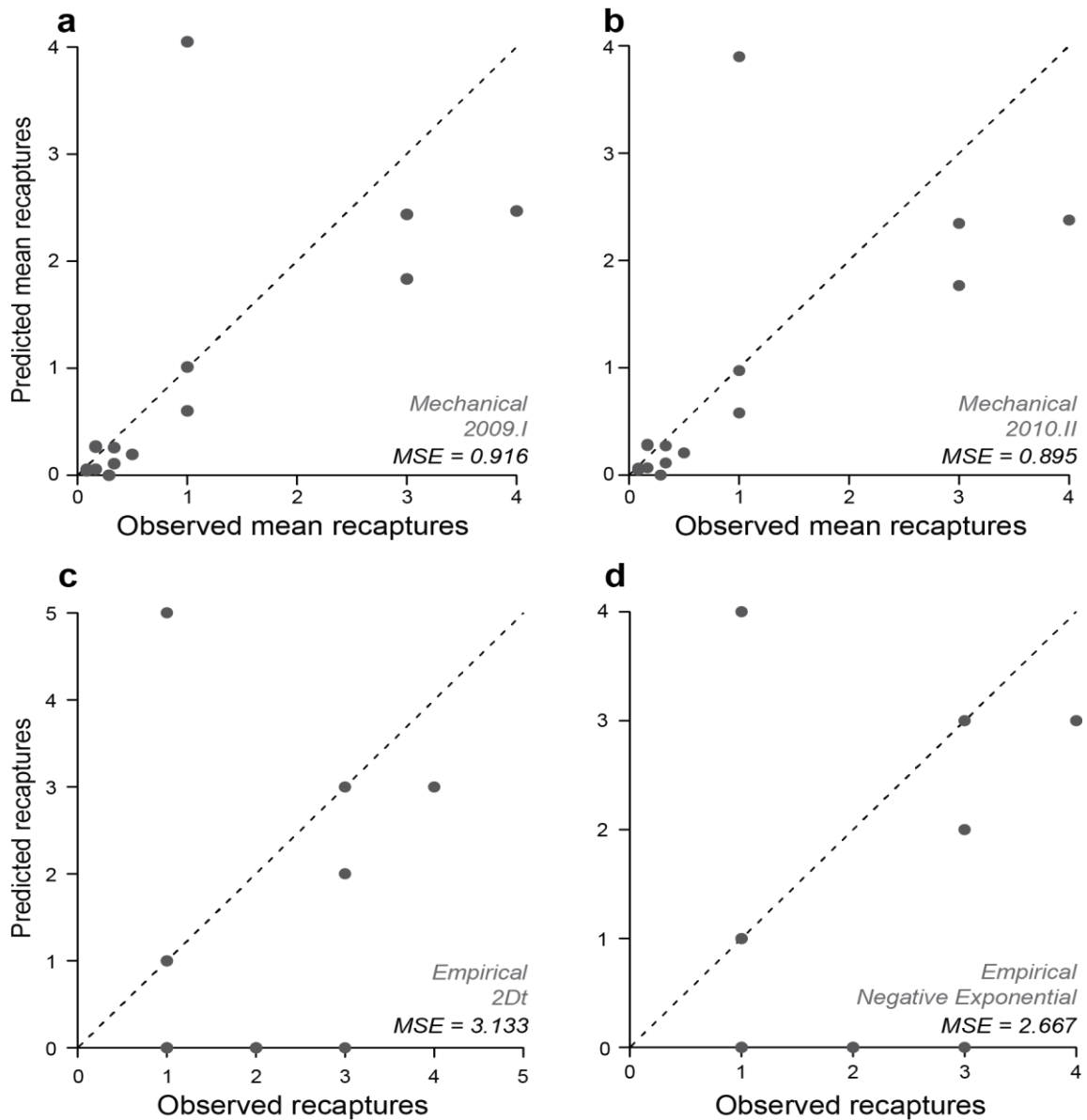


Fig. 3 Comparison of agreements between observed mean and absolute recaptures of *Monochamus galloprovincialis* in Altura (2011) derived from the best fitting mechanical diffusion models and the 2Dt and negative exponential empirical dispersal kernels. The dashed line depicts the line of agreement. Mean squared errors (MSE) for each comparison are provided.

Finally, the effective sampling area (α_{ESA}) and the seasonal sampling range (r_s) among other trapping parameters could be computed using the recaptures of *M. galloprovincialis* in 2009 and 2010. Selected linear mixed models included the zonal effects within the random effects, while the remaining variables evaluated for random effects did not improve fitted models. Derived trapping parameters, along model parameters, are presented in Table 5. Values for the α_{ESA} and r_s were higher in 2010. On the other hand, catch concentration was found to be slightly lower in 2010, meaning that a larger proportion of the beetles caught originated from the outside of the α_{ESA} .

Discussion

The natural spread of PWD is greatly constrained to the dispersal of *Monochamus* beetles and the successful transmission of its causal agent, the PWN. Most of *M. galloprovincialis* resightings were recorded in traps located closest to their origin, irrespective of the study area or experimental year, in accordance to other *Monochamus* observations (Table 1; Gallego et al. 2012; Mas et al. 2013; Shibata 1986; Togashi 1990; Torres-Vila et al. 2015). On the other hand, fitted dispersal kernels under a continuous forest cover locate the median of dispersers at 233-532 m, while 99% of the dispersing *M. galloprovincialis* would not disperse over 2344-3496 m. Recapture rates were very low at a third experiment, yet distance records reached 5000 m on two occasions. No apparent differences between the dispersal behaviour of *M. galloprovincialis* males and females were observed, in agreement with previous reports (David et al. 2014; Mas et al. 2013; Torres-Vila et al. 2015). Torres-Vila et al. (2015) have recently reported an average of 122 m and a maximum 464 m flight-length for wild *M. galloprovincialis* in a study conducted within a 4 ha plot. Similarly, Bonifacio (2009) reported three flights to traps that averaged 237 m, while re-sightings occurred at 5 m from the release site on average when beetles were tracked visually. On the other end, wild-beetles were mainly recaptured in traps at the nearest pine stands, ca. 2 km away of the origin, while one beetle could be recaptured at 8.3 km (Gallego et al. 2012). In addition, Mas et al. (2013) reported recaptures up to 13.6 km from the origin. Both these works included experiments conducted at fragmented forests distributed across hilly terrains. Although our results at the larger scale do agree with them, the dispersal distances of *M. galloprovincialis* under a continuum of forest are longer than previous estimations (Bonifacio 2009; Torres-Vila et al. 2015). Similarly, the studies on the Japanese vector of the PWD, *M. alternatus*, have allocated SDD ranges to a few hundreds of meters (Shibata 1986; Togashi 1990). Yet, at a landscape level, *M. alternatus* was able to disperse up to 3.3 km between forest patches (Togashi 1990 and references therein).

Lab assessments on the flight performance of PWD vectors have shown that their flight potential can in fact be much larger than the distances recorded in the field. Tethered *M. galloprovincialis* can fly 16 km on average during their lifetime (David et al. 2014), 3.7 km long flights were recorded for *M. carolinensis* (Akbulut and Linit 1999), while *M. alternatus* was reported to sustain a maximum 3.3 km flight (Ito 1982; Takasu et al. 2000). Flight-mill records may be biased due to the effects of handling or lab conditions (Taylor et

al. 2010 and references therein). Yet, if net dispersal is compared with flight mill records through random walk or derived diffusion models, they can be regarded as the cumulative distances flown by the insect (Okubo and Levin 2001; Taylor et al. 2010; Turchin 1998).

Attaining reliable measures of dispersal necessarily implies that the method of choice and the size of the study area are providing unbiased data (Franzen and Nilsson 2007; Ranius 2006; Turchin 1998; Yamamura et al. 2003). Applying MRR experiments to describe *M. galloprovincialis* dispersal implicitly requires that a large proportion of the individuals can be tracked down, i.e., recaptured (Franzen and Nilsson 2007; Turchin and Thoeny 1993). Registered rates, at least during 2009 and 2010 (Table 1), fit such scenario. Similar experiments usually result in recapture rates well below 10% (Bancroft and Smith 2005; Franklin et al. 2000; Mas et al. 2013; Ostrand and Anderbrant 2003; Torres-Vila et al. 2015; Turchin and Thoeny 1993; Turchin and Odendaal 1996). The experimental design in 2010 incorporated a lattice pattern design (Fig. S2), which is known to minimize the bias caused by artificial removal of individuals (Yamamura et al. 2003). Also, median dispersal estimates were generally higher in 2010 (Table 4), indicating that *M. galloprovincialis* dispersal was not severely affected by the trapping density. The effect of the presence of artificial sources of infochemicals on the dispersal of *Monochamus* is not known. Bark beetle recapture patterns with distance are known to change in relation to the strength of the lure (Duelli et al. 1997; Franklin et al. 2000; Turchin and Thoeny 1993). However, as *Monochamus* beetles do not need to overcome host defences through aggregation, their responses would not be comparable to bark-beetles. In addition the accumulated sampling area according to the computed α_{ESA} values, represent but a fraction of the study area (Turchin and Thoeny 1993). Taking into account these considerations, we assumed that MRR data using pheromone traps in 2009 and 2010 provided a representative data set for the study of *M. galloprovincialis* dispersal under continuum stands.

The definition of the boundaries between SDD and LDD for *Monochamus* beetles has varied according to the scale of the studies (Bonifacio 2009; Gallego et al. 2012; Mas et al. 2013; Togashi and Shigesada 2006; Torres-Vila et al. 2015; Yoshimura et al. 1999). Following the methodology proposed by Turchin and Thoeny (1993), a simple model for diffusion allowing for disappearances could be applied in six out of eight replicates. One major advantage of mechanical models is that fitted parameters are readily interpretable (Turchin 1998). Consistently, our results showed that while the scale parameter A varied according to corresponding N_0 values per replica, the spatial scale of dispersal (B) did not change much each year, and on average, it was somewhat higher in 2010, likely influenced by the larger study area (Table 2). Variations of the negative exponential kernel have been

previously used to describe *Monochamus* dispersal behaviour (Gallego et al. 2012; Pukkala et al. 2014; Togashi 1990; Torres-Vila et al. 2015). However, the 2Dt kernel should provide a better overall description of both SDD and LDD (Clark et al. 1999). Inverse modelling of observed recaptures in 2009 and 2010 resulted in a better fit of the negative exponential kernel (Table 3; Fig. 2). Yet, even if none of the kernels fitted under continuous forest conditions predicted the 2011 observations accurately (Fig. 3), the 2Dt kernel forecasted the highest probability of LDD events (Table 4).

Proper estimation of LDD requires adjusting the scale of the experiments to the scale of such process, while the sampling effort is held roughly constant (Nathan et al. 2003). In addition, landscape composition is recognized to have a severe effect on dispersal (Clobert et al. 2012), as the degree of fragmentation and connectivity of habitat patches is directly linked to the size of the study areas required for adequate dispersal measurement (Franzen and Nilsson 2007). The experiment in 2011 covered an extensive fragmented area, with a much lower sampling effort, and yet the forecasts using kernels derived from continuous forests were still able to draw the general trends. In fact, the two re-sightings occurring at 5 km took place at the two traps bordering a large host-less area west of the release site (Fig. S1d). The analysis of the recaptures under a framework that considers habitat composition within and between patches was beyond the aim of the present paper, yet our results, and those from similar experiences (Gallego et al. 2012; Mas et al. 2013), suggest that *M. galloprovincialis* dispersal varies according to the landscape, an effect that, to our knowledge, has not been evaluated for PWN spread. Other factors, such host density and susceptibility, vector population level, PWN load or meteorological conditions may also affect the dispersal of *Monochamus* (Robinet et al. 2011).

As other members of the genus, *M. galloprovincialis* requires of a period of about 15 days to mature (Koutroumpa et al. 2008) and to start responding to the pheromonal compounds (Pajares et al. 2010). On average, the median time to recapture was 45.5 days after the release, while the minimum averaged correspondingly to 15 days (Table 1; Fig. S3). Most of successful PWN transmissions occur during the first 49 days after *M. galloprovincialis* emergence (Naves et al. 2007), a period during which ca. 75% of the recaptures occurred in 2009 and 2010. Monitoring or vector population control should be adjusted to emergence patterns in order to maximize the chances that the beetles are trapped when PWN occurrence is highest within the beetles (Koutroumpa et al. 2008; Naves and De Sousa 2009; Sanchez-Husillos et al. 2015). Also, the early removal of beetles carrying PWNs, would add to a lower incidence of LDD events, reducing the rate of successful PWN

transmissions at long distances, altering the reported Allee effects (Takasu et al. 2000; Yoshimura et al. 1999).

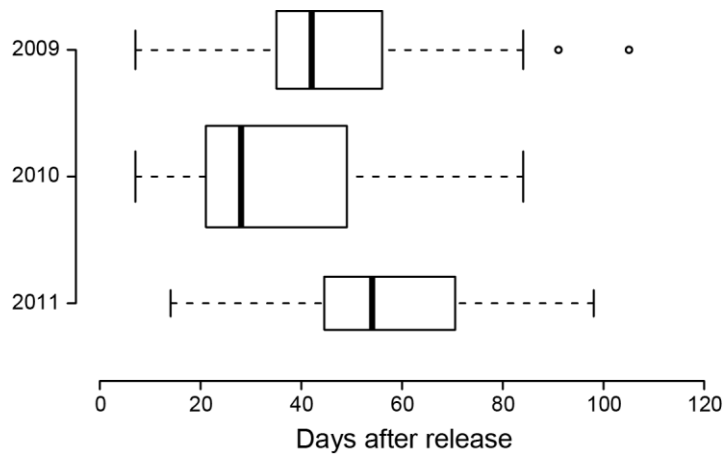


Fig. S3 Boxplots of the number of days between releases and recaptures of *Monochamus 639 galloprovincialis* at Tabuyo del Monte (2009), Villota del Páramo (2010) and Altura (2011). Box widths are proportional to the square-roots of the number of recaptures per year.

Managing of invasive species such as PWN relies on early detections and interventions, but also requires optimization of trapping efforts (Bogich et al. 2008). By analysing the *M. galloprovincialis* recapture data in 2009 and 2010 estimates of relevant trapping parameters could be derived (Table 5; Ostrand and Anderbrant 2003; Turchin and Odendaal 1996). These estimates are readily applicable for managers; for example, according to the r_s values, distances between traps should not exceed 1 km when monitoring for the presence of *M. galloprovincialis*, while α_{ESA} estimates would suggest that population suppression programmes would be maximized at 1.3-1.75 traps per hectare. An assessment of mass trapping on a *M. galloprovincialis* population of an estimated 82 beetles per hectare density, has recently shown that 95% of the population removal would occur at 0.82 traps per hectare (Sanchez-Husillos et al. 2015). Torres-Vila et al. (2015) reported similar sampling area values, even if the parameter was derived from wild-beetles tracked at a much smaller scale. Higher catch-concentration values ($CC=0.32-0.42$) than those reported here ($CC=0.08-0.10$), indicate a possible influence of the scale. These parameters should in any case be interpreted with caution, as the influence of many environmental and design factors might be behind variations in trap performance (Ostrand and Anderbrant 2003).

The latest European Union Commission decision concerning the prevention of PWD spread (2012/535/EU), included mandatory eradication measures establishing “clear-cut” zones of at least 500 m around each susceptible plant in which PWN had been detected.

Both estimated and observed *M. galloprovincialis* dispersal results presented here suggest that such a measure would be ineffective to significantly reduce the probability of spread by beetles emerging from a PWN infected host. Nevertheless, the expected probabilities of LDD events under continuous stands depict an scenario in which 1% of the beetles could disperse beyond 2.5-3.5 km from their emerging host. Furthermore, fragmented habitats would apparently increase those figures, as it has also been observed earlier (Gallego et al. 2012; Mas et al. 2013). On the other hand, diseased trees follow a clumped distribution during the first years of invasion (Bonifacio 2009; Togashi and Shigesada 2006). In theory, a prompt clear-cutting after a very early detection of a new focus could remove all infested-trees, but in practice new detections are likely to occur once beetles have already emerged and dispersed away from PWN infected hosts. However, few detailed studies are available concerning local PWD dynamics or the movement behaviours during feeding and reproduction of *M. galloprovincialis*. Future works should clarify such behaviours, and include in addition the influences of landscape and stand characteristics in the dispersal behaviour of *M. galloprovincialis*. These goals are currently being pursued.

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Chapter 4: *Studying mature and immature Monochamus galloprovincialis dispersal and patch-scale habitat selection using LiDAR techniques.*

Estela Sanchez-Husillos, Iñaki Etxebeste, Juan Pajares

Target journal: *Forest ecology and management*

Introduction

Monochamus galloprovincialis (Olivier, Col.: Cerambycidae) is the native insect vector of the Pine Wilt Disease (PWD) in Europe (Sousa et al., 2001). Recent introduction of the pathogen in Europe (Mota et al., 1999), has led to enormous efforts for controlling the disease, e.g.: clear cutting of infected forest areas, removal and elimination of a of infested material before the emergence of the insect vectors and removal of them by lured traps during their dispersal flight .Dispersal behavior of individuals within between suitable microhabitats is key to understand population distribution, abundance and spatiotemporal structure of populations. *M. galloprovincialis* presents an univoltine life cycle to complete a generation within a short-lived habitat resource (Naves, de Sousa, et al., 2007; Naves et al., 2008). But habitats and resources are changeable in time and space. Immediate after emergence from breeding material, *M. galloprovincialis* adults start feeding on the bark on living conifers shoots until sexual maturation occurs after 12-13 days (Sanchez-Husillos et al. unpublished). Later on, they begin to look for reproducing in recently dead trees, or parts of them. Thus, dispersal behavior plays a key role in ecological processes such as the resource allocation, predator/prey interactions and mating strategies. The host selection process can be subdivided in several sub-process such as habitat localization, host location and host acceptance. Several studies on the later (Naves et al., 2006; Koutroumpa et al., 2009; Sanchez-Husillos et al., 2013) have shown that *M. galloprovincialis* clear prefers *Pinus sylvestris* L. and *P. pinaster* Aiton in Europe. However, it is rare to find empirical data describing key dispersal parameters as microsite selection in habitat location by *Monochamus* spp pine sawyers There is not enough information yet on the habitat selection patterns by *Monochamus* beetles, although some authors have indicated that that they are the earliest colonizers of forests damaged by fire, wind or hailstorm (Gandhi et al.; McCullough et al., 1998). Saint-Germain et al (2004) reported the importance of landscape-scale environmental variables such altitude or distance from nearest unburned areas to a

fire and demonstrated that *M. scutellatus* preferred sites in which both stressed and healthy trees occurred, in order to satisfy nutritional requirements of larvae and adults.

Direct methods for studying insect dispersal behavior involve either characterizing the actual path of the insect movement (Sutcliffe et al., 2003; Brouwers & Newton, 2010), or mark-recapture experiments (Gallego et al., 2012; Sanchez-Husillos et al., 2015). Indirect methods may include genetic markers (Wang et al., 2008), scanning radar (O'Neal et al., 2004), empirical and mechanistic modelling (Etxebeste et al. 2015; Turchin, 1998) or simply modelling the insect movement as a correlated random walk (Kareiva & Shigesada, 1983). Other methodological approaches would include the “resistance” that is the physiological cost of moving through a particular environment. These values are typically used to fill the gap in the knowledge by providing a quantitative estimate of how environmental parameters affect animal movement. However, methods for quantifying resistance surfaces are diverse and there is no general consensus on the appropriate choice of environmental data or analytical approach (Zeller et al., 2012). Other factors, such as host density and susceptibility, vector population level, PWN load or meteorological conditions may also affect dispersal of *Monochamus* beetles (Robinet et al., 2011). Insect home range, predators distribution or the edge effect have been also considered (Vodka & Cizek, 2013). The later would include any environmental attribute that is altered as a result of being at, or in proximity to, the border between two habitats. The edge effect could be provoked by a change in the microhabitat, such as a forest disturbance, or even the setting of pheromone traps that frequently lead to an increase in population density or species richness (Vodka & Cizek, 2013; Navarro-Llopis et al., 2014). Despite the many environmental variables that could affect the insect local distribution, patch-scale distribution patterns of *Monochamus spp.* in microsite selection have received practically no attention.

Light detection and ranging data (LiDAR) based methodologies have proved extremely useful in the accurate three-dimensional (3D) characterization of forest canopy and spatial organization of vegetation within the forest canopy (Lefsky et al., 1999; Suárez et al., 2005; Evans et al., 2009) . The development of new sensor systems, either satellite-borne or airborne, together with the development of the Global Positioning System, are both key issues for the use of remotely sensed data in forest inventories. LiDAR canopy height measures allows for much higher resolution maps, that are calibrated and validated with tree diameter distributions measured within independently geolocated, fixed-radius plots (Hudak et al., 2009). Influences of habitat condition and forest structure on faunal assemblages have been well explored in wildlife studies (Bradbury et al., 2005) and several LiDAR studies have focused on correlations between lidar-derived measures of vegetation, structural diversity and bird species diversity in forests (Goetz et al. 2007). Studies in

agricultural areas using fluorescence LiDAR techniques have also been shown useful for pest monitoring (Mei et al., 2011). Nowadays, applications of LiDAR in pests management, including insect migration and distributions are increasing (Wang et al., 2008; Brydegaard et al., 2009; Müller & Brandl, 2009; Work et al., 2011; Mei et al., 2011; Mueller et al., 2014). LiDAR data can be used as a predictive tool to seek out a given species distribution (Nelson et al., 2005), or can be used too as an exploratory tool to better understanding resource selection by species of known distributions (Broughton et al., 2006). Many studies using LiDAR techniques have focused on mapping, and post-detection and early-warning of insects defoliation (Stone & Coops, 2004; Vastaranta et al., 2013a; b). Also, recent studies have analyzed the impacts of canopy heterogeneity, showing that different components of its structural complexity drive canopy arthropod biodiversity at different spatial scales (Mueller et al., 2014). Another study with LiDAR images performed in boreal forests showed how topography could influence the availability of microhabitats for beetles (Work et al., 2011). Variables such as proximity to burned stands, altitude or tree nutritional condition could be linked to the feeding requirements of *Monochamus scutellatus* (Saint-Germain et al., 2004), thus outlining its importance in the host selection process of the sawyer. Even though a great number of remote-sensing methods are currently available, there are few studies linking the collected information in order to identify the habitat features that are required for *M. galloprovincialis*.

Although many recent studies have dealt with *M. galloprovincialis* dispersal (Mas i Gisbert et al., 2013; David et al., 2013; Etxebeste et al., 2015), none has been carried out to analyze dispersal of immature insects, may be to the difficulty to lure them to traps baited with known attractants (Álvarez et al., 2015a). Recently, Sánchez-Husillos et al. (unpublished) have described *M. galloprovincialis* sexual maturation to be after 12-13 of feeding-days, when which they start to be attracted by pheromone traps. This period is relevant for PWD control since nematode transmission can occur that early (Naves et al., 2007; Dong Soo Kim et al., 2009). Thus, lack of suitable studies limits our knowledge of the mature and immature *M. galloprovincialis* distribution within forests, and thus to make sound decisions for an early detection of PWD.

The present work has been addressed to help defining the environmental variables that motivate *M. galloprovincialis* movement within a forest stand and know if there are different patterns according to the maturation state. Thus, it has been aimed firstly to fit dispersal kernels that could help in the description of the dispersal pattern for both, mature and immature *M. galloprovincialis* beetles and, secondly, to evaluate which forest features

are related to native insect recaptures within a 200 x 200m trap grid and to compare with the variability in the recaptures of lab-released mature and immature beetles at patch scale.

Material and methods

Dispersal study Area

The study area was located in Zarzuela del Pinar (41°17'7"N, 4°13'22"W 854 m a. s. l., Castilla y Leon, Spain). A field trial was conducted in 2011 within an irregular *P. pinaster* pure stand forested by natural regenerated pines, between June 22th and September 28st, covering most of the flight period of the target insect. A capture-mark-recapture assay was performed. Sixty-four teflon coated multiple funnel traps with extended cups (Econex, S L, Murcia, Spain) were placed in the centers of 4 ha square cells (200×200 m; 0.25 traps ha⁻¹; Fig. 1) in a regular grid covering a total of 256ha. Traps were baited with Galloprotect Pack ® (SEDQ, S L, Barceloan, Spain), and lures were replaced every six weeks. All traps were checked weekly during the entire sampling season. During each sampling, freshly cut pine shoots with needles were placed within the cup, to provide food and shelter to trapped *M. galloprovincialis* individuals.

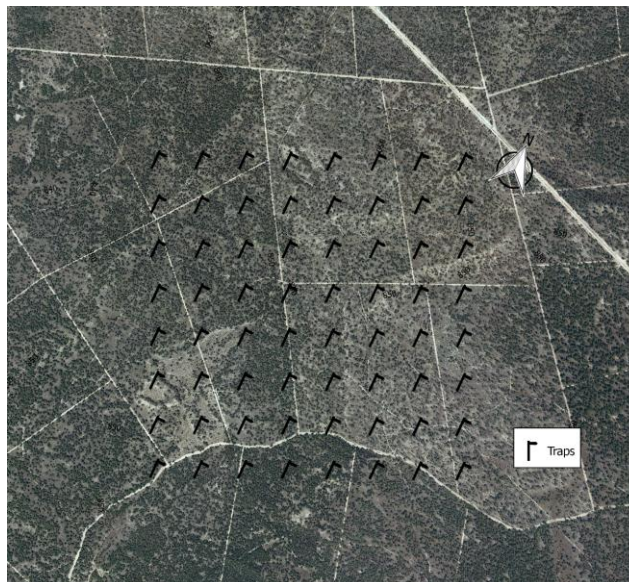


Fig. 1. Trapping set up of the mark-release-recapture dispersal experimnt. Background images are orthophotos (PNOA, Instituto Geográfico Nacional, Spain)

M. galloprovincialis adults were obtained from *P. pinaster* bolts colonized naturally in 2010 after a forest fire in the vicinity of Arenas de San Pedro (40°18'3,71"N, 5°3'W; Castilla y Leon, Spain). Mature-tagged insects were fed during 15 days with fresh *P. pinaster* twigs before release, whereas immature insects were released unfed within two days after emergence. These lab-reared beetles were released at the center of the study area during four consecutive occasions (22th -29th of June and 6th - 13th of July; 111 immature and 112 mature insects). All the insects were individually identified using numbered bee-tags (Opalith Plättchen, Christian Graze KG, Germany), glued at their pronotum.

LiDAR data

LiDAR images acquired in 2010 were obtained from the National Plan for Aerial Orthophotography (PNOA; Spanish National Center for Geographic Information). The average point density was 0.5 pulses/m² with 20 cm in altitude accuracy. An approximate digital terrain model (DTM) was produced by selecting the lowest LiDAR last return in each 5- by 5-m grid cell to be the ground elevation for that cell. All LiDAR returns were assigned to a 5- by 5-m cell in a regular grid oriented parallel with the plot sides. LiDAR heights above the ground (normalized height or canopy height) were calculated by subtracting the ground elevation from the corresponding non-ground LiDAR measurements. Directly from the LiDAR images were obtained the stratified canopy density (Evans et al., 2009), % cover up to 5m (Cover0-5), % cover above 5m (Cover5), and forest canopy cover (FCC)).

Field data were collected during the June-September, 2014. Seven traps were randomly selected and 20m fixed-radius circular plots were set considering the traps as the centers. Trees inside the plot were subdivided in: adult class, including all the trees with a dbh (diameter at breast height) greater than 7.5 cm, and regeneration class comprised of all the trees with dbh less than 7.5 cm. In every tree, the following variables were measured: height to the top (m) (Hf for old trees and Hb for regrowth), height to the lowest living branch (m) (Hc), diameter at breast height (at 1.3 m) (m) (dbh) and total number of trees (N). Diameters were measured with calipers and heights with a VERTEX III digital hypsometer. These data were used for evaluating the differences between field- and LiDAR-derived tree heights. LiDAR images were processed with FugroViewer™, FUSION (Version 3.42) and QGIS (Version 2.6.1).



Fig. 2. LiDAR digital models of calibrated variables. (See text for description of Cover 0-5, Cover 5, FCC, Hf, Hb and Hc)

Statistical analysis

We explored the relationship between LiDAR data and field dasometric variables. Field samples were used for calibration of LiDAR measurements. A scatter plots and stepwise regression analysis were used to obtain the best predictive model. QGIS was used in order to create an accumulate viewshed of each trap in relation to tree height. The seven forest descriptive variables (Hf, Hb, Hc, Cover 0-5, Cover >5, FCC and viewshed) (Fig 2) were averaged for the 200m cells considering each trap as the plot center.

Counts of captured insects were fitted against descriptive variables to a quasi-Poisson (to account for overdispersion) error distribution in a generalized linear model (GLM)(Crawley, 2008). With the aim of obtaining the simplest model, a scatter plot was used and all the non-significant interaction terms were removed. The significance of each term effect was tested with an ANOVA analysis for GLM models (*F-value*). Spatial autocorrelation was analyzed with Moran's Index using the method described by Gittleman and Kot (1990). The edge effect in the catches of external traps was calculated by the Wilcoxon-Wilcox test. The effects of sex and sexual maturation on dispersal distance and dispersal time were compared under GLM Gaussian assumptions and the significance of each term with an ANOVA analysis of the model. Two phenomenological distance location kernels (kL) were

also fitted for the recaptures of mature and immature beetles using two empirical models: 2Dt model and the negative exponential kernel (see details in Etxebeste et al., 2015). All statistical analyses were performed under the statistical programming environment and language R 3.2.0 (The R Development Core Team, 2010).

Results

Dispersion

A total of 1834 native insects were captured (982 males and 852 females). From the 223 lab-released insects, a total of 26 immature (23% of released) and 32 mature (29% of released) insects were recaptured. Of all these 58 lab-released insects recaptured, 46.55 % (11 immature and 16 mature insects) were recovered at 141.42 m distance, followed by 15.51% (5 immature and 4 mature insects) which reached 316.22 m distance from the release center. Recaptures decreased with distance to the release point, so that only 2 immature beetles (0.9% of recaptured) were found 989.95m away of it. There was no significant differences in distance of recapture between maturation stages ($F= 0.587$, $P=0.4469$) or between the interaction of sex with maturation stage ($F=1.107$, $P=0.2975$); there were however significant differences between sexes in distance of recapture ($F=4.872$, $P=0.0316$) and in dispersal time ($F=10.687$, $P=0.001$), being both greater for males. Recapture of mature beetles occurred as soon as 7 and as late as 98 days after release, whereas in immature insects it occurred from 21 to 84 days after release. A significant edge effect was obtained for the captures of natives ($W = 964.5$, $P < 0.001$) in the external traps of the set up.

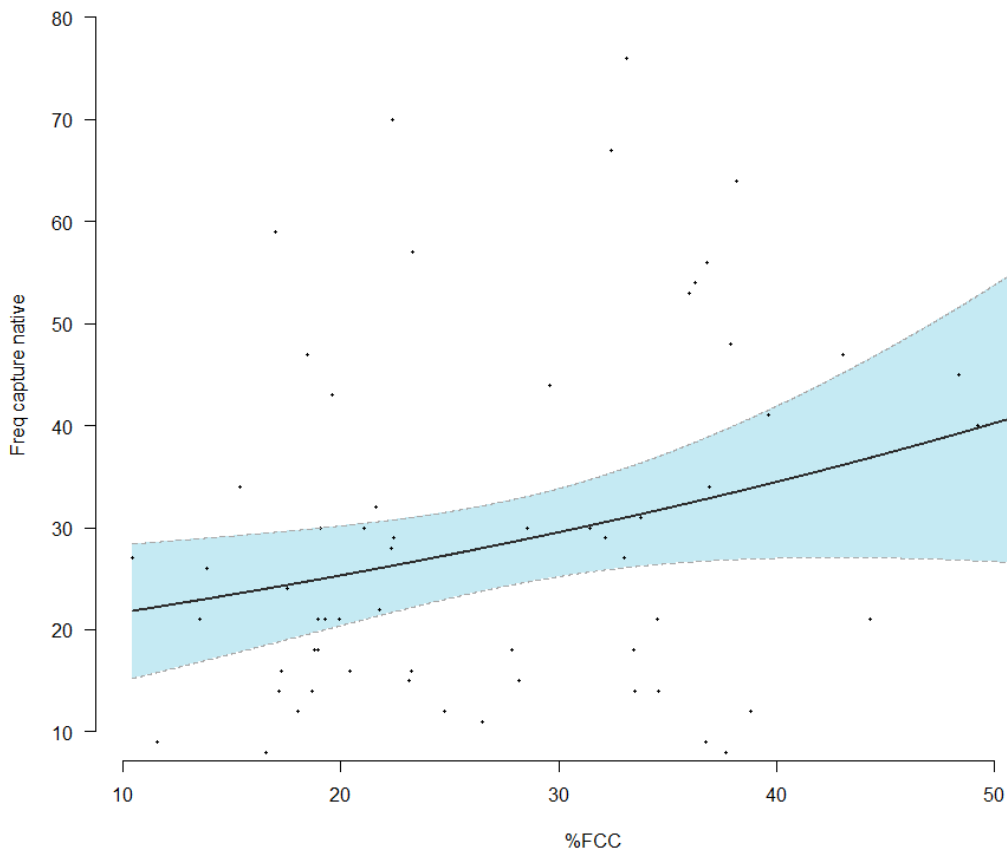


Fig. 3. Effect of FCC (% of forest cover) on number of native insects recaptured. Line corresponds to the model prediction, and shaded area to the standard error. Recaptures = $2.92 + 0.015 \times \text{FCC}$

The negative exponential showed the best fit parameters, providing the lowest AIC value of both models. In table 3, the kernels for location, distances and cumulative probabilities for dispersal computed after the fitted parameters of both models are shown. According to our results, 50% of the immature insects could be found within 347.8-365.4 m range and half of the mature insects would be within 317.7-324 m, whereas practically all the insects (99%) could be found in an interval of 1445-2013m for immatures and 1282-1561m for matures. According to results from the 2Dt and the negative-exponential models, immature beetles could be found over a distance of 1000 m with a probability of 0.07% and 0.06% respectively, whereas only 0.04% and 0.03% respectively of mature insects would be found over such distance. The widest tail was forecasted by the 2Dt model, estimating that 0.001% of the immature *M. galloprovincialis* would fly over 5000 m distance.

	r50	r67	r95	r99	over500	over1000	over5000	df	AIC
2Dt-Inmmatures	347.8	473.8	1132	2013	0.302	0.069	0.001	2	137.1
Neg.exp-Inmmatures	365.4	501.6	1033	1445	0.332	0.057	0	1	126.3
2Dt-Matures	317.7	426.8	946.3	1561	0.248	0.042	0	2	149.5
Neg.exp-Matures	324	444.8	915.8	1282	0.269	0.035	0	1	137.3

Table 3 Estimated radii enclosing 50%, 67%, 95% and 99% of dispersing *Monochamus galloprovincialis* based on fitted distance dispersal kernels (m). Alternatively, probabilities of dispersal events over 500, 1000 and 5000 m are presented.

LiDAR data

Best fitted models showed a significant good predictive behavior of the maximum height variables (Hf and Hb) and of the height of the first living branch (Hc) (Table 1) but due to the lack of adequate data it was not possible to fit a model for the dbh and N variables. Directly from the LiDAR images, the stratified canopy density (% Cover) and the forest canopy cover (FCC) (Fig. 2) were obtained.

MODEL	Adjusted R-squared	p-value
PredHf= $\sqrt{-3.589969 + 23.273839 * \text{Elev.P95} - (1.981176 * \text{Int.mode})}$	0.826	0.013
PredHb= $2.09932145 - (0.10719071 * \text{Elev.mode}) + 0.01516722 * \text{Int.maximum} - (0.54284297 * \text{Elev.MAD.mode})$	0.989	<0.01
PredHc= $3.9980624 - (0.6731551 * \text{Elev.MAD.mode})$	0.531	0.038

Table 1 Prediction models from Lidar variables calibrated with field data.

The spatial autocorrelation of the variables in the 5 by 5-m grid cell dropped within an interval of the Moran's I of 0.25-0.37, resulting into a random association among points. According to the GLM results, FCC had a significant positive effect in the amount of recaptured native insects (F= 4.428, P= 0.034) (Fig. 3). On the other hand, distance and Hb had a significant negative effect in the recapture of released insects (Table 2), while the interaction between distance and the Hb had a light positive effect in recaptures. The ANOVA analysis, however, showed that significantly changes in the number of recaptures were not detected when Hb varied (Table 2).

Class	Intercept	Hb mean	Distance	Hb mean*distance
Immature	15.59 ± 3.9 ***	-6.28 ± 1.7 ***	-0.03 ± 0.006 ***	0.01 ± 4.40 ***
		0,51	12,74***	19,46***
Mature	11.09 ± 3.9 ***	-4.14 ± 1.7*	-0.02 ± 0.007 **	0.007 ± 0.003 *
		2,48	19,16***	8,58**
Release	14.2 ± 2.62 ***	-0.03 ± 0.004***	-5.28 ± 1.1 ***	0.009 ± 0.001 ***
		0,51	23,46***	17,61***

Table 2 Summary of the patch-scale model obtained from GLM with laboratory released *M. galloprovincialis*. First line represents Estimate ± SE of the habitat variables and intercept (columns) on the dependent variable (lines) and second line *F* values. Significant values are in bold (***P < 0.001, **P < 0.01, *P < 0.05)

Discussion

LiDAR is a powerful tool capable of providing a highly accurate geospatial information to characterize forest structure. It can be used to analyze basic landscape structural features affecting species abundance and distribution. Recommendation for vegetation applications is 4–6 pulses/m² as it is well balanced between cost and support (Evans et al., 2009). In our study, however, the existing low point density (0.5 pulses/m², IGN) together with the low number of measured field plots were likely the reasons why only a predictive model of canopy heights could be fitted accurate enough. Even so, FCC could be identified as the main habitat factor affecting abundance of native beetles. Dispersing *M.galloprovincialis* adults should be in search of living trees for feeding, mates, and dying or newly dead pines for egg-laying. It makes sense that insects would look for sites with higher FCC and therefore with a greater variety of suitable host trees. FCC was correlated to maximum tree height ($r^2=0.92$, $p<0.01$) which it is usually an indicator of old trees, suggesting that in these areas native beetles would best find trees or dead branches for reproduction. Several studies on the influence of lures, type, and placement of traps, and habitat type on cerambycid catches have shown that the viewshed of the trap affected the number of captured insects (Dodds et al., 2010; Dodds, 2011). In our case, trap viewshed was negatively correlated with FCC ($r^2= -0.35$, $p<0.01$), pointing to that, even if trap viewshed is smaller with high FCC values, native captures were still greater in these areas, highlighting its importance in beetle habitat selection.

Best habitat prediction model for lab-released insects indicated that distance was an important factor. Thus, according to the fitted 2Dt and the negative exponential models, 50% of recaptures should be observed within a maximum distance of 317-365m. Impact of landscape features on pest abundance could be influenced by edge effect (Costa et al., 2013). Then, traps along the edge of the set up would capture a disproportionate amount of insects,

even if stand features were not most suitable and this may be happening for laboratory insects that were released in the center of the plot. Here, models indicated that distance affected negatively abundance (table 2), seen as a higher proportion of recaptures in the traps nearest to the plot center. Therefore, high captures may occur in these center traps in areas without optimal habitat conditions. Thus, the fact that higher recaptures were associated to lower values for regrowth height could be an artifact since in this central area regrowth was of low height. The effect of distance on recaptures can generate pseudo-absence data in traps placed further inland inducing errors in the models. Native beetles, in the other hand, were in their natural habitat when the trapping set up was installed and, even if accounting for a potential sink effect, pheromone-traps, would capture insects proportionally to the suitability of natural characteristics in the area, not being influenced by distance of release. In addition, other factors such as predator occurrence, temperature or wind speed and direction would determine transportation, deposition and establishment of insect populations where there are suitable hosts and climate conditions (de la Giroday et al., 2011).

Density of pheromone traps was selected to both, maximize recapture and minimize trap interference, in accordance to previous studies, and obtained recaptures, 23-29% of released insects, laid well within the range reported by them (Etxebeste et al, 2015, Sanchez-Husillos et al., 2015). Several studies on *M. galloprovincialis* dispersal have reported that most recaptures of released beetles were recorded in traps closest to release point (Gallego et al., 2012; Mas i Gisbert et al., 2013; Torres-Vila et al., 2014; Etxebeste et al., 2015). Our results are in agreement, with it, but, distance for 99% probability of recapture resulted lower than the expected distance (2344-3495m upon on replica and model) pointed by Etxebeste et al (2015). In the present study, values for 99% probability of recapture ranged from 1282m (mature beetles, negative exponential model) to 2013m (immatures, 2Dt model). Beetle initial stage of maturation did not influenced dispersal, though immatures appeared to disperse somewhat further. This, result does not agree with that reported by David et al(2013, 2015) of increasing flight distance with age of *M.galloprovincialis* in a flight mill. It must be bared in mind that lab released immature beetles only held such condition during their first 12-13 days after release. It would be then most interesting to differentiate dispersal during this short immature period from dispersal once beetles became mature, but lack of response of immatures to know attractants (Álvarez et al., 2015b) made it impossible to know. The fact that most mature and immature insects were recaptured in the traps closest to the center is interfering with the observation of beetle habitat selection. Similarly, lack of response of immatures increases the

uncertainty for predictions on possible differential habitat selection by these beetles. We found that *M. galloprovincialis* males dispersed further away and during more time than females and this, together with its known earlier emergence (Naves et al.2008; Togashi & Magira, 1981; Shibata, 1999), may point to an strategy of finding suitable hosts before the later arrival of females.

Our understanding of the influence of forest structure on *M.galloprovincialis* is still limited, but results reported here illustrate how habitat differences at patch-scale may exert a strong influence on beetle abundance. Remote sensing measurements used to model insect distributions should ideally be related to the scale at which beetles discriminate habitat characteristics (Scott et al. 2002), as well as the scale at which managers make conservation priority decisions.

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Chapter 5: Effectiveness of mass-trapping in the reduction of *Monochamus galloprovincialis* Olivier (Col.: Cerambycidae) populations.

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Introduction

The pine wood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner and Bührer; Nematoda: Aphelenchoididae) is considered a weak pathogen in its original range in North America (Wingfield et al. 1982). PWN was introduced in Asia causing massive tree mortalities (Zhao et al. 2008). In Europe, it was first reported on a *Pinus pinaster* Aiton tree in Portugal (Mota et al. 1999). Thereafter, three distant infestation foci in areas close to Portugal are under eradication in Spain (e. g. Abelleira et al. 2011). The spread of the PWN might occur via the transportation of infested woody material, or through the activity of the adult stages of beetles of the genus *Monochamus* (Coleoptera: Cerambycidae) through feeding on shoots of susceptible living trees, or oviposition on dying or recently dead trees (EPPO 2009; Futai 2013; Linit 1988).

Soon after the detection of the PWN in Portugal, *Monochamus galloprovincialis* (Olivier) was shown to be its vectoring agent (Sousa et al. 2001). Although *Monochamus* beetles had previously been considered secondary pests, the introduction of the PWN, has instigated increased efforts devoted to understanding its biology and ecology (Akbulut and Stamps 2012). With this, the study of their chemical ecology has shown how host and bark beetle kairomones can be used to attract them (Allison et al. 2001; Francardi et al. 2009; Ibeas et al. 2007). Furthermore, a male-specific pheromone, 2-undecyloxy-1-ethanol, has been shown to be very attractive for both sexes of *M. galloprovincialis* (Pajares et al. 2010), for *M. sutor* (Pajares et al. 2013) and for other members of the genus (Macias-Samano et al. 2012). A very attractive pheromone-kairomone lure has since been available for trapping *Monochamus* beetles. The combination of this lure and the availability of enhanced trap designs (Álvarez et al. 2014) represents one effective method for monitoring the vectors of PWD, but might also be used as a direct control method of the vector population. In fact, the European Union implemented measures to control the disease that include the felling and destruction of all susceptible plants in a minimum radius of 500m around the infected trees

and intensified surveillance for its presence in a demarcated area that covers 20km around the infestation focus. (EC, Decision 2012/535/EU).

So far, the control of the disease has proved difficult once *B. xylophilus* has been introduced into a susceptible tree species. Removal of symptomatic trees after intensive monitoring is the only practical method that has shown some success in the control of PWD. Other methods such as tree injection or preventive pesticide sprays (Zhao et al. 2008) are either impractical on a whole-forest scale or environmentally unacceptable. Mass trapping of the vector might provide a more proactive management strategy for the eradication or the containment of the PWD. European foresters are reported to have used 'trap-trees' for over 200 hundred years to massively remove damaging bark beetles (Coleoptera: Scolytinae), taking indirect advantage of the attractive compounds released by piled host logs (Niemeyer 1997). Nevertheless, substitute use of traps baited with infochemicals to reduce bark beetle population levels was not considered as an alternative to trap-trees until the discovery of their pheromone blends (El-Sayed et al. 2006). To date, mass trapping has been studied as a control tool for a wide range of insect pests but results have not always been successful. Examples within Coleoptera include the first reported mass trapping attempt carried out in 1970 on bark beetles (Coleoptera:Scolytidae), showing promising results (Bedard and Wood 1974). A large scale mass trapping campaign was also conducted in Scandinavia with the aim of controlling *Ips typographus* (L.) (Weslien 1992), and the effect of a long term mass trapping campaign on an isolated stand was also studied (e. g. Schlyter et al. 2001).. In the case of long-horned beetles, very few studies have been reported. Maki et al. (2011) showed that both mass trapping and mate disruption could help lower the population densities of *Prionius californicus* Motschulsky in hop production yards. On the other hand, two works have shown how mark recapture studies can be used to evaluate mass trapping of *Cerambyx welensii* Küster through comparisons of estimated population densities and trap catch levels (Torres-Vila et al. 2012; Torres-Vila et al. 2013). El-Sayed et al. (2006) concluded that mass trapping has the potential to suppress or eradicate low-density, isolated pest populations. In fact, this effect on the target population is known to increase as its density decreases (Barclay and Chao 1991). This scenario fits the case of newly detected PWD foci well, in which vector population build-up has not occurred yet, and the removal of many individuals would decisively help suppress the infestation of new hosts. Furthermore, *M. galloprovincialis* reproduces in univoltine life cycles in southern Europe, and semivoltine in the north (Akbulut and Stamps 2012), which is a recognized advantage of mass trapping (El-Sayed et al. 2006).

However, factors related to the target insect or to the trapping methodology might severely affect the success of mass trapping. Population density, rates and patterns of population increase, mobility, migration, and dispersal capacities of the target insect are key factors that can drastically reduce the success of mass trapping. Hence, in order to evaluate the real effect of mass trapping, estimates of the initial population density are mandatory. Trap catches of *M. galloprovincialis* during monitoring and research trials suggest that this beetle generally occurs at low densities (e. g. Álvarez et al. 2014; Francardi et al. 2009; Pajares et al. 2010), but absolute estimates are lacking. Demographic studies of insects using mark-recapture procedures have shown promising results, and examples of studies of long-horned beetles are available (e. g. Drag et al. 2011; Tikkamäki and Komonen 2011; Torres-Vila et al. 2012; Torres-Vila et al. 2013), including an early work that studied the abundance of *M. alternatus* Hope in a small PWD affected *P. thumbergii* Parl stand in Japan (Shibata 1985).

On the other end, technical details behind mass trapping campaigns are also very important. Works evaluating the design of the trapping device have shown that modified multiple funnel (Lindgren 1983) or cross vane traps are well suited for the catch of *M. galloprovincialis* (Álvarez et al. 2014; Rassati et al. 2012). Also, lures available for *M. galloprovincialis* (Pajares et al. 2010) have been shown to obtain high catches in the field (Álvarez et al. 2014; Pajares et al. 2010; Rassati et al. 2012). Yet, the effect that different trapping densities could have on *M. galloprovincialis* populations is not known precisely, nor have other technical details been studied.

Thus, considering the trapping technology available, two field trials were conducted in order to assess the effect of mass trapping on *M. galloprovincialis* populations with the aim of developing a method for reducing the vector population as support for the eradication or containment of the disease in PWD affected areas. For this, our major goals were (i) to evaluate a valid method to estimate population abundances of this insect, so that we could (ii) study mass trapping efficiency, i.e. the proportion of adults that could be removed from the target population, and (iii) to estimate the relationship between trap density and vector removal so forest health managers could choose the optimal trap density suited to their objectives.

Materials and Methods

***Monochamus galloprovincialis* population estimates.**

The first experimental approach to the estimation of population densities of *M. galloprovincialis* was carried out along side other capture-mark-recapture (CMR) experiments, also currently in the process of publication, studying the dispersal capacity of this insect. Within the trial, both native and lab-reared beetles were tracked down using a grid of infochemical baited traps. The study was conducted in a 224 ha pine stand located in Villota del Páramo (42°32'26"N, 4°51'56"W 1003 m a. s. l., Castilla y Leon, Spain), from the 18th of June to the 30th of September 2010. The study area (Fig. 1a) was composed of reforested stands of *P. pinaster* (8%), *Pinus nigra salzmannii* Arnold (78%) and *Pinus sylvestris* L. (14%), with *Quercus pyrenaica* Willd. growing patchily in the understory. Multiple funnel traps were modified to prevent the escape of *M. galloprovincialis* by applying a Teflon® coating to the collection cup and the inner face of the lowest funnel (ECONEX S.L., Murcia, Spain; Álvarez et al. 2014). Fifty-two traps were placed in the centres of 4 ha square cells (200×200 m; 0.25 traps ha⁻¹ ; Fig. 1a).

In addition four additional traps covered the closest 1 ha cells away from the center with the aim of detecting individuals that could have remained close to the release point. Traps were hung from ropes between trees with the collection cup placed at least 50 cm from the ground. The commercial *M. galloprovincialis* lure kit (Gallopprotect 2D®, SEDQ S. L., Barcelona, Spain), containing two bark beetle kairomones (ipsenol and 2-methyl-3-buten-2-ol) and the pheromone compound (2-undecyloxy-1-ethanol) identified by Pajares et al.(2-undecyloxy-1-ethanol; 2010) was complemented with (-) α -pinene (Gallopprotect Plus ®, SEDQ S. L.). Lures were replaced every six weeks.

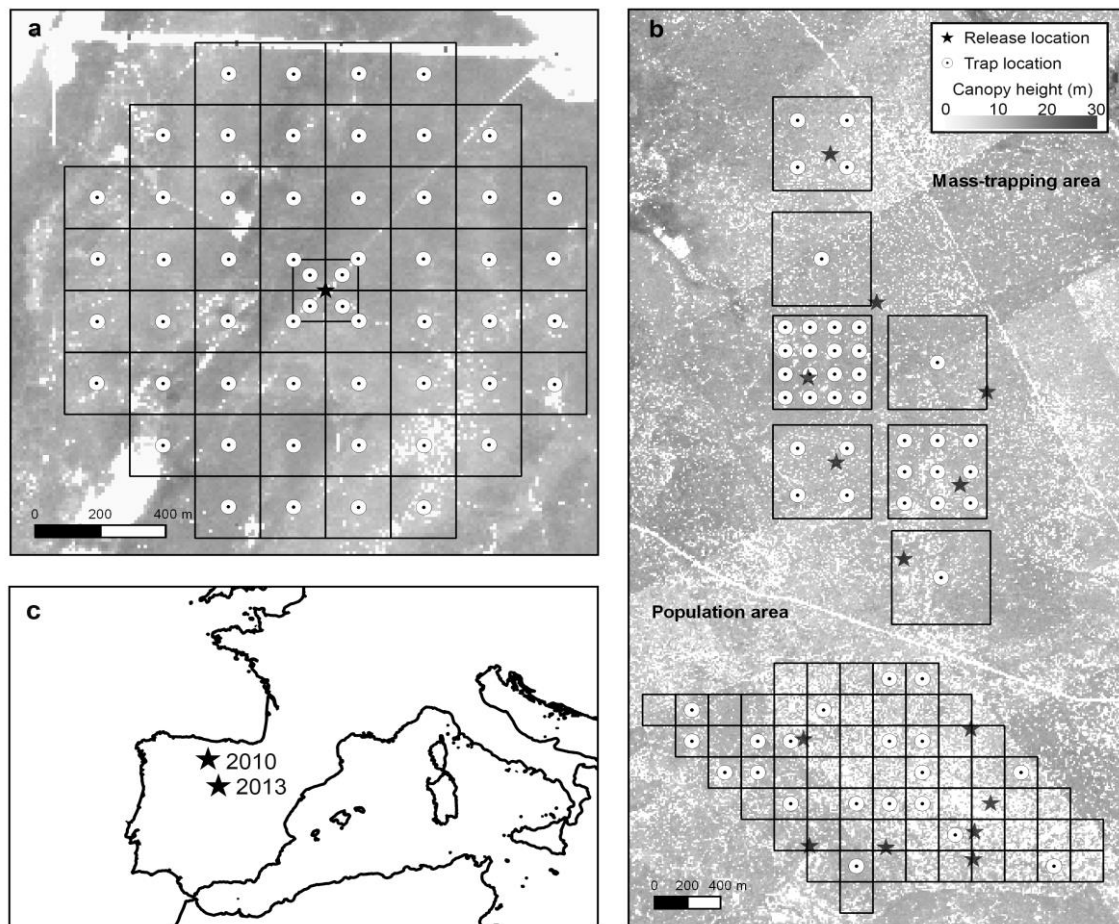


Fig. 1 Spatial disposition of traps and experimental plots at the field trials carried out in (a) Villota del Páramo (Palencia, Spain) in 2010 and (b) Cuellar (Segovia, Spain) in 2013. Circles depict trapping positions, while stars refer to lab-reared beetle initial release points. Background raster depicts canopy height models derived from airborne LiDAR data (PNOA, Instituto Geográfico Nacional, Spain). See Materials and Methods for further details regarding sampling design. The geographical location of the experiments by years is also presented (c).

Lab-reared beetles were released at the centre of the study area with the intention to serve as a positive control of the evaluation of the population estimates. These beetles were collected during early spring, 2010 in the vicinity of Arenas de San Pedro (Castilla y Leon, Spain; 40°11'02"N, 5°02'22"W) from *P. pinaster* bolts infested with *M. galloprovincialis*. Upon emergence (from mid-June to early-August), collected beetles were marked, using coloured, sequentially numbered bee tags glued at their pronotum and then placed individually in glass jars. Beetles were not fed and were kept in growth chambers for a maximum of three days until their release (15L:9D, 25°C:20°C). With this, 353 lab reared *M. galloprovincialis* imagoes were released from the middle of the set up (Fig. 1a) four weeks in a row, rendering batches of 39 (22♀,17♂), 106 (53♀,53♂), 142 (66♀,76♂) and 66 (34♀,32♂) individuals released on the 29th of June, and the 6th, 13th and 20th of July respectively.

Traps were then checked weekly for lab-reared and native beetles. In the case that any native *M. galloprovincialis* individual was caught, if it was judged to be in good shape to disperse further, a number was painted on its elytrae using fine-tipped waterproof paint markers. These and any recaptured beetles were released at least 100m away from the source traps to minimize the interference of the traps with their dispersal. Individuals found dead or in bad shape were tallied as losses during sampling occasions.

In 2013, a second experiment simultaneously evaluated the native population density and the removal of *M. galloprovincialis* by using different trap densities within a large continuous stand of *P. pinaster*. The area was located in the municipality of Cuellar (41°17'7"N, 4°13'22"W 854 m a. s. l., Castilla y Leon, Spain). Although the experimental area was almost completely covered by *P. pinaster*, scattered *Pinus pinea* L. trees were present throughout the stand. Special care was taken to select forest units that were of similar tree age and density (Fig. 1b). The field trials were conducted between the 4th of June and the 21st of October 2013, covering the entire flight period of the target insect.

The experimental site was subdivided into two similar contiguous zones so that the effect of different trap densities on mass trapping could be assessed. For this purpose in mind, the southern sector was used to estimate the local population density of *M. galloprovincialis* ("Population area", Fig. 1b), while in the northern area seven square subplots (600×600 m), with ca. 100 m wide buffer zones between them were used to test four trapping densities ("Mass-trapping area", ca. 296 ha; Fig. 1b). The population area (ca. 260 ha) was established using six contiguous forest units of similar stand characteristics. A regular grid of 4 ha cells were defined over the area, and then 20 cells were selected randomly. Multiple funnel traps were placed in the centre of the selected cells. This time, traps in both areas were hung from inverted "L" shaped metal poles. Also, taking into account the improvements suggested for the trap design, ECONEX S. L. (Murcia, Spain) provided traps that had all its surfaces coated, including the collection cups, with Teflon®. The length of the collection cup was doubled in relation to the original one, and the bottom of it had been converted to a wire mesh, enhancing air circulation within the cup (Álvarez et al. 2014). In addition, during each sampling newly cut pine shoots with needles were placed within the cup in such a way that food and shelter would be provided for trapped *M. galloprovincialis* individuals. Traps were baited with Galloprotect Pack®, and lures were replaced every six weeks. All traps were checked weekly during the entire sampling season. Trapped individuals were marked and released following the previously described procedure in the 2010 experiment.

As in the previous trial, lab-reared beetles were released as a positive control of the method. To rear the beetles, 140 cm long *P. sylvestris* logs sections were placed at the edge of fire cuts and were baited with the commercial lure Galloprotect 2D® at Tabuyo del Monte during the spring of 2012 (Castilla y Leon, Spain; 42°17'45"N, 6°10'52"W). Colonized logs were brought to the lab and stored in an outdoor cage over winter. As in 2010, seven batches were released in the "Population area" on the 18th of June (9 and 20 individuals), 25th of June (20 individuals), and on the 26th of July (20, 21, 24 and 15 individuals). In addition, batches of 20 to 40 lab-reared beetles were released on the 9th, 16th and 23rd of July at random locations at the "Mass-trapping area" sub-plots. In total, 188 females and 211 males were released in from 14 randomly selected sites (Fig. 1b).

The effect of trapping densities on population removal

As introduced above, the northern area of the 2013 experimental site ("Mass-trapping area", Fig. 1b) contained seven 36 ha subplots, each of them assigned one of four tested trap densities. Three subplots had only one trap in the centre, corresponding to a trap density of 0.02 traps/ha. In two subplots four traps were installed following a regular grid (300 m inter-trap distance; 0.11 traps/ha). The remaining subplots had nine traps (200 m inter-trap distance; 0.25 traps/ha) and 16 traps in a regular grid (150 m inter-trap distance; 0.44 traps/ha) respectively (Fig. 1b). Insects trapped in the mass-trapping area were removed.

Statistical analyses

All analyses and calculations were performed using the R statistical environment and language (The R Development Core Team 2014). Demographic analyses were conducted using the RMark library as an interface for the MARK package (Ivan 2008), under the assumption that *M. galloprovincialis* populations during the sampling periods were of the open type, i. e. births, deaths, emigration and immigration occurred during those periods. By following the Jolly-Seber methodology under the POPAN parameterisation, three primary parameter groups can be estimated which lead to three obtained parameters: births (B_i), population size (N_i) at the sampling interval, and the total population size (superpopulation, \hat{N}_{Tot}). The primary parameters represent: p_i , the probability of capture at occasion i ; ϕ_i , the probability of an insect surviving between occasions i and $i + 1$; and b_i , the probability that an insect from the superpopulation would enter the population between occasions i and $i + 1$ (also referred as p_{enti} , probability of entrance). These primary

parameters were modelled as to be constant, (\cdot) in MARK notation, linearly dependent on time (T), to differ between sexes (Sex), or have additive or multiplicative interactions between sampling occasion and sex (T×Sex). A range of models for each primary parameter were defined and then run. Factorial treatment of time was not considered due to the fact that more than one sampling occasion lacked recaptures in all data-sets. Best models were selected using the quasi-Akaike information criterion (AICc). \hat{N}_{Tot} estimates were derived from weighted average parameters of best models ($\Delta AICc < 10$). Losses-on-capture, i. e. beetles found dead or in bad shape, were accounted for in the computation. In addition, for the analysis of lab-reared beetles, a second range of models was adjusted by fixing parameter b_i to zero, i. e. considering that no new individuals were recruited by the superpopulation during the experimental period.

To evaluate the effect of trapping on the *M. galloprovincialis* population, N_{Tot} estimates needed to be transformed into density (D) units, putting the state of the population into a common currency. The estimation of D implies assuming some sort of geographical closure, which in turn likely violates the openness of the population under study (Ivan 2008). Yet, for evaluation purposes, three different effective sampling area calculations were considered. These were the area defined by our own seasonal sampling range calculation for the used traps and lures (sensu Turchin and Odendaal 1996; 561.76 ha), the mean maximum distance covered by the beetles between trapping events (see Ivan 2008, and reference therein; 517.43 ha; Wilson and Anderson 1985) and the defined study area (the naive density estimator, Wilson and Anderson 1985; 260 ha). Then, the last one was chosen as the smallest and hence the most conservative in terms of pest management, i. e. it would yield the highest attributable D value and hence the lowest proportion of beetles removed.

Accumulated trap catches at the mass-trapping area in 2013 were analysed in three distinct ways. First, absolute catches per trap were fitted against treatment (trap density) and block factors (the subplot at which they were placed) in a generalized linear model (GLM) with a Poisson error distribution. Means were later separated using Tukey's HSD test with Bonferroni's adjustment. Second, accumulated catches per experimental plot for each tested trap density was plotted. Due to the low number of replicates, parametric tests were not applied. Yet, when those values were put in relation to the estimated D value, a logistic regression could be fit relating the estimated percentage of removed population and the trap density used. The regression was fit under GLM assumptions and a quasibinomial error distribution to account for overdispersion. Trap densities at which 50% and 95% of the population could be removed (named TD50 and TD95) were computed on the fitted GLM.

Finally the effect on the *M. galloprovincialis* population of 5 successive years of mass-trapping carried out at the four tested trap densities was simulated. For this purpose a stochastic stationary Gompertz model was defined and iterated 1000 times with or without mass-trapping (Holmes et al. 2014). The hypothetical carrying capacity was arbitrarily set to 100 individuals per hectare with a 100% population growth rate, although the initial population density was set to 82 individuals per hectare, deriving from the estimated actual population in 2013. Weak density dependence was assumed for this hypothetical *M. galloprovincialis* population (0.78). The process variance was set to 0.01. Although the effectiveness of mass-trapping is known to increase with lowered population densities (Barclay and Chao 1991), for illustration purposes, it was held constant during simulations.

Results

***Monochamus galloprovincialis* population estimates**

The field trial in 2010 resulted in 102 lab-reared beetles that were recaptured once (28.89% of the released beetles; 60.53% females and 39.47% males), of which 12 could be recaptured at least a second time. During trap emptying, 22.8% of lab-reared beetles were found dead or dying (losses on capture). Altogether, CMR resulted in 114 detections during 13 sampling occasions that allowed model fitting. The best-fitting Jolly-Seber models (Table 1) showed time dependent survival probabilities (φ), whilst the capture probability (p) either remained constant or was time and sex dependent, when entrance probability (b) was allowed to be computed or was fixed at zero. Although the derived population sizes under both modelling scenarios overestimated the actual figures, they fell within one standard error of the released 175 female and 178 male lab-reared beetles (Table 1), and hence the accuracy of the abundance estimates derived from CMR data was considered appropriate.

Regarding the native insects, 41 individuals were captured in 2010. Only eight were recaptured, yielding a total of 49 detections that could be used for the fitting. Furthermore, 28.6% were lost on capture. The mean distance between consecutive capture locations was 680.10m, with a maximum distance of 1280.78m and a minimum distance of 100m between detections of each individual. A local superpopulation of 116 individuals could be derived from the best Jolly-Seber models (Table 1). The best-fitting model pointed to a time dependent capture probability, whereas remaining parameters held constant.

Year	Insect Source	$N_{released}$	Best models	No. P.	AIC _c	Abundance ($\hat{N}_{Tot} \pm SE$)		
						Females	Males	Total
2010	Lab-reared	♀175; ♂178	$\{p., \varphi_T, b_{T \times Sex}, N.\}$	8	217.97	296±123	277±127	573
			$\{p_{T \times Sex}, \varphi_T, b_{fixed=0}, N.\}$	7	230.49	242±85	216±85	458
	Native	$\{p_T, \varphi., b., N.\}$	5	109.01	-	-	115.98±61.49	
2013	Lab-reared	♀188; ♂211	$\{p_{T \times Sex}, \varphi_T, b_{fixed=0}, N.\}$	9	135.66	143±151	132±151	275
	Native		$\{p_{T \times Sex}, \varphi_T, b_T, N.\}$	9	2468.38	10835±1289	10484±1282	21319

Table 1 Summary of best fitting Jolly-Seber models (POPAN parameterisation) and derived population abundance ($\hat{N}_{Tot} \pm SE$) estimates of lab-reared and native *Monochamus galloprovincialis* at the field trials carried out in Villota del Páramo (Palencia, Spain) in 2010 and Cuellar (Segovia, Spain) in 2013. Model parameters were held constant (.), fixed (e.g. 0), differed between sexes (Sex), responded to time in a linear (T) manners or could present interactions (e.g. T×Sex). No. P, stands for number of parameters in the model. \hat{N}_{Tot} estimates derived from weighted average parameters of best models ($\Delta AIC_c < 10$). $N_{released}$ summarize the number of lab-reared beetles released at each study area.

During the second experimental season in 2013 a total of 2836 native insects were registered in 2968 detections and 19 occasions. Of these 50.11% corresponded to female and 49.89% to male *M. galloprovincialis* individuals. Losses on capture accounted for 54.2% of total registered beetles. The mean distance between consecutive capture locations was 518.90m with a maximum distance between detections of each individual of 694.55m and a minimum distance of 100m. Mean maximum distance covered by the beetles between trapping events was computed at 523.02m. The best-fitting Jolly-Seber model revealed that survival and entrance probabilities for native beetles in 2013 were time dependent (Table 1). A time-sex interaction could be derived in the capture probability as well. Weighted averaging of models under POPAN parameterisation resulted in the superpopulation estimation size of 21319 *M. galloprovincialis* individuals. Weekly estimates ranged between 291 and 1138 individuals with a slight bias towards females (Fig. 2c).

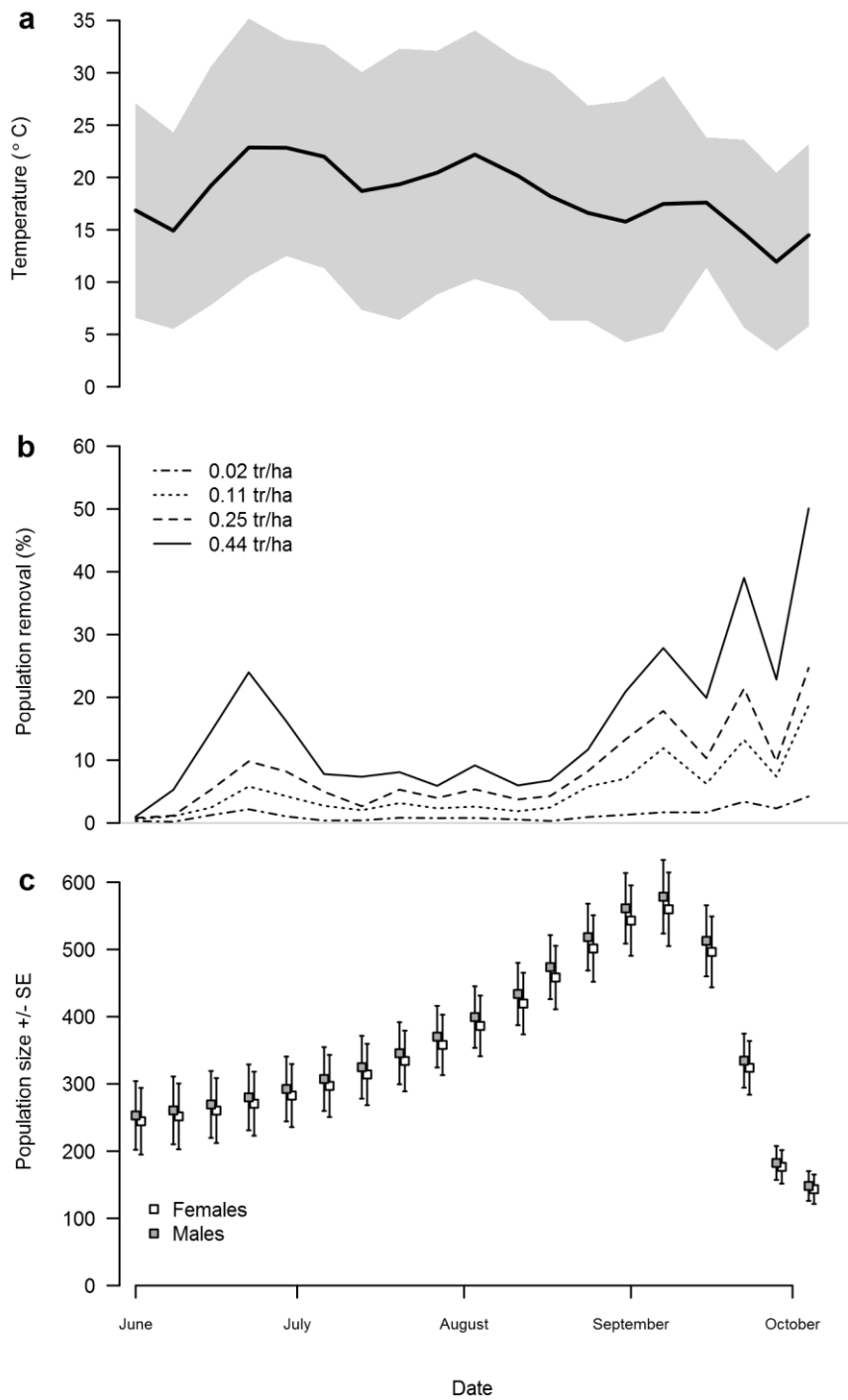


Fig. 2. Temporal evolution of (a) mean temperatures, (b) estimated population removals by trap densities, and (c) *Monochamus galloprovincialis* population abundance estimates by sexes per week in the 2013 experiment at Cuellar (Segovia, Spain). Temperatures were retrieved from the Spanish Meteorological Agency's station located at the same municipality. The polygon depicts the mean of daily maximum and minimum temperatures per week, whilst the weekly mean for the daily average temperature is represented by the black line. Estimation of population removal was based on the *M. galloprovincialis* population density corresponding to the study area (260 ha). Means per trap densities and sampling week are presented. Weekly population densities derived from the mark recapture data modelled under POPAN method and from the weighted average of best model parameters.

On the other hand, only 44 lab-reared insects were recaptured (36.67%) in 47 detections (54.17% females and 45.83% males), yielding 3 net recaptures that could be used for fitting demographic models. Most of fitted models with this data had parameters that did not converge or resulted in singular parameters probably due to the extremely low second recapture figures. Table 1 presents the most accurate of them, modelled under no-new-entrance probability. The figure of 399 beetles released in the study area was underestimated by the model (275) yet remained realistic.

The effect of trapping densities on population removal

A total of 5113 native insects were captured during the whole trapping period at the mass trapping area in 2013. Of these, 47.8% were males and 52.2% were females. The abundance figure calculated for the nearby “population area” was transformed to density considering the size of the design area (260 ha), yielding a reference of 82 *M. galloprovincialis* individuals ha⁻¹ density value for the superpopulation. Weekly abundance estimates transformed to densities were used to calculate the population removal percentages during the sampling period (Fig. 2b). A noticeable reduction in trap catches occurred after the fourth sampling week, and these did not increase again until mean maximum temperatures fell below 30 °C (Fig. 2a). Significant differences in accumulated mean catches per trap were found between different trapping density plots (Analysis of variance of fitted GLM on trapping density effect, F_{3, 32} 5.38, P(>F) 0.004; Fig. 3a).

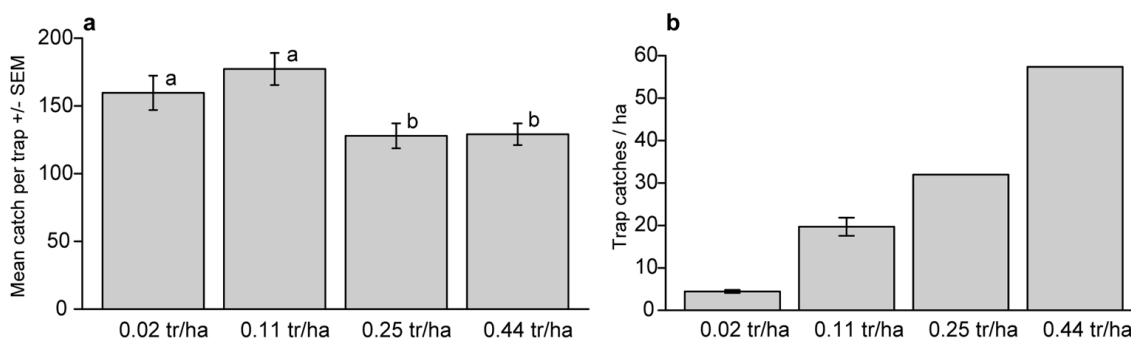


Fig. 3 Bar plots representing mean *Monochamus galloprovincialis* catches ±SEM per trap and trapping densities (a) and mean *M. galloprovincialis* catches per hectare ±SEM at Cuellar (Segovia, Spain) during the 2013 experimental season. Bars sharing the same letter are not significantly different (Tukey’s HSD, Bonferroni’s adjustment, $P < 0.05$).

Although the highest value per trap was recorded at a trapping density of 0.11 traps ha⁻¹ (177.25±11.84 SEM *M. galloprovincialis* individuals per trap), the plot with 0.44 traps ha⁻¹ caught a total of 2065 individuals (129.06±8.01 SEM *M. galloprovincialis* individuals per trap), providing the highest catch per unit area (57.36 insects ha⁻¹; Fig. 3b).

Fig. 4 shows the values for the estimated population removal of tested trapping densities, and the curve fitted after the logistic regression. Calculations were done using the estimated 82 individuals ha⁻¹ D value for native beetles. The slope parameter was found to be significantly higher than zero ($P > |t| 0.025$), and the computation of the TD50 and TD95 values, the trap densities required to remove 50% and 95% of the estimated population for the sampling plot, forecasted values of 0.37 ± 0.04 SE traps ha⁻¹ and 0.82 ± 0.11 SE traps ha⁻¹ respectively. More specifically, the highest density of traps removed 59.80% of the estimated population, and, as the density of traps decreased, the percentage of extracted population exponentially decreased to 4.66% of the extracted population at the density of 0.02 traps ha⁻¹ (Fig. 4).

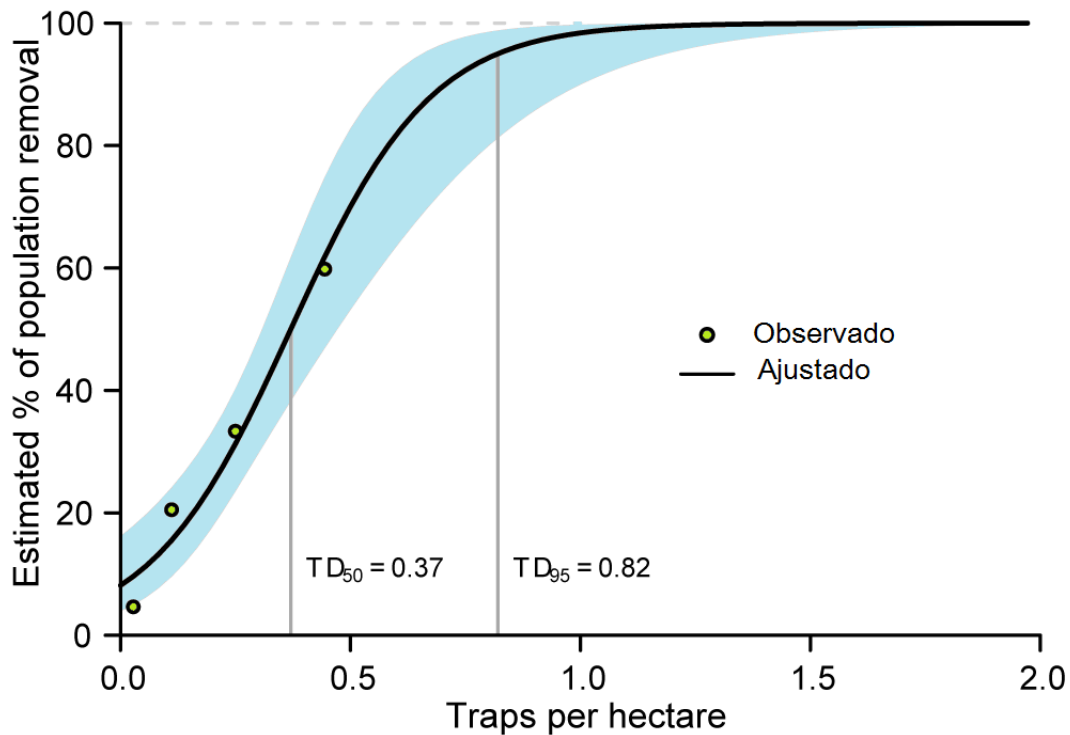


Fig. 4 Logistic regression fit of the estimated removed number of *Monochamus galloprovincialis* in relation to increasing trapping densities within the mass trapping area in Cuellar (Segovia, Spain) during the 2013 experimental season. Shaded region represents 95% confidence interval of the fitted response. Estimated TD₅₀ and TD₉₅ values of traps per hectare required to remove 50 and 95% of the local populations are presented

An additional figure to show the mass-trapping effectiveness could be computed using the estimated abundance of native *M. galloprovincialis* in 2010. The capture of 41 native insects would have resulted in the reduction of 35.34% of the population at the trapping density of 0.25 traps ha⁻¹. In 2013, the estimated removal at the same trapping density was slightly lower, accounting for a 33.33% reduction in the local plot abundance.

Discussion

M. galloprovincialis was considered a secondary pest until its association with *B. xylophilus* was proven (Sousa et al. 2001). Yet, this saproxylic beetle causes little, if any, disturbance to management goals in forestry. Furthermore, the boring activity by its larvae occurs within the early successional stages of the saproxylic habitat, and it is very likely that, as has been shown for other beetles of the same genera (e. g. Saint-Germain et al. 2004), *M. galloprovincialis* provides valuable ecosystem services during the decomposition process of conifer wood. It is only in PWD affected areas where *M. galloprovincialis* reaches pest status. It must be stressed that the population control measures discussed within this work only apply to those areas and should be considered within the framework of integrated pest management directed, in this case, to the containment and eradication of PWD.

Direct pest control by mass trapping depends on the availability of suitable trapping systems. Very efficient lures and traps are currently available for mass trapping of several *Monochamus* species (Álvarez et al. 2014; Macias-Samano et al. 2012; Pajares et al. 2010; Pajares et al. 2013; Rassati et al. 2012). Furthermore, and as shown by our results, the commercial pheromone-kairomone lure attracted both sexes of *M. galloprovincialis* equally (see also Álvarez et al. 2014; Pajares et al. 2010) making direct control of the PWN vector more feasible. Recent developments in multiple funnel and in cross vane traps have greatly improved trap performance (Álvarez et al. 2014). Yet, other factors must be accounted for in order to evaluate the effectiveness of mass trapping in population removal. Among these, an accurate estimate of the target population and sound knowledge of the pests' dynamics are of particular importance.

Population abundance estimates derived from CMR data of lab-reared *M. galloprovincialis* were fairly consistent with the true values (Table 1), indicating that estimated abundances for native beetles would also fall within their true values. If anything, values in 2010 indicated slightly overestimated abundances. Contrary to precautionary principles in conservation biology (Tikkamäki and Komonen 2011), overestimated values are less of a problem for integrated pest management. Best supported models for native *M. galloprovincialis* in 2013 pointed towards linear relationships with sampling occasions in all three primary Jolly-Seber parameters. Furthermore, the interaction between sex and time in probability of capture (π_i) was highlighted by this model. Trap catches have been reported to be slightly female biased for *M. galloprovincialis* (Pajares et al. 2010), and, as

shown in Fig. 2, traps tended to remove higher proportions of the estimated population towards the end of the summer. Hence, the model structure for the capture probability parameter seems to reflect observed phenomena. Similarly, individual recruitments occurring both through immigration and new beetle emergences are thought to have occurred during the sampling period. In fact, beetle emergences at the outdoor lab extended until mid-August, which is also reflected in the recruitment parameter. Finally, survival and emigration patterns of *M. galloprovincialis* populations are unknown. Yet, high rates of losses of captured beetles, especially during the warmest sampling occasions, were observed.

Estimated absolute population sizes needed to be transformed to density units in order to evaluate the effect of trapping density on the *M. galloprovincialis* population. Yet defining the geography that encloses the estimated population abundances (N^{\wedge}) is generally difficult to attain (Wilson and Anderson 1985). The native density estimator, $D^{\wedge}=N^{\wedge}/A$ using the area covered by the trapping grid, results in the overestimation of D^{\wedge} as a consequence of ignoring edge effects (Wilson and Anderson 1985). The area derived from the mean maximum distance covered by the recaptures is a common measurement used to reduce such bias, as it approximates the home range of the recaptured individuals (Ivan 2008). In this study, the area derived from the mean maximum distance covered by the beetles between trapping events (517.43 ha) would have meant an estimated superpopulation density of 41 individuals ha⁻¹, which, in turn, would have doubled the effectiveness of mass-trapping. Assuming the effective sampling area to be the experimental area (260 ha) increases such density and provides the most conservative estimation. Jolly-Seber models have been used for the estimation of PWN vector population sizes. Shibata (1985) reported *M. alternatus* population estimates that reached densities over 3500 individuals ha⁻¹ in a stand sustaining 48% of *P. thumbergii* Parl tree mortality in Japan. Similarly, Togashi (1988), studying the link between *M. alternatus* populations and the incidence of PWD in a 0.05 ha *P. thumbergii* stand, computed densities of native *M. alternatus* of up to 1176 individuals ha⁻¹.

Increased trap densities resulted in increased catches per plot (Fig. 2b and Fig. 3). Thus traps deployed at the highest trap density (0.44 traps ha⁻¹) were able to catch 57.36 beetles ha⁻¹ (2065 within the 36 ha plot), or 59.80% of the estimated resident population. The corresponding adjusted logistic model (Fig. 4) predicted that a trap density ca. 1.5tr/ha would remove almost 100% of the population. However, deploying only half the traps (0.82 traps ha⁻¹) would result in a 95% reduction of the population density. Trapping results in 2010 compared to 2013 did not provide any further support to the hypothesis that mass

trapping is beetle density-dependent, as reported earlier by Barclay and Chao (1991), and population removal was estimated to be very similar at equal trapping densities but very different *M. galloprovincialis* population levels. Under normal circumstances, *Monochamus* beetles are secondary insects that reproduce on dying or freshly dead trees and branches. The availability of such host material is dependent on stand conditions that may vary strongly spatially and temporarily (Grove 2002). Under such assumption, *M. galloprovincialis* populations would be expected to exist mainly in low densities. Yet, according to our results, the estimated native beetle densities can still vary strongly (0.52 and 82 individuals ha⁻¹ in 2010 and 2013 respectively). Regardless, the numbers of removed beetles suggest that mass trapping can be implemented operationally even in apparently moderate density populations. The population levels reported for *M. alternatus* (Shibata 1985; Togashi 1988) raise the question of what population levels are reached within PWD affected stands in which *M. galloprovincialis* is the vectoring agent. A deeper knowledge of the dynamics of *Monochamus* populations under different scenarios is required. Alternatively, reduction of stand damage has been commonly regarded as a proxy for population reduction and has served as a way of measuring mass trapping success (Alpizar et al. 2012; Faccoli and Stergulc 2008; Oehlschlager et al. 2002; Schlyter et al. 2001; Weslien 1992). *Monochamus* beetles, when not vectoring *B. xylophilus*, are secondary conifer borers that do not cause any damage. However, these beetles thrive on hosts that have been damaged by other agents therefore, intensive sanitation of freshly dead or dying trees is recommended in high PWD risk areas.

Simulations of mean reverting dynamics of a moderate density *M. galloprovincialis* population (82 beetles ha⁻¹) either under five consecutive years of mass trapping or not are presented in Fig. 5. In the case of removal efficiency of a fixed population, a mass trapping campaign conducted at 0.44 traps ha⁻¹ should drive local populations to near extinction levels. If local extinction did not occur, populations would gradually reach their initial densities ten years after the mass trapping had ceased. Simulations have used a constant population growth rate, as could be expected for stable *M. galloprovincialis* populations. Such rate, however, would be much higher if destabilizing positive density-dependent feedback occurred due to uncontrolled nematode inoculations, resulting in a great increase in suitable breeding material. In such a scenario, growth rate would tend to increase exponentially so population would recover much faster once trapping were cancelled.

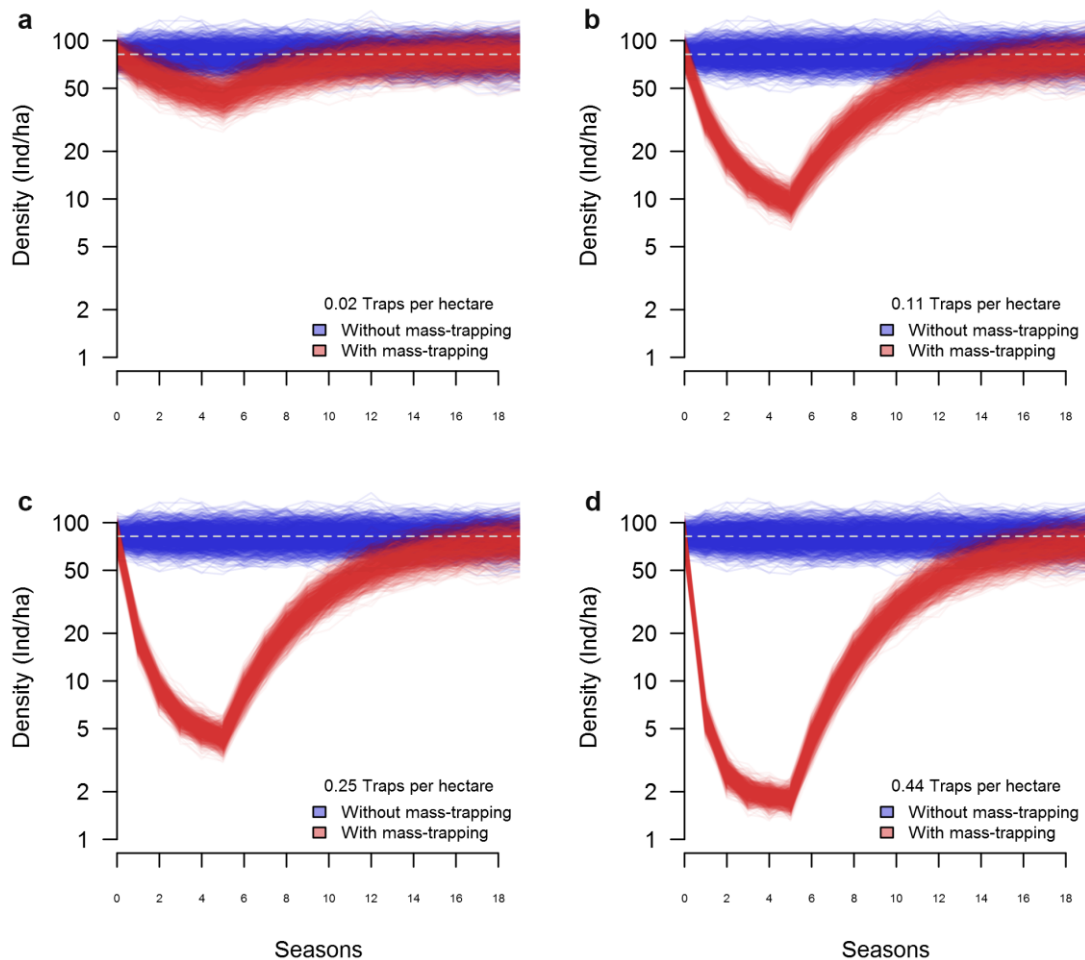


Fig. 5 *Monochamus galloprovincialis* population trends under different trapping intensities. The results of stochastic stationary Gompertz model simulations iterated 1000 times with (red), or without (blue) mass-trapping during the first five seasons are shown. Simulated trapping densities correspond to those tested in the 2013 field experiment: (a) 0.02, (b) 0.11, (c) 0.25 and (d) 0.44 traps per hectare.

High costs are a relevant constraint for operational mass trapping management tactics. Costs incurred could vary greatly between programmes intended for preventive control or for eradication of the target insect. Hence, forest health managers must decide the optimal trap density, balancing the desired results with the costs of the required trapping effort. Other than the cost of lures and traps, manpower likely accounts for the highest proportion of the costs of mass trapping programmes. These costs are related to site features, number of traps, distances between traps, the area of the target forest, and target population density (Barclay and Chao 1991). As shown by Bogich et al.(2008), it is important to balance the costs and benefits in both the detection and the eradication of invasive species, as optimal trap densities are most influenced by growth rate of the infestation. Figure 4 could help forest health managers by providing a sound correlation between trap densities, thus expected costs, and the desired removal of the estimated

population. Isolation of the treatment area is also a critical issue, since isolated populations are easier to eliminate (Barclay 1984).

The by-catch of non-target organisms may be another constraint to mass trapping programmes. Even if the *M. galloprovincialis* aggregation pheromone compound in the commercial lure is specific to *Monochamus* beetles, the host and bark beetle kairomones in the operational lures attract several saproxylic insects, including some bark beetle predators, and these are undesirably removed from the forest (Francardi et al. 2009; Ibeas et al. 2007; Rassati et al. 2012). Similarly, a large number of saproxylic species have been shown to be attracted to bark beetle pheromones (Etxebeste et al. 2013), and trap modifications have minimized the catch of beneficial insects (Martín et al. 2013). Even though research efforts are focused on improving the trapping of *Monochamus* beetles specifically (Álvarez et al. 2014), the effects of large scale mass trapping operations on non-target entomofauna should be taken into consideration.

Mass trapping should be regarded as a tool complemented with other control measures aimed at reducing vector populations, and not as a stand-alone control method. Sanitation of breeding material is imperative in any operational bark beetle control programme using mass trapping (Borden 1993; Wermelinger 2004), and should necessarily be carried out if effective control of PWD is the aim. Biological control of *M. galloprovincialis* using natural enemies could be regarded as a complementary strategy to mass trapping as well. The release of *M. alternatus* parasitoids has been quite successful in China and Japan (Zhao et al. 2008), but studies on *M. galloprovincialis* parasitoids are still preliminary (Naves et al. 2005). Entomopathogenic organisms are of particular interest within the options of biological control of the vectors of PWD. Considering the fact that a very effective lure is available, pathogenic organisms could be inoculated into the vector population by means of integrated methods, such as the 'lure and infect' tactic that has been developed for other pest systems (Jackson et al. 2005). In this sense, some fungal strains isolated from *M. galloprovincialis* adults and larvae in Spain have demonstrated high potential for horizontal transmission in adults and might become available for practical implementation in the near future (Álvarez-Baz, pers. comm.). Furthermore, the identification of the reported contact-pheromone in *M. galloprovincialis* (Ibeas et al. 2008), could further increase the chances of successful application of this tactic. The combined usage of attractant and repellent infochemicals ('push and pull') in pest management represents yet another alternative for the integration of mass trapping with other techniques (Cook et al. 2007). Infochemicals with known repellent effect on bark beetles, such as verbenone (Etxebeste and Pajares 2011; Etxebeste et al. 2013), had been tested earlier on *M. galloprovincialis* with little

success (Ibeas et al. 2007). Further research should be done to identify and evaluate other candidate repellents that could be used in the management of the PWD.

With current high risk of PWD expansion within Europe, the need for control measures keeps increasing. Yet, the general public supports environmentally friendly pest management alternatives. The present paper shows that mass trapping of *M. galloprovincialis*, the European vector of the PWN, represents a valuable tool in the integrated management of this serious disease. Mark-recapture studies have allowed assessing this tool under two different population densities, and managers are provided with a relationship between the implemented trap density and the expected proportion of *M. galloprovincialis* individuals removed. According to our results, mass trapping may effectively help stopping the spread of PWD, both in eradication programmes in areas where the disease has recently been detected and in border areas or other buffer zones where containment is strongly required.

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- **Synthesis**

Host preferences and the role of *Pinus pinea* during host selection by *Monochamus galloprovincialis*

The pine wood nematode (PWN), the causal agent of the pine wilt disease (PWD), was introduced in Portugal in 1999 (Mota et al., 1999). So far, only the cerambycid *Monochamus galloprovincialis* (Olivier) has been shown to vectorize the PWN in Europe (Sousa et al. 2001). The record on the evolution and expansion of PWD throughout the demarcated areas in this country showed that the nematode infections and tree mortality have been restricted to *Pinus pinaster* Aiton and *Pinus nigra* Arnold (Inácio et al., 2015) whereas stone pine, *Pinus pinea* L., which frequently occurs in the infested zone, has to date, not shown symptoms of decay (Naves et al., 2006). The first aim of this work was to determine the host spectrum of *M. galloprovincialis* in the Iberian Peninsula, specially focusing on the insect's behavior in relation to *P. pinea*.

According to the results derived from Chapter, *M. galloprovincialis* is able to feed, lay eggs, and develop on *P. pinea*, at least under laboratory conditions. Although it was not the preferred host when compared with other Iberian *Pinus*, the beetle performed best on *P. pinaster*, no significant detrimental effects on feeding or reprocuting on *P. pinea* were observed. The influence of pine volatiles that are more abundant on different host species have on the attraction of *Monochamus* to traps has been studied in detail (Pajares et al., 2004, 2010; Ibeas et al., 2007, 2008; Álvarez et al., 2015). In the case of *M. galloprovincialis*, limonene, the most abundant terpene in *P. pinea* (Santos et al., 2006; Martini et al., 2010) has been suggested as one of the most important chemical cues that might influence the process of host selection (Naves et al., 2006), as has also been suggested for *Monochamus alternatus* Hope (Fan & Sun, 2006). As it has been shown in Chapter 1, a progressive decrease in feeding was detected as limonene dosage increased, which could indicate a probable deterrent effect of limonene in the diet of adult *M. galloprovincialis*. Overall, the results on the host preferences of the vector do not explain the observed absence of nematode killed *P. pinea* trees in the field and further research should be focused on the study of *P. pinea* in order to developed possible new options to control the disease.

Dispersal of the European vector Monochamus galloprovincialis and immature dispersal capacity.

Although transportation of PWN and the beetles over very long distances by humans is considered the single-most important factor in spreading the PWD, the natural *Monochamus* dispersal behavior plays a very important role too, as the short-distance propagation of the PWD is mainly dominated by this process. With the development of effective traps and a highly attractive commercial lures for *M. galloprovincialis* (Pajares et al., 2004, 2010; Ibeas et al., 2007, 2008; Rassati et al., 2012; Álvarez et al., 2015), mark-release-recapture (MRR) assays began to be feasible for this species (Gallego et al., 2012; Mas i Gisbert et al., 2013; Torres-Vila et al., 2014; Mas i Gisbert, 2015). However, accumulating knowledge shows that immature *M. galloprovincialis* are not detected in traps baited with the standard lure (Ibeas et al., 2008; Pajares et al., 2010; Álvarez et al., 2015). On the other hand, knowledge on the survival, physiological development and dispersal capabilities of *M. galloprovincialis* constitutes a key factor in the development of adequate management policies of the PWD. With this mind, results presented in the Chapter 2, indicate that *M. galloprovincialis* depends on shoot feeding for sexual maturation for a period averaging 12-13 feeding days. Beyond that moment, they respond to the commercial pheromone lures. Remarkably, *M. galloprovincialis* emerged with enough lipid reserves, and muscular fractions to faces dispersal flight, regardless of the size or sex. Furthermore, even if the immature adults do not survive long without food, they would live long enough to successfully transmit the PWN to susceptible hosts. In order to gain empirical evidence on how eventual translocation of PWN infested material into host deprived areas could lead to a further expansion of the disease, batches of immature beetles were released at increasing distances from a *P. pinaster* stand from locations within a cropfield. Overall, our results show that newly emerged *M. galloprovincialis* are able to sustain long flights in the field without requiring hosts for feeding. In addition, the registered recapture percentages over release distances of lab-fed, 15-day-old mature *M. galloprovincialis* followed did not significantly differ.

The natural spread of PWD is greatly constrained to the dispersal of *Monochamus* beetles and the successful transmission of its causal agent, the PWN. Chapter 3 presented several studies focused in describing the dispersal kernels, i. e. the probability density function of the distribution of the dispersal distance travelled by a disperser that could assist in the description of the PWD expansion when vectored by *M. galloprovincialis*. Three experiments were held under two landscape scenarios, fragmented and continuous pine stands. Our result showed that although median recaptures occurred at the closest distance to the release point, recaptured beetles were found across all the experimental setup, in agreement with other dispersal assays (Gallego et al., 2012; Mas i Gisbert et al., 2013). Dispersal kernels deriving from mechanical and empirical

models fitted to observed *M. galloprovincialis* recaptures predicted that the 50% of the recaptured insects would occur at distances within 250-532 m from the source, and that the 99% of the dispersers would remain within 2344-3495 m. The most conservative kernel (bivariate t), forecasted that 0.6% of dispersers would fly beyond 5000m. Yet, our recapture results under the fragmented landscape scenario suggest that such habitats would apparently enhance the dispersal behaviour, as was also observed by Gallego et al (2012). These results, clearly call into question the relevance of the precautionary clear-cuts recommended by the latest European Union Commission decision concerning the prevention of PWD spread (2012/535/EU), which obliged member states to remove all susceptible hosts 500 m around new infestation foci.

The same experimental set-ups provided results that helped computing trapping parameters by regressing the proportions of recaptured insects over the distances to the release point (Turchin & Odendaal, 1996; Östrand & Anderbrant, 2003). This way the effective sampling area was derived to be between 0.57-0.76 ha, while the seasonal sampling range oscilated between 426 and 645m. Although these figures might vary in relation to environmental or technical parameters and apply to the evaluated trap and lures, they by themselves constitute a reference from which design management strategies such as early detection surveys, or ways of lowering the populations of the PWN vector.

Net dispersal is defined as the movement of an individual from site of birth to site of reproduction. Accordingly, the foremost reasons for dispersal in *Monochamus* beetles include looking for host material for maturation feeding, for sexual mates, and for dying or newly dead conifers suitable for egg-laying (Naves et al., 2008). The search of resources to meet these physiological needs could be affected by the habitat structure, which would in turn oblige *M. galloprovincialis* to move looking for optimal patches in the forest. However, with a few exceptions (Saint-Germain et al., 2004), empirical data that describes *Monochamus* spp. habitat location preferences are rarely found. The development of new techniques that allow mapping quantitative variables that can greatly enhance the characterization of the habitat. For example, LiDAR images have led to improved measurements of tree and forest variables and the characterization spatial structure of the stands (Hudak et al., 2009). Correspondingly, this information is increasingly used on studies dealing with pest management (Brydegaard et al., 2009; Mei et al., 2011; Vastaranta et al., 2013a; b). By studying the abundance of *M. galloprovincialis* across the experimental site in 2011 in relation to LiDAR derived stand characteristics, canopy cover could be identified as the principal habitat factor that quantitatively affected the presence of native beetles. On the other hand, not surprisingly, distance to the release site was the main factor affecting their abundance. This result is in agreement with the predictions by the dispersal kernels fitted in Chapter 3, and correspondingly insects would be recaptured at the closest distances to their release site. In addition, another factor was found to significantly affect lab-reared *Monochamus* habitat selection, the maximum height of regeneration (i. e. the

height of pines in the understory). Yet, this result could have been caused by the overestimated impact of the height of the regeneration at the nearest distances, caused by a very high number of recaptures in less of 300 m of the release site (62 % of recaptures), where regeneration was lower than in the rest of the site.

One of the main drawbacks in the development of methods for the early detection of PWD, is the lack of information on how immature *M. galloprovincialis* distribute within the forest. The same dispersal kernels as above were fitted to the recaptures of beetles released at mature or immature stages of their life cycle during Chapter 4. Fitted models, predicted similar values for the probability of dispersal, yet, the kernel based on the bivariate *t* distribution predicted that 0.001% of immature *M. galloprovincialis* would fly over 5000 m, when such events were not foreseen for mature beetles. Overall, such results are highly comparable to those obtained in Chapter 2, i. e. dispersal of matures and immatures under a scenario deprived of hosts. Keeping in mind that mature insects were 15 days-old when they were released, while immature insects reached their sexual maturation only after 12 feeding days, the displacements by immature beetles during their feeding flights do not seem to modify the dispersal behavior observed by the recapture of marked *M. galloprovincialis* by pheromone baited traps. Anyhow, keeping in mind that PWN primary transmission occurs as they feed, future studies aiming for the identification of active compounds for trapping immature pine sawyers may help understanding this initial feeding dispersal behaviour.

Mass-trapping as control method of Monochamus galloprovincialis

Mark-recapture methods have been widely used to quantify animal abundance in the field (Seber, 1982). On the other hand, the development of effective traps and lures for *Monochamus* spp. (Pajares et al., 2004, 2010; Álvarez et al., 2014, 2015) opened the question whether mass trapping could be used to reduce the population levels of the vector of the PWN, which might provide a more proactive management strategy for the eradication or containment of the PWD than the current management practices. But first, the methodology by which mark-release-recapture data would be use to estimate the size of the target population abundance needed to be evaluated. The data used to fit the dispersal kernels in 2010, allowed validating the POPAN parametrization of Jolly-Seber models for such purpose. A large scale experiment conducted in 2013, showed that mass-trapping might help reducing the population level of *M. galloprovincialis* in the studied area. However, special attention is needed in a scenario where the highest population levels would be reached, as could be the case of PWD-affected stands (Togashi, 1988). In such case, a destabilizing positive density-dependent feedback could occur due to uncontrolled nematode inoculations, resulting in a great increase in suitable breeding material. Evaluated trapping densities removed were 4.66%, 20.50%, 33.33% and 59.80% of *M. galloprovincialis*

estimated population at 0.02, 0.11, 0.25 and 0.44 traps/ha, respectively. Accordingly, thus the estimated 95% of removal would occur at 0.82 traps/ha⁻¹.

Yet, as discussed by Bogich et al. (2008), it is important to balance the costs and benefits in both, the detection and the eradication of invasive species, as optimal trap densities are most influenced by growth rate of the infestation. It is also important to consider the natural role of *M. galloprovincialis* globally, as under normal circumstances, this saproxylic insect provides, as many other members of the same guild, very important ecosystem services as primary decomposers of wood (Vives, 2000; Saint-Germain et al., 2004). In addition the effect on by-caught non-targeted species must also be taken into account. Overall, and according to our results, mass trapping may help stopping PWD spread, and could potentially be applied in eradication programs in areas where the disease has recently been detected and in border areas or other buffer zones where containment strategies are strongly required.

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• Conclusions

Shoot feeding and oviposition preferences by Monochamus galloprovincialis (Chapter 1)

1. Scots pine was the most preferred host by *M. galloprovincialis* for feeding, followed by maritime pine. All tested Iberian pines were accepted by the beetles for feeding.
2. *M. galloprovincialis* was able to feed, lay eggs, and complete all its developmental stages on *P. pinea*, under laboratory conditions.
3. Insect feeding decreased as limonene dose increased, suggesting that an inhibitory effect by this terpene may occur.
4. Oviposition wounds occurred more frequently on *P. pinea* than on *P. sylvestris* or *P. pinaster*, though the proportion of successful egg laying was significantly lower.
5. *M. galloprovincialis* successfully completed progeny development on *P. pinea*, but survival was lower than on *P. sylvestris*.

Physiology and dispersal of immature Monochamus galloprovincialis (Chapter 2)

6. Sexual maturation took an average of 11.66 ± 1 and 12.86 ± 1 days of feeding for *M. galloprovincialis* males and females respectively.
7. *M. galloprovincialis* newly emerged adults had lipid and pterothorax (i.e. flight muscles) contents enough for dispersal flight, regardless of size, age or sex.
8. Unfed adults survived 12 days on average (range 6-20 days).
9. Newly emerged *M. galloprovincialis* were able to flight 2 km over areas deprived of hosts. Longest flight distance by these adults was estimated at 3109 m.

Monochamus galloprovincialis dispersal under continuous and fragmented pine stands (Chapter 3)

10. The negative exponential model was best fitted to *M.galloprovincialis* dispersal data
11. Derived estimated radii enclosing 50% and 99% of dispersing *M. galloprovincialis* under continuous pine stands ranged between 250-532m and 2344-3495m, depending on replicate and model.
12. The widest tail was forecasted by the 2Dt model, predicting that 0.6% of *M. galloprovincialis* adults would fly over 5000 m.
13. *M. galloprovincialis* dispersal distance is affected by landscape structure, being longer in areas fragmented areas.
14. No differences in dispersal behaviour between *M. galloprovincialis* males and females were observed, nor there was significant influences of beetle size in distance of recapture.
15. The effective sampling area for *M.galloprovincialis* resulted 0.57-0.76ha, and the seasonal sampling range was 426-645m.

Monochamus galloprovincialis abundance and dispersal in relation to stand characteristics (Chapter 4)

16. Forest canopy cover was the main variable that positively affected native *M.galloprovincialis* abundance.
17. Distance from the release point and maximum height of regrowth negatively affected distribution of both mature and immature released insects. Interaction of these variables had a light positive effect on abundance.
18. Immature and immature beetles had similar dispersal kernels when data was fitted to 2Dt and negative exponential models. Maximum distance probability resulted in that 0.001% of immature *M. galloprovincialis* would fly over 5000 m.
19. *M. galloprovincialis* males dispersed further away and during more time than females.

Monochamus galloprovincialis population reduction for PWD management (Chapter 5)

20. Capture-mark-recapture resulted an accurate method to estimate *M. galloprovincialis* population abundance. Conservative estimate of population density in the study are was 82 beetles/ha
21. As *M. galloprovincialis* population can be classed as one of the open type, abundance estimates had to be calculated weekly and different percentages of population removal were obtained during the study period.
22. Mass-trapping was a valid method for reducing *M.galloprovincialis* population. Trapping densities at 0.02, 0.11, 0.25 and 0.44 traps/ha removed 4.66%, 20.50%, 33.33% and 59.80% of *M. galloprovincialis* population, respectively. 95% removal was estimated to occur at 0.82 traps/ha.

• Conclusiones

Preferencias de alimentación y oviposición Monochamus galloprovincialis (Capítulo 1)

1. El pino sivestre fue el más preferido por *M.galloprovincialis* para su alimentación, seguido del pino resinero. Todas las especies de pinos ibéricos testadas fueron aceptadas
2. *M. galloprovincialis* fue capaz de alimentarse, hacer la puesta y desarrollar su progenie en pino piñonero, en condiciones de laboratorio.
3. La alimentación de los insectos disminuyó según se aumentó la dosis de limoneno, lo cual sugiere podría indicar un efecto disuasorio de este terpeno.
4. Las mordeduras de puesta fueron mayores en *P. pinea* que en *P. sylvestris* o *P. pinaster*, si bien la proporción de ellas que contenía huevo fue significativamente menor.
5. completó con éxito el desarrollo de su progenie en *P. pinea*, aunque la supervivencia fue menor que en *P. sylvestris*.

Fisiología y dispersión de Monochamus galloprovincialis (Capítulo 2)

6. La duración media de maduración para los machos y las hembras de *M. galloprovincialis* fue de 11.66 ± 1 y 12.86 ± 1 días de alimentación respectivamente.
7. Los adultos recién emergidos de *M. galloprovincialis* tenían suficiente contenido de lípidos y musculatura alar para emprender el vuelo de dispersión, independientemente de las diferencias en tamaño, edad o sexo.
8. La supervivencia de los adultos sin alimentarse fue 12 días de media (entre 6-20 días).
9. Los adultos recién emergidos de *M.galloprovincialis* fueron capaces de volar 2 km sobre un área sin hospedantes. La mayor distancia de vuelo para estos adultos fue estimada en 3109m.

Dispersión de Monochamus galloprovincialis en masas continuas y fragmentadas. (Capítulo 3)

10. El modelo exponencial negativo resultó el mejor ajustado a los datos de dispersión de *M.galloprovincialis*
11. Los radios que engloban el 50% y el 99% de los *M. galloprovincialis* dispersantes s fueron estimadas por los modelos entre 0-532m y 2344-3495m respectivamente, dependiendo de la réplica y del modelo.
12. Las predicciones de máximas distancia estiman que el 0.6% de los adultos de *M. galloprovincialis* se dispersarían hasta los 5000 m.
13. La dispersión de *M. galloprovincialis* depende de la estructura del paisaje, siendo mayor en paisajes con mayor grado de fragmentación.
14. No existieron diferencias significativas entre la dispersion de machos y hembras de *M. galloprovincialis*, ni ésta se vió influida por el tamaño del insecto.
15. El área efectiva de muestreo de la trampa utilizadas fue de 0.57-0.76ha, y su rango estacional de muestreo de 426-645m.

Abundancia y dispersion de Monochamus galloprovincialis en relación con las características de la masa (Capítulo 4).

16. La abundancia de los insectos nativos se vió influída positivamente por la fracción de cabida cubierta.
17. La distancia al punto de liberación y la maxima altura del regenerado afectaron negativamente a la distribución de las capturas, mientras que la interacción de ambas lo hizo de forma ligeramente positiva.
18. Ambos modelos 2Dt y exponencial negativo obtuvieron similares kernel de dipersión para los individuos maduros e inmaduros. Se estimó una probabilidad de que el 0.001% de los insectos inmaduros se dispersaría por encima de los 5000m.
19. Los machos de *M. galloprovincialis* se dispersaron más lejos y durante más tiempo que las hembras.

Reducción de la población de Monochamus galloprovincialis como método para el manejo de la enfermedad del marchitamiento del pino (Capítulo 5)

20. La Captura-marcado-recaptura resultó un método preciso para estimar la abundancia de población de *M. galloprovincialis*. La estimación conservadora de la densidad poblacional en el área de estudio fue de 82 insectos/ha.
21. Al ser la población de *M. galloprovincialis* de tipo abierto, las estimaciones de abundancia tienen que ser calculadas semanalmente y por tanto los porcentajes de recaptura obtenidos fueron diferentes a lo largo del período muestreado.
22. La captura masiva es un método válido para reducir la población de *M. galloprovincialis*. Las densidades de 0.02, 0.11, 0.25 y 0.44 trampas/ha retiraron el 4.66%, 20.50%, 33.33% y 59.80% respectivamente de la población de *M. galloprovincialis*. Se estimó que el 95% de reducción ocurrirá con una densidad de 82 trampas/ha.