

Impact and ecological adaptation of *Leptoglossus occidentalis* (Hemiptera, Coreidae) on *Pinus pinea*

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À Cátia e à pequena Catarina
Somos todas raízes da mesma árvore!

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ABSTRACT

Leptoglossus occidentalis Heidemann (Heteroptera: Coreidae) is an invasive pest seriously damaging conifer seeds. Native from North America, the insect was first detected in Europe in 1999, and recorded in Portugal in 2010. Both nymphs and adults feed on seeds of several conifer species. Bug impact on seed production of Stone pine, *Pinus pinea*, is of major concern in the Mediterranean Basin countries because cone production and seed yield have decreased during the last years quite simultaneously to the records of *L. occidentalis*. Thus, the insect has been considered the most plausible cause of this decrease. However, there was still a substantial lack of knowledge about the effective impact of bugs and their ecological adaptations on Stone pine.

My main goal was to add valuable and pertinent knowledge to understand the interactions between seed bugs and Stone pine. At first, a careful revision of all the literature available about *Leptoglossus occidentalis* was carried out, together with discussions with other European researchers working on this pest, in order to define the PhD aims. The PhD plan was then divided into three main issues. I first characterized and measured the importance of bug damage on seeds of Stone pine. In a second part, I investigated the ecological interactions between invasive bugs and Stone pine cones and seeds. Bug host preference was tested between Stone pine and the other two main native pine species growing in southern Europe (*P. pinaster* and *P. halepensis*), and cues possibly underlying such preferences were suggested. I also evaluated the impact of the bug in Stone pine seed orchards under two different management strategies. In a third part, I suggested possible invasion routes of *L. occidentalis* in the Iberian Peninsula, using genetic data and field records.

Trials of the bug damage on matured seeds of Stone pine gave an estimate of consumption of about a fifth of a seed kernel per bug per month with the consumed kernels appearing skunked and wrinkled. In addition to this result, these first trials revealed (and it was later confirmed) a collaborative feeding behaviour since all bugs of the same box fed through the same hole of the seed coat in most of the cases.

When testing the bug host preferences, results showed that individual seeds and cones of *P. pinea* were 2 to 3 times more consumed than those of the two other pine species. I assumed that stone pine greater seeds and cones are highly rewarding for a polyphagous bug like *L. occidentalis*.

Field trials in pure stone pine seed orchards indicated that fertilized and irrigated trees are more susceptible to both conelet mortality and seed damage by biotic agents compared to trees with no treatment. Two adults enclosed in a bag for one month (August) damaged an average of 6% of seeds per cone. The partial damage of the kernel revealed to be a signature of *L. occidentalis* feeding in mid-summer.

Lastly, my genetic results support a stratified expansion of *L. occidentalis* invasion in the Iberian Peninsula with at least three independent introductions: one in Barcelona, one in Valencia, or in the South, probably in Almeria and another in the North of the peninsula.

In the end of the thesis, I present cues to help control this pest and suggest directions for further studies.

Keywords: invasive insect species, Stone pine, pest control, seed damage, forest management

RESUMO

Leptoglossus occidentalis Heidemann 1910 (Heteroptera: Coreidae), também conhecido como sugador de pinhas, é uma praga invasora nativa da zona Oeste da América do Norte de onde se expandiu até à costa atlântica dos Unidos da América (Gall, 1992) e daí para a Europa (Lesieur *et al.*, 2018). O seu primeiro registo no continente europeu data de 1999 na cidade de Vicenza do norte de Itália (Tescari, 2001). Nos dez anos que se seguiram foi observado na maioria dos países europeus, desde Portugal a Oeste, Rússia a Este, Suécia a Norte e a ilha italiana de Sicília a sul (Fent & Kment, 2011).

O sugador de pinhas é um insecto picador-sugador, isto é, possui um conjunto de estiletos que insere entre as brácteas das pinhas até atingir as sementes sugando o seu interior. As pinhas atacadas não apresentam estragos externos aparentes. Tanto as ninfas como os adultos alimentam-se de pinhas de todas as fases de desenvolvimento. Alimenta-se de cones e sementes de coníferas de vários géneros: *Pinus*, *Picea*, *Abies*, *Cedrus*, *Tsuga* e *Pseudotsuga* (EPPO 2009). No **Capítulo 1** apresento uma introdução geral ao tema da tese, descrevendo a biologia e ecologia do insecto, esta última focando no seu hospedeiro mais importante no país, o pinheiro manso (*Pinus pinea*). De facto, nos países mediterrânicos a preocupação recai no impacto que este insecto poderá ter na produção do pinhão de Pinheiro manso, *Pinus pinea*. A produção de pinhão é, juntamente com a cortiça, uma das explorações florestais de produtos não lenhosos economicamente mais importantes em Portugal. Relatórios da indústria de processamento de pinhão bem como de alguns investigadores reportam um decréscimo no rendimento em pinhão na última década (Roversi, 2009; Mutkle *et al.*, 2014) apontando *L. occidentalis* como uma das causas mais prováveis. Contudo, ainda não existe uma quantificação precisa do impacto deste insecto nem conhecimento da sua ecologia no novo hospedeiro, *P. pinea*. Ciente destas lacunas, comecei por conduzir, em equipa, uma revisão exaustiva ao estado actual da investigação deste insecto e da sua interacção com o pinheiro manso, **Capítulo 2** desta tese. Para além de identificar falhas no conhecimento e apontar linhas de foco futuros esta revisão juntou uma equipa de investigadores de vários países da Bacia mediterrânea que partilham a preocupação pela acção deste insecto.

Este projecto de doutoramento procurou responder a questões relacionadas com o impacto, e ecologia desta espécie invasora em relação ao novo hospedeiro *P. pinea* bem como a sua

história de invasão na Península Ibérica. Para a questão do impacto comecei por caracterizar e avaliar impacto deste insecto nos pinhões de pinheiro manso.

Dois outros objectivos foram definidos na questão relacionada com a ecologia: investigar se este insecto tem uma preferência de hospedeiro entre os pinheiros mais comuns na região da Bacia do Mediterrâneo e se selecciona árvores sob diferentes estratégias de gestão. Por último, procurei descodificar as rotas de invasão e a estrutura populacional de *L. occidentalis* na Península Ibérica;

Relativamente ao cálculo do impacto do insecto no pinheiro manso, identifiquei logo no início do projecto uma lacuna que seria necessário colmatar. Para poder estimar este impacto era crucial caracterizar o estrago nos pinhões, i.e., identificar e separar o estrago causado pelo *L. occidentalis* de outros estragos possíveis. No **Capítulo 3**, apresento uma caracterização do estrago em pinhões negros, obtida em ensaios laboratoriais. Para uma caracterização precisa usei uma abordagem com três metodologias diferentes: raios-X, Micro-Tomografia Computorizada (Micro-CT) e observação directa. Os pinhões onde o insecto se alimentou apresentaram um endosperma enrugado e seco. A morfologia do estrago depende da duração do período de alimentação podendo ir de meio-consumido (Estrago tipo I) até totalmente seco e enrugado (Estrago tipo II).

Para além da caracterização do estrago, estes ensaios laboratoriais revelaram um comportamento cooperativo de alimentação. Tendo vários pinhões negros à disposição, os insetos, de uma mesma caixa, consumiam um único pinhão usando, para tal, um único furo na casca. Este comportamento aparentemente cooperante leva a uma concentração do estrago o que, a acontecer em condições naturais, pode trazer benefícios na gestão das plantações.

Com a invasão da Europa, o sugador de pinhas encontrou novas espécies de hospedeiros, que no nosso território são essencialmente o pinheiro bravo *P. pinaster* e o pinheiro manso *P. pinea*. Devido à relevância económica das sementes comestíveis de pinheiro manso, torna-se premente avaliar possíveis preferências do *L. occidentalis* entre vários hospedeiros. Assim, no **Capítulo 4** testei a preferência deste insecto pelas três espécies de pinheiros mais importantes da Bacia do Mediterrâneo: o manso, o bravo e o de Alepo. São apresentados resultados de estudos de laboratório usando ramos, cones e sementes como substrato de escolha. Os ensaios usando ramos com pinhas revelaram que o comportamento gregário deste insecto prevalece sobre a taxonomia do ramo. O estado fisiológico do ramo é também sugerido como tendo sido um factor predominante na escolha dos insectos. Posteriormente ficou demonstrado num ensaio de selecção de hospedeiro em

campo que estes insectos são efectivamente sensíveis à qualidade do hospedeiro (Capítulo 6). Quando o substrato de escolha foram as pinhas, o insecto visitou e alimentou-se significativamente mais vezes nas pinhas de manso que das outras duas espécies de pinheiros. O sugador também consumiu sempre uma percentagem muito superior de miolo de pinhões de manso (cerca de 90%) relativas às outras espécies. Encontrei evidência para a escolha dos cones e sementes estar relacionada com a maior recompensa que o pinheiro manso oferece. De facto, as pinhas de manso são 2 a 3x maiores que as de bravo e Alepo, respectivamente. O mesmo acontece com as sementes em que o miolo de manso é 4 x maior que o de bravo e 13x maior que o de Alepo.

A interacção entre as práticas de gestão florestal e o impacto de *L. occidentalis* é discutida no **Capítulo 5**. Os ensaios foram conduzidos em plantações enxertadas de pinheiros mansos. Numa mesma área comparámos o estrago em pinheiros submetidos a fertilização e irrigação (FR) e pinheiros sem tratamento (C). Adicionalmente, usámos mangas de rede em ramos seleccionados ao acaso onde colocámos insectos adultos ou ninfas de forma a seguir o seu estrago em condições de campo. Os resultados mostraram haver diferenças no estrago entre adultos e ninfas. A mortalidade de pinhas jovens de 2º ano foi 63% superior nas mangas com ninfas em comparação com as com adultos ou controlo. No geral as árvores FR foram mais susceptíveis à mortalidade de pinhas jovens e ao estrago em sementes. Contudo, esta diferença só foi significativa para as ninfas e ramos expostos.

Um estudo genético recente mostrou que a invasão da Europa teve origem em populações da região oriental da América do Norte e não da região oeste, de onde é nativa, num mecanismo denominado de *bridghead effect* (Lesieur *et al.*, 2018). A invasão de *L. occidentalis* na Europa foi ainda acelerada por múltiplas introduções independentes provenientes de populações invasivas europeias ou novas introduções da região oriental da América (Lesieur *et al.*, 2018). A sua boa capacidade de voo e hábitos polípagos contribuíram para o sucesso da expansão dessa praga. Lesieur *et al.* (2018) identificou duas introduções independentes em Espanha, em Barcelona e em Valência as quais corresponderam às duas únicas amostras Ibéricas analisadas. Assim, a história completa da invasão deste percevejo na Península Ibérica ficou por clarificar. No **Capítulo 6**, usei marcadores genéticos, mitDNA e microsatélites, em conjunto com dados observacionais de primeiros registos de campo para analisar a estrutura genética e a diversidade das populações de *L. occidentalis* na Península Ibérica e inferir a sua rota de invasão. Os resultados comprovam a existência de, pelo menos mais uma introdução independente na costa norte ou oeste da Península Ibérica. O mercado intensivo de cones de *P. pinea* na Península Ibérica pode ser uma das principais causas de novas introduções. Este mercado

abrange o transporte de cones de plantações para fábricas a distância relativamente curta, mas também a longa distância com a exportação de cones de proprietários de terras ou fábricas para outros países, como a Turquia ou a Itália. Análises adicionais, por métodos Bayesianos deverão ajudar a decifrar os cenários de invasão.

Os vários ensaios desenvolvidos nestes quatro anos revelam factos novos relativos ao impacto e ecologia deste insecto. A morfologia do estrago causado por *L. occidentalis* em pinhões negros de pinheiro manso desenvolvida neste projecto foi crucial como conhecimento base para o desenvolvimento de uma metodologia de estimativa de estrago a partir de amostras de campo.

Relativamente ao estudo da sua ecologia, *L. occidentalis* mostrou uma tendência para escolher pinhas e sementes de pinheiro manso em detrimento do pinheiro bravo e do Alepo. Este resultado justifica-se pela maior recompensa que o pinheiro manso oferece. Do ponto de vista nutricional, podemos assim esperar que as árvores e plantações de manso possam favorecer o crescimento populacional de *L. occidentalis*.

Dentro de uma plantação pura de pinheiro manso o insecto também mostrou uma tendência para causar mais estrago em árvores em melhor estado fisiológico, isto é, árvores fertilizadas e irrigadas em comparação com árvores sem qualquer tratamento. Uma selecção activa de árvores por parte de *L. occidentalis* já havia sido demonstrada noutros hospedeiros (Blatt, 1997; Blatt & Borden, 1996; Richardson *et al.*, 2017). Estes resultados têm implicações na gestão dos povoamentos de pinheiro manso. Deverá ser conduzida uma análise de custo-benefício de forma a ponderar os custos da gestão e o aumento do estrago com a produção de pinha.

O estudo genético das populações Ibéricas revelou que o *L. occidentalis* invadiu a Península Ibérica segundo um mecanismo de dispersão estratificada, isto é, usando a dispersão a curta-distância (dispersão contínua) como a longa-distância, usufruindo oportunisticamente de transportes casuais mediados pelo homem. É sugerido que o comércio intenso da pinha seja responsável pela translocação de muitos indivíduos.

Com este projecto foram colmatadas algumas das lacunas detectadas no início. Os resultados apontam tendências que deverão ser agora integradas num futuro plano de gestão desta praga no pinheiro manso. Este plano tem de obrigatoriamente resultar de um esforço conjunto entre todos os países mediterrâneos de forma a realizar uma abordagem integrativa desta praga.

CHAPTER 1

General Introduction and

Thesis outline



Chapter 1 - General introduction and Thesis outline

Invasive species are generally considered as the third-leading threat to biological diversity behind, only, habitat loss and fragmentation (Baillie *et al.*, 2004). This expansion of alien insects into new areas is likely to cause serious economic or ecological hazards (Rabitsch, 2008) and actions must be taken promptly on the path to mitigate their impacts (Junker & Lupi, 2011). Biological invasions are constantly increasing with globalization without any signs of saturation (Seebens *et al.*, 2017), especially in insects (Roques, 2010), and most of the recently-arrived invaders (i.e. since the 1990s) are spreading across Europe faster than before (Roques *et al.*, 2016). The seed bug is representative of such an invasive history and fast spread.

The Western Conifer Seed Bug, *Leptoglossus occidentalis*, is a Western North America cone and seed insect native from Western North America where it is a relatively serious pest of conifer seed orchards (Koerber, 1963; Hedlin *et al.*, 1980; Bates *et al.*, 2000). In the middle of the last century it started spreading across America in the West-East direction (Gall, 1992). During the 1950's and 1960s it was newly recorded in Mid-West (Indiana, Iowa, Montana, Nebraska, Kansas), and in the 1970's it was discovered in Connecticut on the Atlantic coast (Gall, 1992). In the 1990's it extended its range to North-Eastern USA and South-Eastern Canada (McPherson *et al.*, 1990; Gall, 1992). Human-mediated transportations were indicated as the main responsible for the spreading of this species (Gall, 1992).

The European invasion began in Italy where it was first recorded in 1999 (Tescari, 2001). Only ten years after, it can be observed in most European countries (Fent & Kment, 2011) (Figure 1). In Portugal, its first record dated from 2010 (Grosso-Silva, 2010; Sousa & Naves, 2011). Then, this species also invaded other continents; i.e., North Africa (Gapon, 2015; Jamâa *et al.*, 2013), Asia (Korea, Japan and China; Ishikawa & Kikuhara, 2009; Zhu, 2010; Ahn *et al.*, 2013), and South America (Chile; Faúndez & Rocca, 2017).

The arrival of this seed feeder to Mediterranean Basin countries where Stone pine is distributed raised serious concerns. This pest was observed feeding on Stone pine cones in several regions (Pimpão, 2014; Bracalini *et al.*, 2015; Calama *et al.*, 2016; Ponce *et al.*, 2017a; Farinha *et al.*, 2018b). There have been reports suggesting an important impact of this bug on the profitable Stone pine edible nuts (Roversi, 2009; Bracalini *et al.*, 2013; Mutke *et al.*, 2014, 2017; Ponce *et al.*, 2017a; Farinha *et al.*, 2018a, 2018b) but an

accurate impact estimation as well as studies on the ecology and adaptation of this bug to this recent host are lacking.



Figure 1. World distribution of *L. occidentalis* in CABI (2018) (www.cabi.org/isc) and first records of *L. occidentalis* in Europe (in Fent & Kment, 2011)

In **Chapter 2**, I am presenting a review article on the current state of the knowledge of *L. occidentalis* interactions with Stone pine. This review article is an attempt to create a much-needed baseline for future research and focus on this issue. Moreover, it also serves as a compiled background for the reader to better understand the gaps and challenges related to this recent invasive pest.

Can we measure the effective impact of the bug on Stone pine seeds?

Regarding its impact, *L. occidentalis* is considered a significant pest in some European countries and held responsible for a decrease in Stone pine nut production in Italy and Spain (Roversi, 2009; Bracalini *et al.*, 2013; Mutke *et al.*, 2014; Farinha *et al.*, 2018b) and in natural regeneration of native pine stands in France (Lesieur *et al.*, 2014). Beyond the ecological impacts, this species poses a serious threat to pine nut production (Farinha *et al.*,

2018b). Stone pine in Portugal occupies an area close to 176,000 hectares corresponding to 6% of the forest area of the country. Pine nut production is currently one of the major economic activities of Portuguese forests representing 4-5% of all country exports with a total value of 60-80 Million of euros each year and 13.3% of the employment related to forest (ICNF 2013).

Despite this bug being considered the most probably cause for the decrease production of the Mediterranean pine nut, there is still a substantial lack of knowledge on the quantification of its impact on Stone pine seeds as well as some debate about the bug relative impact compared to that of other biotic and abiotic factors. In **Chapter 3**, I aimed at characterizing the impact of LO on cones and seeds of Stone pine. The use of X-raying technique in force feeding trials allowed me to follow *L. occidentalis* damage on seeds week by week. Previous studies have shown the impact of this insect on other *Pinus* spp. but in which *P. pinea* was not included (Bates *et al.*, 2000; Lesieur *et al.*, 2014). A full characterization of the type of damage on *P. pinea* seeds caused by this insect was then a critical first-step to allow the development of an accurate impact quantification methodology for field-based studies.

Comparing life history and ecology in the native range and the invaded Portugal

The Western Conifer Seed Bug, *L. occidentalis*, is a hemipteran insect in the family Coreidae characterized by a brownish colour, hind tibia with expansions resembling a leaf and a zig-zag white line across the hemelytra (Koerber, 1963). Adults leave their winter shelters in April / May, depending on the climatic conditions (Barta, 2016) and fly to a pine stand to feed and mate. Eggs are laid on pine needles from which first instar nymphs emerge. This species has five instar nymphs all aptera. Young nymphs are orange and brown becoming reddish-brown as they develop (Bernardelli & Zandigiaco, 2001). The total developmental time from egg to adult depends on external temperature ranging from 78 days at 20°C to 25 days at 30°C (Barta, 2016). The number of generations of this bug varies with latitude from one to several. Barta (2016) using climatic data and laboratory studies to infer *L. occidentalis* life cycle at different temperatures predicted the number of generations for most of the European countries. In general, in central Europe the bug would complete one generation, two in the south, three in Spain and Malta and four in Cyprus (Barta, 2016). Field data is now needed to corroborate and calibrate these results.

This bug is a polyphagous sucking insect that feeds on pine cones of various host species, namely those belonging to the genera *Pinus*, *Picea*, *Abies*, *Cedrus*, *Tsuga* and *Pseudotsuga*. Both adults and nymphs feed on cones and seeds in all development stages.

With the invasion of Europe, *L. occidentalis* expanded its host range to new pine species such as the Maritime pine, *P. pinaster*, and Stone pine, among other European pine species. Due to the economic relevance of Stone pine edible seeds it became preeminent to assess *L. occidentalis* host preferences in the new invasive continent. Therefore, **Chapter 4** explores the preference by *L. occidentalis* for the three most important pine species growing in the Mediterranean Basin: Stone pine, Maritime pine and Aleppo pine *P. halepensis*. Results from laboratory studies using branches, cones and mature seeds are presented and discussed.

Besides preference among host species, preference between trees of one given host (host selection) is also pertinent to assess since it has implications in control and management measures. It has been demonstrated that *L. occidentalis* is capable of distinguishing between clones of *P. contorta* Douglas in its native range of Western North America (Blatt & Borden, 1996; Blatt, 1997; Richardson *et al.*, 2017). Suggested cues for this selection are the size of the cones, chemical volatiles profile of the tree and cone reflectance (Blatt & Borden, 1996, Richardson *et al.*, 2017) which can be related to the tree physiological status. We can then hypothesize that the physiological status of the tree is probably an important cue for *L. occidentalis* when choosing a tree to colonize. In fact, experiments with a close species, *L. phyllopus*, demonstrated that the quality of the host prevailed in all times over the host species (Mitchell, 2006). Resourcing to seed orchards under different management practices we evaluate the cue of the tree physiological status in Stone pine. I compared the impact of *L. occidentalis* on trees that were fertilized and irrigated and trees that were not. Results and conclusions can be found in **Chapter 5**. All trials were conducted in one Stone pine seed orchard of grafted trees.

Can we reconstruct the invasion history of *L. occidentalis* in the Iberian Peninsula?

A recent genetic study suggested that the source population of the primary European invasion in Italy originated from Eastern America, a primary invaded region, and not from the native Western America, a phenomenon named as "bridgehead effect" (Lesieur *et al.*, 2018). It was also hypothesized that *L. occidentalis* invasion of Europe was further accelerated by several other independent introductions from Eastern America, combined to long-range translocations of European invasive populations (Lesieur *et al.*, 2018) and to good flight capacities and polyphagous habits which contributed to the successful expansion

of this pest. Additional introductions in Spain, namely in Barcelona and Valencia, were thus suggested by Lesieur *et al.* (2018) but the complete invasion history of the Iberian Peninsula still needed to be clarified. A multi-marker strategy combining molecular markers, mitochondrial and nuclear, has been widely used to analyse populations' structure and have been proved to be useful for elucidating the invasion history and the reconstruction of the invasion routes (Kirk *et al.* 2013). Lesieur (2014) had already developed 11 microsatellites to *L. occidentalis* that now can be used to disentangle the invasion route of the Iberian populations. In **Chapter 6** of this thesis I present insights of how the invasion of *L. occidentalis* in the Iberian Peninsula may have occurred taking into analysis new genetic data of mtDNA and microsatellites and observational data of field first records.

Project plan and objectives

The overall objective of this PhD project was to characterize the impact and ecology of the invasive seed feeder *L. occidentalis* in Portugal. Emphasis was given to the interaction with Mediterranean pines, especially with Stone pine due to its major importance for nut production. First, I start by (1) do an exhaustive review of what we know and what we still need to know about *L. occidentalis* and its host Stone pine (Task 1 - Figure 2).

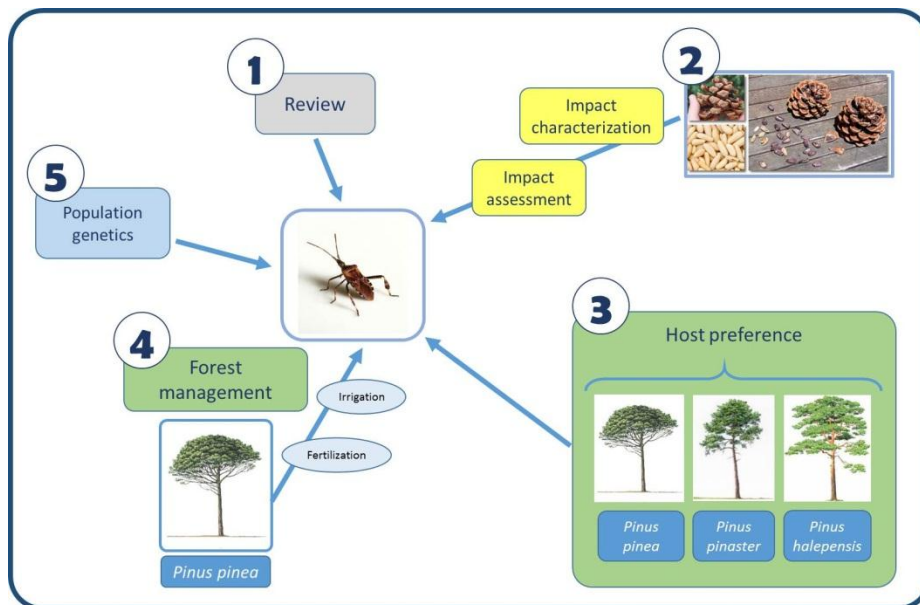


Figure 2. Concept model of the PhD plan. Review (1) and the three parts of the PhD: impact, ecology and invasion history. Yellow task (2) is impact- related; green tasks (3 and 4) are ecology- related; blue task (5) is invasion history- related.

After, the PhD plan was divided into three big issues: the impact, the ecology of *L. occidentalis* on Stone pine in Portugal and the invasion history of the bug in the Iberian

Chapter 1 - Introduction

Peninsula. For the impact I began by (2) characterizing and assessing the impact of the bug on seeds of Stone pine. The second big issue related to ecology had two tasks: (3) to investigate if this bug has a host preference among the most common pines in the Mediterranean Basin region and (4) to analyze if it selects differently trees of Stone pine under different management strategies (Figure 2). Finally, I studied the (5) invasive pathway and population structure of *L. occidentalis* in the Iberian Peninsula.

Research questions and hypothesis were set for each objective (Table 1).

This PhD project will present insights on important issues related to this serious invasive pest which will help decipher its ecology and impact on Stone pine.

Three scientific publications were accepted in indexed journals reporting the work that I developed during this Ph.D.

Table 1. Research questions and hypothesis of the PhD project. Hypothesis were formulated based on the available literature results.

Objective	Research question	Research hypothesis
1 Review	What do we know and what are the major knowledge gaps in the interaction of <i>L. occidentalis</i> with the host <i>Pinus pinea</i> ?	n.a.
2 Impact characterization	How can we discriminate <i>L. occidentalis</i> feeding on cones and seeds of <i>P. pinea</i> ?	<i>L. occidentalis</i> has a damage signature in cones and pines of <i>P. pinea</i>
3 Host preferences	Does <i>L. occidentalis</i> have a preference among the most common pines in south Europe (stone pine, maritime pine and aleppo pine)?	<i>L. occidentalis</i> prefers Stone pine compared to other Mediterranean pines
4 Host management	Will <i>P. pinea</i> management in grafted seed orchards influence <i>L. occidentalis</i> impact?	Fertilized and irrigated trees have higher losses of pine nuts and cones due to <i>L. occidentalis</i> .
5 Population genetics	Does the Iberian invasion proceed from the introductions in Barcelona and Valencia? Was there any other introduction in the Peninsula?	The invasion of the Iberian Peninsula resulted from the spread of bugs from Barcelona and Valencia
	Can we find a genetic structure in the Iberian populations of <i>L. occidentalis</i> ?	There is no genetic differentiation in the populations of <i>L. occidentalis</i> in the Iberian Peninsula

CHAPTER 2

**How important is the non-native seed feeder
Leptoglossus occidentalis on the sustainability of
the Mediterranean Stone pine?**

State of the art



Review manuscript in preparation

Chapter 2 – How important is the non-native seed feeder *L. occidentalis* on the sustainability of the Mediterranean Stone pine? A review

Farinha, A., Mutke S., Lesieur, V., Calama, R., Roques, A., Sousa, E., Branco, M.

Abstract

Stone pine edible seeds market is an important sector across the Mediterranean Basin countries where this pine is distributed. There is an increased investment in new plantations by landowners, mainly in Portugal, Spain and Turkey. However, evidence of a decrease in cone production and in seed yield has alarmed producers, researchers and the industry. In the last five years much has been hypothesized about the responsibility of the invasive insect *L. occidentalis* on the losses of *P. pinea*. Research should be oriented towards clarifying the determinants of this decrease.

Here we gather information on the insect *L. occidentalis*, the host Stone pine (*Pinus pinea*) and the insect-plant interaction in the Mediterranean Basin countries. It reviews the literature on these subjects focusing on the recent breakthroughs in the bug ecology and impact.

We start by describing the Mediterranean Stone pine and its edible seeds presenting data on cone and seed economy as well. Losses on production of cones and seed yield are then discussed. After, it follows a chapter on the bug. General biology and ecology of *L. occidentalis* is presented. A review on this bug impact estimations and control measures are also presented. We then identify important research gaps and suggest future research lines that could help understand the interaction between this bug and the Stone pine. An integrative management plan of Stone pine forests combining nut production with *L. occidentalis* impact and market demands on a Mediterranean scale it is urgently needed in the near future.

Keywords: stone pine, seed bug, Hemiptera, pine nut

Mediterranean Stone pine and its edible pine nut kernels

1. Distribution and silvicultural management of Stone pine

Native to the Iberian Peninsula, the Stone Pine, *Pinus pinea* L., has been an important planted forest tree in Mediterranean countries since the Antiquity for provision of timber, firewood and pine nuts (Thirgood, 1981; Agrimi & Ciancio, 1993; Prada *et al.*, 1997; Gil, 1999). In the last decades, the increasing world market demand on its nuts has led to increased demand for this species as alternative crop on farmland of Mediterranean climates. The tree performs well on poor soils even with reduced cultural practices, and it resists well to climate adversities such as droughts or late frosts (Bilgin *et al.*, 2000; Sülüşoğlu, 2004; Mutke *et al.*, 2007b; Loewe & Delard, 2015; Guadaño *et al.*, 2016; Guadaño & Mutke, 2016).

Stone pine is a conifer tree up to 25-35 m high and with 1-2 m stem diameter in monumental trees. In forests Stone pines more likely culminate at 12–20 m height and 40–50 cm diameters at the end of the silvicultural rotation (80–120 years). Open-grown Stone pines present a characteristic umbrella-like crown shape, often overtopping a lower layer of Mediterranean shrubs and oak woodlands (Lanner, 1989; Fady *et al.*, 2004; Mutke *et al.*, 2005b, 2012). Native to the Mediterranean Basin, Stone pine is distributed sparsely from the Portuguese Atlantic coast to the shores of the Black Sea and the slopes of Mount Lebanon, growing from sea level up to 1 000 m, occasionally to 1 400 m a.s.l. (Blanco *et al.*, 1997; Prada *et al.*, 1997; Quézel & Médail, 2003) (Figure 1). Since 1900, its forest area has more than doubled to more than 0.7 million hectares as results of a series of afforestation initiatives, aiming often for soil protection, especially in case of bare dunes or former estuary marshes where other tree species cannot grow. Other objectives for afforestation were ecosystem restoration on degraded pastures or scrubland, landscaping, as well as the expected production of both timber and pine nuts (Feinbrun, 1959; Agrimi & Ciancio, 1993; Prada *et al.*, 1997; Gil 1999; Scarascia-Mugnozza *et al.*, 2000; Fady *et al.*, 2004; Konstantinidis & Tsiourlis 2011; Loewe *et al.*, 2012; Mutke, 2013; Yilmaz *et al.* 2013).

2. Phenology of Pinus pinea

Within the taxonomic group of Mediterranean pines, Stone pine is the only species with seeds larger than 15 mm, reaching up to 20 mm, and ripening only two and a half years after pollination, a year later than in most other pine species (Klaus & Ehrendorfer, 1989;

Montero *et al.*, 2004). Annual shoot growth and cone formation is verticiled, and this 2.5-year time lag implies that up to three consecutive cone cohorts can be found simultaneously on the same tree, placed in three consecutive whorls. In spring, the new receptive female conelets emerge from canopy on the tips of vertical, recently elongated spring shoots, sticking in mid-air to be pollinated by wind before new needles grow and involve the shoots (Abellanas & Pardos, 1989; Abellanas, 1990).

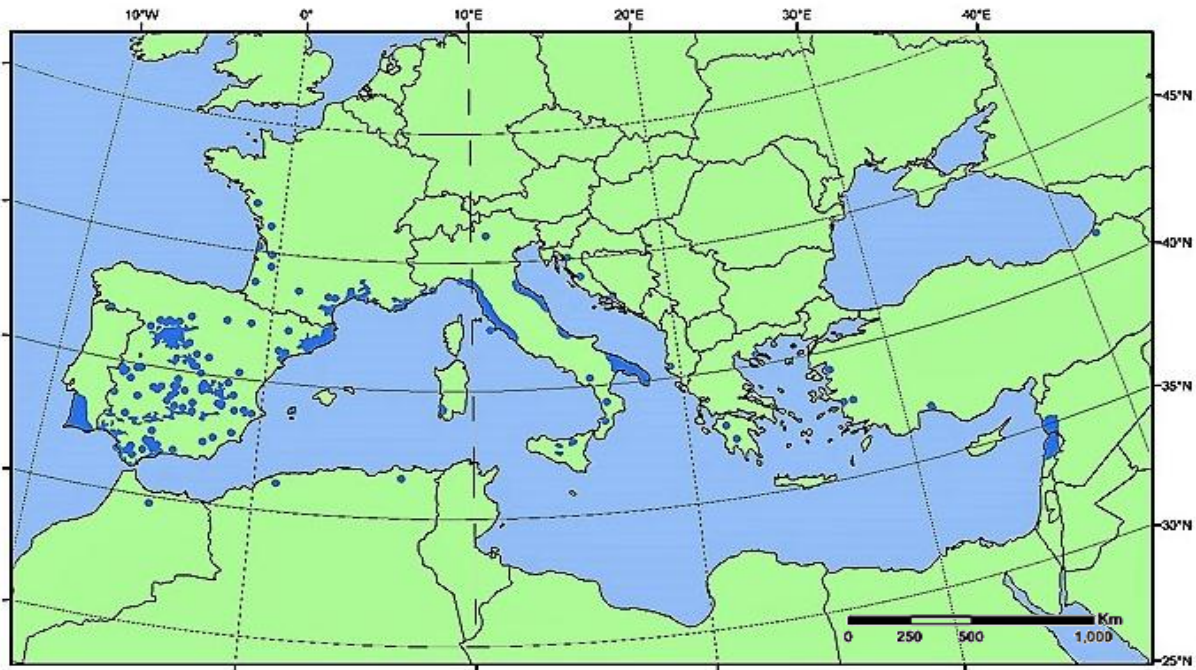


Figure 1. Natural distributions of *P. pinea* (EUFORGEN 2009).

Timing of pollination phenology varies between climatic zones. It can happen from April to June depending on the accumulated degree day sums, knowing that it requires about 1,000-degree days above a threshold of 1°C (Mutke *et al.*, 2003). At the same time and just one whorl beneath the shoot apex, last year's conelets are growing between new lateral shoots, to reach about 2-3 cm until summer. Meanwhile, those cones pollinated two springs ago, placed at the second whorl from the shoot tip, are finally ripening. After fertilization and start of embryogenesis in late spring, cones will grow to a final size of 12-15 cm length until mid-July. While internal seed development will further progress until maturity in autumn. This upcoming cone yield will be harvested in the following winter. Considering that also primordia of next year's strobili are already being formed within the new shoot buds in

late spring, even four consecutive cohorts do coincide on the tree. For instance, studies of cone yield time series at forest, stand, tree and even shoot scale have observed a negative autocorrelation with a lag of 3 years. Indeed, heavy cone crop ripening in one spring does reduce the number of induced primordia, that is, the conelet number for next spring and the resulting cone crop three yields later (Mutke *et al.* 2005a; Calama *et al.*, 2008, 2016a).

3. *The edible seed*

Edible conifer seeds are extracted from cones that are usually gathered from trees in forests rather than in agronomic plantations. In case of Mediterranean Stone pine however, ongoing domestication is aiming at orchard production of Mediterranean pine seed, a traditional ingredient of many Mediterranean and Arabian dishes (Mutke *et al.*, 2007b, 2012; Salas-Salvado *et al.*, 2011). Stone pine seeds are large (around 2 centimetres long) and pale brown with a powdery black coating. They consist of a thick seed coat (around 1.4 mm) and an ivory-white edible kernel. In general usage, when a seed has an edible kernel, the seed coat plus the kernel, can also be called nut. These nuts are considered one of the most emblematic and economically relevant non-wood forest products of Mediterranean forests and woodlands (Sheppard *et al.*, 2016). Stone pine nut kernels are among the most expensive nuts in the world, highly esteemed as gourmet and health food. They are rich in unsaturated fatty acids (mainly omega-9 and omega-6) and proteins (35%), doubling the protein content of other traded pine nuts species, mainly the Asiatic *Pinus koraiensis*, *P. gerardiana* or *P. sibirica*, which differ clearly in taste, dietetic values and lower wholesale prices from genuine Mediterranean pine nuts. Human consumption of Mediterranean pine nuts benefits health due to high contents in bioactive carbohydrates, e.g. low-molecular weight carbohydrates such as fagopyritol in concentrations similar to that found in buckwheat; and richness in minerals (phosphorus, iron, zinc, magnesium), thiamine and riboflavin (vitamine B1/B2), tocopherols (vitamine E), phytosterols and polyphenols (Carrascal, 1994; Cañellas *et al.*, 2000; Ferreira & Pestana, 2000; Nasri *et al.* 2005, 2007, 2009; Costa & Evaristo, 2008; Evaristo *et al.*, 2010, 2012; González *et al.*, 2012; Ruiz-Aceituno *et al.*, 2012; Özcan *et al.*, 2013). Kernels are obtained by shelling pine nuts after extraction from cones. The seed coat is thick and woody, pine nuts yielding only 25% kernel in weight, i.e. in average 0.15 g out of 0.6 g per unit, respectively. Mean cone weight is 250-350 g when harvested in winter (relative humidity 50%), yielding 15-20% seeds and only 3.5-4% kernels in weight. Cone picking has traditionally been done by tree climbers using long, hooked poles, although during the last decades, the use of specific mechanical

tree shakers has been generalised where tractors can access tree stems, reducing occupational risks associated with manual cone harvest (Martínez-Zurimendi *et al.*, 2009; Castro-García *et al.*, 2011; Mutke *et al.* 2012).

4. Cone and seed economy

In the last 30 years private land owners have been investing directly into more than 0.25 million hectares of new *P. pinea* orchard-like plantations, often on farmland that renders higher cone crops than poor sandy sites where traditional pine forests grow. Portugal and Turkey are leading this evolution, both having multiplied nearly fourfold their historic Stone pine area since the 1980's. Also, in Chile and New Zealand, there are now ongoing initiatives of planting Stone pine for pine nut production (Loewe & Delard, 2016, 2017; Küçüker & Baskent, 2017). Management of these plantations does focus on optimised cone production rather than on multipurpose forestry (DGF, 1985; Acar, 1995; Calado, 2012; Mutke *et al.*, 2012; Kilci *et al.*, 2014; Santos, 2015; Can, 2016; Loewe & Delard, 2016). Mechanic harvesting, as well as modern automatized cone processing facilities, has strongly lowered associated labour and costs (Mutke *et al.*, 2000a, 2000b, 2007a, 2012; Loewe & Delard, 2012). Together with a sustained high price for kernels, exceeding 60 €/kg in retail, lately even 100 €/kg, it has made cone harvesting more profitable, giving higher revenues to forest owners than the slow-growing, low-value timber of Stone pine (Mutke *et al.*, 2000b; Gordo, 2004; Ovando *et al.*, 2010; Pasalodos *et al.*, 2016; Gordo *et al.*, 2016).

World market demand for pine nut kernel moves several hundred million euros annually (Awan & Pettenella, 2017; EUROSTAT, 2017; Küçüker & Baskent, 2017; Mutke *et al.*, 2015, 2017b), but production in each country varies among years from one-half to five times the average yield, owing to mast synchronising of regional crops mainly by weather cues (Mutke *et al.*, 2005a; Gonçalves & Pommerening, 2012; Calama *et al.*, 2008, 2016b). In Portugal, mean production of cones is about 700 kg/ha and in Spain 100 Kg/ha (Mutke *et al.*, 2012). Considering that kernel-per-cone yields are around 2-4%, one hectare may generate 2 - 28 Kg of kernel sold later by the industry at a price between 25 and 45 €/kg (Mutke & Calama, 2016). Global Mediterranean pine nut production is about 16,000-20,000 metric tons of pine nuts annually, i.e. 4,000-5,000 tons of shelled kernels (FAO 2010; INC, 2012).

Nevertheless, the bases of national and European import-export data are not sound, because declaration starts often only with kernels obtained in factory and does not reflect quantity and actual origin of cones processed. Combined nomenclature trade codes subsume within the same code *NC 0802 90 50* any kind of 'pine nuts, fresh or dried,

whether or not shelled' (Council Regulation EEC 2658/87, Annex I; Mutke *et al.*, 2013; Awan & Pettenella, 2017). From per kilogramme prices it can be deduced that data for shelled kernel (25-45 €/kg), pine nuts in shell (2-5 €/kg) and occasionally even closed cone exports (less than 1 €/kg) are mingled. E.g., part of export from Portugal to Spain has been traditionally in form of unprocessed cones, not pine nuts, and hence added to apparently "Spanish" pine nut kernel production that is (re)exported. Moreover, code NC 0802 90 50 doesn't even allow for distinction between genuine Mediterranean pine nuts (*Pinus pinea*) and imports of lower-priced seeds of other, Asiatic pine species, such as *P. koraiensis*, *P. sibirica* or *P. gerardiana*, whose volumes (re)exported by Germany or the Netherlands exceed the genuine Mediterranean pine nut exports widely (Agri-Ciência, 2014; Pastor, 2014; Mutke *et al.*, 2015; EUROSTAT, 2017).

5. Conelet abortion and seed yield loss – The dry cone syndrome (DCS)

On the background of this short overview for pine nut production from Mediterranean forests and orchards, during the last few years alarms have been raised by actors along the supply chain that production has dropped drastically in most Mediterranean countries. Ongoing climate change and increased cone pests' prevalence have been named as putative causes, reducing severely the per-hectare number of cones harvested in the last few years (Tiberi, 2007; Sousa *et al.*, 2012; Parlak *et al.*, 2013, 2017; Mutke *et al.*, 2014; Can, 2016; INC, 2016). Occurrence of low cone crops is relatively frequent related to the effect of severe droughts and can be neatly modelled using deficient rainfall sums during cone induction and development as predictive variables (Mutke *et al.*, 2005a; Calama *et al.*, 2008, 2011, 2016b). However, in the last years, cone pickers have reported an unusual high number of aborted conelets i.e. unripe first and second year conelets which dry before maturation. This phenomenon has contributed to decrease the final cone yield. Additionally, processing industries have reported an alarming drop in kernel-per-cone yield obtained in factory for Stone pine cones collected in main producing countries, namely Turkey, Portugal, Spain, Italy, and Lebanon. When cracking apparently sane cones, up to half of the seeds are empty or contain only withered remains of the kernel. As consequence, income for forest owners, cone pickers and processors has plumped, and economical sustainability of the forest system and value chain is seriously jeopardised (Mutke *et al.*, 2014; Mutke & Roques, 2015).

The fast-coincident rise and expansion of both phenomena, some ten years ago in Italy, 2008 in Catalonia, and since 2011/12 generalised in the rest of the countries, has coined

the common name *Dry Cone Syndrome (DCS)*, suggesting a possible common agent (Tiberi, 2007; Mutke *et al.*, 2014, 2017a). Only in these last five years, awareness has grown in the respective national pine nut value chains about the emergence of *DCS*. Similar to the case of other wild collected Non-Wood Forest products (NWFP), the lack of sound official statistics on yields has been hindering to back up the severity of the problem. Though in several countries, statistics of non-wood forest products are published by Ministries of Agriculture, they are often mere estimates for number of annual crops, based on processing industries' declarations or extrapolated from public forests sales, lacking information for private forests. One direct source for information about the incidence of the *DCS* has been a survey in processing industries about the seed and kernel-per-cone yield, conducted since 2014, to assess the impact of *DCS* (Mutke & Roques, 2015). Though only a short number of responses were obtained from cone processors, their cone supply comes from a wide geographic range and includes all relevant Stone pine growing areas in Spain and Portugal. Also, the Lebanese Pine Farmers Association contributed with yield series of forest management units reported by 15 forest owners who harvest and process their own cones and have registered the proportion of empty seeds in the last 7 years (Mutke *et al.*, 2017a). Available time series of seed per cone yield data from Iberian cone processors showed that average pine nut per cone weight yield had dropped from a stable yield of 17 % before 2010 to 5-12 % since 2012. Average kernel per cone weight yield had decreased from stable 3.8 % before 2008 to 2.2-2.8% since 2011. Unusual increases in percentage of empty or internally damaged among normal-sized seeds were specified as main reason: historic values were less than 10%, but currently proportions of 30-50% are observed. The same increase of empty seed proportion has been observed in the Lebanese data series (Mutke *et al.*, 2017a).

As putative causes for conelet abortion and empty or damaged seeds, different biotic agents or abiotic factors have been discussed (e.g. droughts & climate change, poor pollinization, cone pests and the fungus *Diplodia pinea*). One special clear hint was the coincidence of the appearance of the Dry Cone Syndrome with the parallel expansion of an invasive exotic seed pest, *Leptoglossus occidentalis*. This bug was introduced accidentally in Italy probably during the late 1990s and has quickly spread since across all Europe and the Mediterranean countries. Evidence that the seed bug damage can result in abortion of seeds or even conelets in several conifer species, including those of genus *Pinus* (Bracalini *et al.*, 2013; Lesieur *et al.*, 2014; Farinha *et al.*, 2018a, 2018b) points even more strongly for this insect to be a major agent responsible for decreased seed production observed in *P. pinea*.

The Western Conifer Seed Bug, *Leptoglossus occidentalis*

1. Origin, history of invasion (record and genetic data), current distribution

The Western conifer seed bug, *Leptoglossus occidentalis* Heidemann 1910 (Heteroptera, Coreidae) is a conifer seed feeder native to western North America (wNA). This species is widely found from British Columbia to Mexico in the North-South direction and from the Pacific coast to Colorado in the West-East direction (Figure 2; Koerber, 1963; Hedlin *et al.*, 1980; Zhu *et al.*, 2013). However, its range probably corresponds more accurately to the western distribution of the Pinaceae, its host plants. Probably accidentally introduced by human activities, the bug was discovered outside its native range to eastern North America (eNA) with a first record in Iowa in the middle of the 1950s (Schaffner, 1967). Since then, *L. occidentalis* spread eastwards and its eastern invasion has been largely documented. In the 1970s, it was observed in Wisconsin and Illinois and the bug was reported to reach the Atlantic coast in the 1990s (McPherson *et al.*, 1990; Marshall, 1991; Gall, 1992; Wheeler, 1992). It further spread in eNA as far east as Nova Scotia (Ridge-O'Connor, 2001; O'Shea *et al.*, 2005; Scudder, 2008). In eNA, the species is now established in four Canadian provinces and 18 US states (Figure 2).

In Europe, the species was first reported in 1999, in northern Italy, in Vicenza area (Tescari, 2001). This first observation was followed by many records in Italy (Bernardinelli & Zandigiacomo, 2001; Olivieri, 2004; Taylor *et al.*, 2001; Villa *et al.*, 2001) until Sicily, where the species was reported in 2002 (Maltese *et al.*, 2009). Shortly thereafter, the species was observed in southern Switzerland (Colombi & Brunetti, 2002) and Slovenia (Gogala, 2003) and in 2003, the species was reported for the first time in Spain, near Barcelona in Catalonia (Ribes & Escolà, 2005). The bug continued to expand its range and, in a very short time frame, *L. occidentalis* has conquered, if not all, almost the entire European continent (Figure 2). The bug has now been observed from Portugal (Sousa & Naves, 2011) to Ukraine and Russia (Gapon, 2012), and from Norway and Sweden (Lindelöw & Bergsten, 2012; Mjøs *et al.*, 2010) to Sicily and Turkey (Maltese *et al.*, 2009; Fent & Kment, 2011).

Following the European invasion, *L. occidentalis* has become a highly successful worldwide invader with observations in Asia (China; Zhu (2010), Japan; Ishikawa & Kikuhara (2009) and South Korea; Ahn *et al.* (2013)) but also in North Africa (Morocco; Gapon (2015)),

Tunisia; Jamâa *et al.* (2013)) and the most recent record, in Chile, South America (Faúndez & Rocca (2017).

When considering the edible pine seed production of the Stone pine, *P. pinea*, the species seems to be well established in all main producing countries (Figure 2) namely Portugal (Pimpão, 2014; Grosso-Silva, 2010; Farinha *et al.*, 2018b), Spain (Pérez Valcárcel & Prieto Piloña, 2010; Mutke *et al.*, 2015a), Italy (Roversi *et al.*, 2011; Bracalini *et al.*, 2013), Turkey (Fent & Kment, 2011; Özgen *et al.*, 2017), France (Dusoulier *et al.*, 2007; Lesieur *et al.*, 2014) and Greece (Petraakis, 2011; Van der Heyden, 2017) but also in Morocco (Gapon, 2015) and Lebanon (Nemer, 2015).

The European invasion of *L. occidentalis* is a complex scenario. The fast colonization of Europe is likely the result of multiple introductions combined with spread from established populations (Lesieur *et al.*, 2018). Indeed, in addition to the first Italian outbreak, several introductions were suspected because of spatially disconnected first records in Spain (Ribes & Escolà, 2005; Pérez Valcárcel & Prieto Piloña, 2010), France (Dusoulier *et al.*, 2007), Belgium (Aukema & Libeer, 2007) and Great Britain (Malumphy *et al.*, 2008). Recent advances confirmed that European invasion results from multiple independent introductions in Europe originating from eNA (the North American invaded area) coupled with secondary spread within Europe (Lesieur *et al.*, 2018). At least, two independent introductions are confirmed by molecular data; the first one in northern Italy and the second one in Barcelona's area in Spain. Additional introductions from eNA in Spain, France and Austria are also highly suggested by the results. Likewise, this study also shed light on movements of individuals within Europe by their own dispersal or by anthropogenic activities which likely enhanced the rapid spread of this invasive species (Lesieur *et al.*, 2018).

Many observations confirmed that *L. occidentalis* seems to be a "perfect hitchhiker" (Dusoulier *et al.*, 2007; Malumphy *et al.*, 2008; Sciberras & Sciberras, 2010; Lesieur, 2014; Endrestøl & Hveding, 2017). Records near important harbor areas (*e.g.* Venice, Barcelona, Le Havre, Ostend or Weymouth) suggested that the propagules may have been transported by ships in containers. Moreover, interceptions of adults with timber shipments suggested that the introduction pathway could be related to such shipments (*e.g.* timber logs or wood panels) from the USA, associated with the bug habits to aggregate inside man-made structures in the fall to seek shelter for overwintering (Dusoulier *et al.*, 2007; Malumphy *et al.*, 2008). Furthermore, individuals (eggs, nymphs or adults) may also spread with the trade of their host plants, *e.g.* with the intense Stone pine cone trade along the Mediterranean basin region or with commercial Christmas trees or other ornamental trees (Gall, 1992; Sciberras & Sciberras, 2010; Gapon, 2012). For instance, Sciberras & Sciberras

(2010) noted that the first specimens detected in Malta were observed in 2004 when a small group of trees were being loaded out of a container arriving from Italy. A genetic study aiming at deciphering *L. occidentalis* invasion routes in the Iberian Peninsula found evidence for a stratified dispersal mechanism which encompasses short diffusion of individuals and long-distance dispersal probably human-mediated (Farinha *et al.*, in prep). Such mechanism of dispersal greatly accelerates the rate of invasion and leads to intraspecific hybridization (admixture) in populations. Both the greater speed and the constant entry of new individuals into a given location and also the increased admixture are difficult challenges to overcome when designing successful management strategies to this pest. Stone pine cone trade is probably an important human-mediated transportation of bugs across the peninsula and the rest of Europe.

In addition to long-distance dispersal, the rapid expansion of the range over a very broad front may also be due to the insect's own dispersive capabilities. Adults of *L. occidentalis* are considered as strong fliers (Koerber, 1963; Ridge-O'Connor, 2001; Malumphy *et al.*, 2008;) and the evaluation of flight capabilities in laboratory conditions using flight mills confirmed previous observations; some individuals were able to cover distances of more than 20 km (Lesieur, 2014).

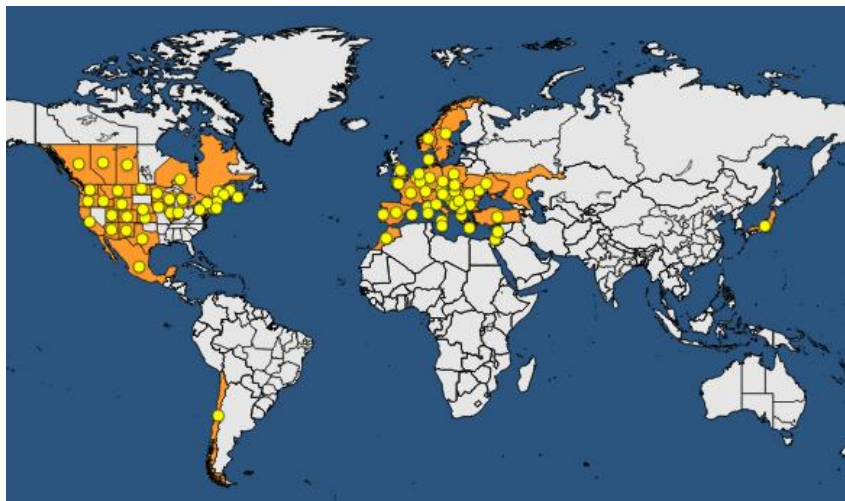


Figure 2. Currently world distributions of *L. occidentalis*.
(<https://gd.eppo.int/taxon/LEPLOC/distribution> consulted in 5/2018).

2. *LO* biology and ecology (life cycle, hosts, feeding activity)

The bug *L. occidentalis*, is a leaf-footed bug for having expansion on the hind tibia that resemble leafs (Koerber, 1963). Adults are 9 to 18 mm long (Figure 3) with females being

larger than males. The life cycle of the bug is represented in Figure 3. Females lay eggs in rows on the ventral side of the host needles from late April / May (Barta, 2016) depending on the climatic region. A single female lay on average 30 eggs in the field during its lifetime but in laboratory conditions it can be up to nearly 80 eggs (Bates & Borden 2005; Barta, 2016). Eggs are semi-cylindrical, barrel shape and measuring about 2 mm long. The colour is light brown after deposition becoming dark brown along the development of the nymph inside which takes about two weeks (Koerber, 1963). There are five nymphal instars. Young nymphs are orange and brown becoming reddish-brown as they develop (Bernardelli & Zandigiaco, 2001) until reaching adult stage. Total developmental time from egg to adult depends on external temperature ranging from 78 days at 20°C to 25 days at 30°C (Barta, 2016).

The number of generations that this bug has per year varies with latitude. It is described as univoltine in North America (Koerber, 1963) but multivoltine in Mexico (Cibrián-Tovar *et al.*, 1986). In Europe, it has been observed to complete one to three generations depending on location: one generation in an Alpine region of Northern Italy (Tamburini *et al.*, 2012), two generations in South Italy, in areas of low altitude level in Northern Italy and in South Spain (Maltese *et al.*, 2009; Tamburini *et al.*, 2012; Mas *et al.*, 2013) and three generations in North-eastern Italy (Bernardinelli *et al.*, 2006). Phenology of this insect is highly dependent on weather conditions (Barta, 2016). In southern Europe countries overwintered adults emerge from overwintering sites as soon as late March / April (Tamburini *et al.*, 2012; Mas *et al.*, 2013; and personal observations) when the mean daily air temperature starts to exceed 14.5°C (Bernardinelli *et al.*, 2006; Barta, 2016). Second generation of adults emerges in late July/August when a peak of the bug density can be observed in the field. Adults can be spotted on trees until October (Mas *et al.*, 2013) or even November depending on air temperatures (personal observation).

As other Coreidae, *L. occidentalis* has a gregarious behaviour that is more notorious in the younger development stages (Mitchell, 2000; Wertheim *et al.*, 2005) that are often observed to rest and eat in groups in the same branch. Studies on *L. occidentalis* ecology have showed that the bug tends to colonize always the same trees in a given orchard (Blatt, 1997; personal observations). Protection from the enemies and efficiency in resource exploitation has been pointed out as benefits associated with the gregarious strategy (Mitchell, 2000; Wertheim *et al.*, 2005; Mitchell, 2006; Fernandes *et al.*, 2015). Cues for the selection of trees can be related to the number of cones, chemical volatiles, tree physiological status and type of clone (Blatt & Borden, 1996; Richardson *et al.*, 2017;

Farinha *et al.*, 2018b). Furthermore, adults form large aggregations for overwinter purposes (Blatt, 1994).

The population dynamics of this species is classified as chaotic (*i.e.* high inter-annual population fluctuations) both in the native area (W. Strong, comm. pers.) and in the invaded areas (Tamburini *et al.* 2012; Lesieur *et al.* 2014;). So far, the reasons underlying these high population fluctuations are still poorly understood. Future research must focus on this key-stone issue.

L. occidentalis is a pine seed feeder. It is frequently observed on top of the cones inserting its stylets between the cone scales to reach the content of seeds. It has sucking mouthparts composed by modified mandibles and maxillae to form a stylet of two channels, the salivary and the food channel, sheathed within a modified labium (Cobben, 1978). When feeding, first the salivary channel injects two types of saliva, gelling saliva to line the path of the stylets and watery saliva with enzymes to digest tissues exteriorly. The liquefied food is then sucked through the food channel and ingested (Cobben, 1978). Although, feeding mainly on seeds it also feed on xylem for hydration (Mitchell, 2000) as previously described in a related species, *L. phyllopus* (Linnaeus,1767) (Mitchell, 2006).

Both adults and nymphs feed on cones and seeds of several coniferous. In the field this bug can feed upon cones in all development stages. When feeding on young conelets *L. occidentalis* can cause abortion compromising the seed production of the followed years.

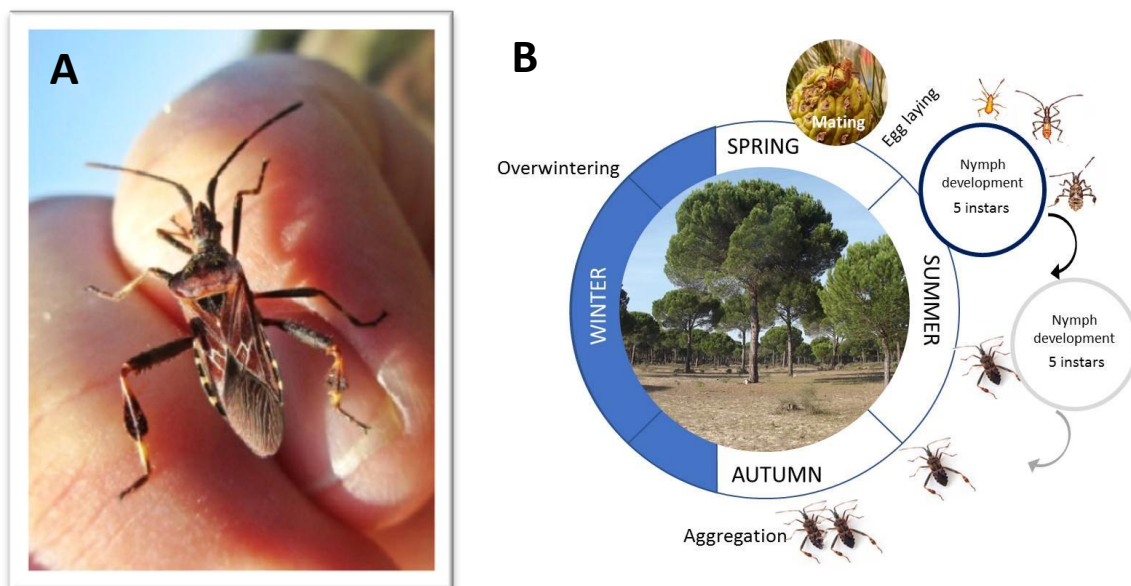


Figure 3. (A) Adult *L. occidentalis* and its' (B) life cycle. Photo: *Leptoglossus occidentalis* at Mulderskop zuid, the Netherlands. Photo by B. Schoenmakers at waarneming.nl, a source of nature observations in the Netherlands (uploaded number 15027646). <https://commons.wikimedia.org/w/index.php?curid=62780536>

3. *L. occidentalis* impact on cones and seeds

As said, both adults and nymphs feed on cones and can potentially create significant economic losses in high-value seed orchards for regenerations and afforestation proposes (Strong *et al.*, 2001; Bates *et al.*, 2002b; Bates & Borden, 2005) or for edible seed production (Calama *et al.*, 2016a, 2017b; Ponce *et al.*, 2017a; Farinha *et al.*, 2018b). Feeding activity extends from late spring, when adults leave overwinter sites, until the onset of the hibernation conditions, that can range from September to November depending on the latitude (Barta, 2016) and altitude (Tamburini *et al.*, 2012). Damage estimations are scarce mainly because *L. occidentalis* leaves no visible external signs on cones (Lait *et al.*, 2001). Moreover, pine cones have a maturation time that can be of one, two or even three years depending on species (Owens & Blake, 1985; Kolotelo, 1997). Thus, damage assessment is hard to follow, from flowering up to ripen cone, specially in three-year development cones like the ones of *P. pinea* (Mutke *et al.*, 2003; Valdivieso *et al.*, 2017). Differences on the impact of adults and nymphs of *L. occidentalis* in conelets are not consistent between hosts. In *Pinus contorta* var. *latifolia*, experiments showed that the majority of nymphs fed only on conelets fail to develop to the next instar (Bates *et al.*, 2002a). In *Pinus monticola*, both nymphs and adults cause considerable damage to conelets (Bates *et al.*, 2002a) but in *Pinus sylvestris* mortality of conelets did not differ among bags implemented with adults, with nymphs and control ones without insects (Lesieur *et al.*, 2014). All three pine species, *P. contorta* var. *latifolia*, *P. monticola* and *P. sylvestris* have cones that take two years to develop; conelets refers to first year cones (Bates *et al.*, 2002a; Lesieur *et al.*, 2014). A recent study in *P. pinea* suggested that adults choose mature cones over young ones and that nymphs fed on both young and mature cones although no significant preference was noted (Farinha *et al.*, 2018b). However, it is important to highlight that *L. occidentalis* has different feeding behavior according to the season (Koerber, 1963; Schowalter & Sexton, 1990; Connelly & Schowalter, 1991; Schowalter 1994; Bates *et al.*, 2001, 2002a, 2002b, 2005; Strong *et al.*, 2001; Strong 2006; Calama *et al.*, 2016a, 2017b; Ponce *et al.*, 2017a). In field experiments using insects in caged branches of *P. pinea*, Ponce *et al.* (2017a) observed a high mortality of first-year conelets in the beginning of summer (around 90%) while second-year conelets mortality extend until August.

Concerning damage on seeds of matured cones, it has been characterized in several conifer species in both the native and invaded range using X-rays (Bates *et al.*, 2000b; Lesieur *et al.*, 2014; Farinha *et al.*, 2018a, 2018b). This methodology is expeditious and allows a fast

damage diagnosis. However, damage can be underestimated because seeds with light damage can appear sound in X-ray, especially when analyzing small seeds like the ones of *Pinus pinaster* or *Pinus halepensis* (Farinha *et al.*, 2018c). Opening seeds is a more reliable method for damage assessment although underestimation have also been reported due to the similarity between aborted seeds because of environmental or genetic reasons and aborted seeds because of *L. occidentalis* feeding in early stage of seed development (Schowalter & Sexton 1990). In its native range, estimate losses caused by *L. occidentalis* range from < 5 to 50% in Douglas-fir *Pseudotsuga menziesii* (Schowalter & Sexton, 1990; Blatt & Borden, 1996). In *P. contorta*, Bates & Borden (2005) estimated that for a hypothetical density of one seed bug per tree early in the season the expected seed loss would be of 310 seeds. In *P. pinea* seed damage estimations are clearly season dependent. In the beginning of summer, the higher percentage of damaged seeds are characterized as empty seeds, i.e., seeds that fail to develop while as the summer continues most of damaged seeds in cones present a kernel partially damaged or with a different colour (Calama *et al.*, 2016a, 2017b; Ponce *et al.*, 2017a).

Besides the fully developed seeds that are damaged, *L. occidentalis* is also believed to be the agent responsible for fused seeds, i.e., seeds that do not grow and get fused to the cone scale of ripen cones due to a feeding in an early stage of the seed development (Bates *et al.*, 2000, 2002b; Strong *et al.*, 2001; Lesieur *et al.*, 2014). This phenomenon leads to the reduction in the total number of extractable seeds per cone.

Laboratory force feeding experiments have showed that Stone pine mature seeds fed upon by *L. occidentalis* became shrunken and totally dry or only partially damaged according to the duration of feeding (Farinha *et al.*, 2018a). The same damage morphology was detected in force feeding studies englobing also seeds of *P. pinaster* and *P. halepensis* (Farinha *et al.*, 2018c) (Figure 4).

The notable variation of impact estimations and feeding behavior reveal great adaptability of *L. occidentalis* to different hosts, climates and environments.

An accurate impact estimation of this bug in *P. pinea* is mandatory to define economic thresholds before applying any control measure. Data from processing industries for Stone pine cones collected in the main producing countries, namely Portugal, Spain, Italy, and Turkey have showed an increase of 50% in the percentage of empty seeds from 2011 to 2014 (Mutke *et al.*, 2014). No data could be found to the followed years. Although *L. occidentalis* is pointed as the main biotic agent causing this loss (Mutke *et al.*, 2014, 2017a; Mutke & Roques, 2015; Calama *et al.*, 2016a), doubts still exist on the possible implications of increasing draughts and phenological shifts due to climatic change (Mutke *et al.*, 2005a;

Calama *et al.*, 2011) or even other biotic agents such as *Dioryctria mendacella* (Calama *et al.*, 2017a) and pathogen fungi like *Diplodia* spp. (Luchi *et al.*, 2011). The latter is a serious pest of Stone pine in Italy (Feducci *et al.*, 2009). Molecular studies (real-time PCR) proved that *L. occidentalis* is a potential vector for *D. pinea* in Stone pine forests (Luchi *et al.*, 2011). On this work, the fungi were found simultaneously on the bodies of *L. occidentalis* and on the symptomless *P. pinea* cones where the insect fed. Other study showed an average of 38% infected bugs in three different regions of North Italy (Tamburini *et al.*, 2012).

Both the bug and the fungi share similar habitat conditions. The hypothesis for the increase in damage in Stone pine cones be related to this fungi-insect interaction must be seriously study.

In conclusion, increased damage to Stone pine cones and seeds is a reality. However, discrimination of damage causalities in *P. pinea* cones is blurred and must be elucidated by ongoing research.

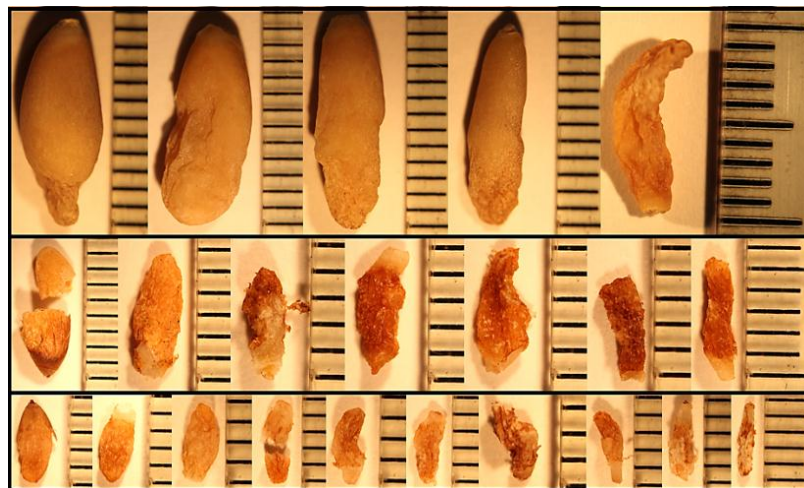


Figure 4. Photos of damage caused by *Leptoglossus occidentalis* on seeds of (from top) *P. pinea*, *P. pinaster* and *P. halepensis*. Seeds arranged in each row from the less (left) to the more damaged (right). Photographs taken by Canon 1100 D. The marks on the scale correspond to 1mm. (Photos were taken by Charlene Durpoix).

4. Host preference and selection

Information on the plant preferences of *L. occidentalis* is of central importance for pest management. This bug is known as a polyphagous insect quickly adapting to new hosts

when expanding its geographic range. More than 30 different conifer species belonging to several genera (*Abies*, *Calocedrus*, *Cedrus*, *Cupressus*, *Picea*, *Pinus*, *Pseudotsuga* and *Tsuga*) (EPPO, 2018) and even the pistachio tree, are described as hosts of this pest (Rice *et al.*, 1985). These polyphagous habits have been pointed out as crucial for the success of its world expansion. To our knowledge, only two studies have been conducted on *L. occidentalis* host preferences and both concerned the European invaded region (Lesieur *et al.*, 2014; Farinha *et al.*, 2018c). Comparison of bug preferences among mature seeds of several European conifers (*P. sylvestris*, *Pinus nigra*, *Abies nordmanniana* subsp. *bornmuelleriana*, *Larix decidua* and *Picea abies*) and those of the native pine *P. menziesii* var. *glauca*, did not reveal any clear preference among these host species (Lesieur *et al.*, 2014). Yet, on another study using cones instead of mature seeds the insect expressed a preference for *P. pinea* compared to *P. pinaster* and *P. halepensis* (Farinha *et al.*, 2018c). In this study, the bug spent more time feeding and visit more frequently the cones of *P. pinea*. The larger cones with consequently greater seeds, providing higher reward to the bug, were hypothesized to justify *L. occidentalis* preference on *P. pinea* cones. More literature is available on *L. occidentalis* preference between trees of a same host (host selection) than between different host species. In its native range, it has been demonstrated that *L. occidentalis* is capable of distinguish between clones of *P. contorta* (Blatt & Borden, 1996; Blatt, 1997; Richardson *et al.*, 2017). The cues explaining this selection are the size of the cones, chemical volatiles profile of the tree and cone reflectance (Blatt & Borden, 1996, Richardson *et al.*, 2017).

Concerning *P. pinea*, studies on orchards with trees under different management regimes revealed higher percentage of damage on conelets and ripen cones on trees that were fertilized and irrigated compared to control ones (Farinha *et al.*, 2018b). The physiological status of the tree may be a cue for *L. occidentalis* when it needs to choose a tree to colonize. Similar results were already demonstrated for a close species, *L. phyllopus*, to which the quality of the host had always prevailed over the host species in multiple experiments (Mitchell, 2006). Assessing *L. occidentalis* host preferences and host selection is pertinent both for ecological and economic reasons, especially having in mind the differentiate value of *P. pinea* seeds.

***Leptoglossus occidentalis* control**

1. Natural enemies and biological control

Natural enemies are most relevant for the control and regulation of prey populations. By reducing prey populations, natural enemies frequently help to keep other organisms at sustaining low levels. Yet, when organisms establish themselves outside their native range they benefit from the resulting isolation from the natural enemies existing in the native range. In consequence, non-native populations may have larger growth rates, as predicted by the enemy-release hypothesis (Keane & Crawley, 2002; Shea & Chesson, 2002).

However, the reverse may also happen, that is, native natural enemies of similar species may further shift to invasive species widening its feeding range (Mack *et al.*, 2000). In this context, it is very pertinent to test if native predators and parasitoids may shift to *L. occidentalis*. This could widen opportunities for the biological control of the bug.

Few studies document the activity of native predators and parasitoids of *L. occidentalis*. The impact of the natural enemies on the mortality of different developmental stages of the bug and its consequence on the population dynamics is poorly studied in its native range. Therefore, a complete depiction is required for the natural enemy's complex of *L. occidentalis*. On the other hand, very few studies address the impact of natural enemies on *L. occidentalis* mortality on the invaded range. Nevertheless, this information is most crucial for the development of control strategies, including the use of classical and conservation biological tactics. Here, we review current knowledge on the predators and parasitoids of *L. occidentalis*, on both its native and introduced range, and on the different developmental stages.

Eggs

Like many other bugs of the Coreidae family, the eggs of *L. occidentalis* are predated by generalist predators. Ants and orthopterans are considered common egg predators of *Leptoglossus* species. Signs of egg predation may be assigned to specific groups of predators. Missing eggs are mostly attributed to ant predation. Chorion with chewing damage can be a predation sign from orthopterans. Shrunken eggs may be charged to sucking activity by hemipteran predators, although egg desiccation may be also a result of

abiotic factors (Bates & Borden, 2005). Among these groups, ants are probably the major predators of the eggs. Still, egg mortality by predation is overall low. In a study conducted by Maltese *et al.* (2012) predation contributed to about 2% of egg mortality. Still, for the congeneric species *L. phyllopus*, also present in eastern North America, predation was observed to account to 28% of egg masses mortality (Mitchell & Mitchell, 1986). At the extent of our knowledge, there are no studies of potential predation rates of the egg masses of *L. occidentalis* by generalist predators in Europe. Since the egg masses of *L. occidentalis* are exposed in the needles of conifers, particularly pines, we hypothesize those eggs might be preyed also by ants and orthopteran present on this habitat. There is information that the eggs of the pine processionary moth, also positioned on pine needles, are commonly preyed by ants and tettigoniid orthopterans (Way *et al.*, 1999; López-Sebastián *et al.*, 2004; Mirchev *et al.*, 2015). We presume that these predators may also feed on the eggs of *L. occidentalis*. Still, the predation rates by generalist predators may be highly variable, depending on local populations of the predators, further depending on habitat and alternative prey available. It would be interesting to address the impact of the habitat diversity and landscape heterogeneity on the putative predators of *L. occidentalis* in Europe. In North America, the egg mortality of *L. occidentalis* is major due to the activity of parasitoids. The solitary parasitoid, *Gryon pennsylvanicum* (Hymenoptera: Platygasteridae) (Ashmead) is the main responsible for the egg parasitism of *L. occidentalis*. This parasitoid species frequently represents 80% or more of the eggs parasitized (Bates & Borden, 2004; Maltese *et al.*, 2012). Still, egg parasitism rates are highly variable and moderate. Using sentinel egg masses Bates & Borden (2004) found c.a. 30% egg mortality from parasitism. In another study, egg parasitism varied seasonally from 3% in June till a peak of 25% in July (Maltese *et al.*, 2012).

G. pennsylvanicum is polyphagous; it parasitizes several species within the Coreidae family from the genera *Leptoglossus*, *Narnia* and *Anasa* (Masner, 1983; Mitchell & Mitchell, 1986; Yasuda, 1990). In a study with another host, *Leptoglossus australis*, Yasuda & Tsurumachi (1995) found that *G. pennsylvanicum* uses volatile chemicals released from the male bugs as kairomone to find the substrate where eggs are laid. It might happen a similar kairomonal attraction for *L. occidentalis*. All known hosts for *G. pennsylvanicum* are great sized bugs, from North America or Japan. European coreids are smaller and therefore Roversi *et al.* (2011), considered that *G. pennsylvanicum* would not represent a risk to European species within the Coreidae family. This parasitoid species was thus proposed to be introduced in Italy, to controls *L. occidentalis* through a classical biological control program (Roversi *et al.*, 2011). A laboratory population of *G. pennsylvanicum* was kept in

quarantine conditions to perform rearing and risk assessment studies (Peverieri *et al.*, 2015). So far, there are no records of field releases.

Other egg parasitoids were also found associated with *L. occidentalis* in North America, mainly from genus *Anastatus* (Hymenoptera: Eupelmidae) and *Ooencyrtus* (Hymenoptera: Encyrtidae), but always emerging in lower numbers in comparison with *G. pennsylvanicum* (Bates & Borden, 2004; Maltese *et al.*, 2012). Two species of egg parasitoids were reported: *Anastatus pearsalli* and *Ooencyrtus johnsoni*. The former species is widely distributed in the Nearctic region and is highly polyphagous, parasitizing host from different orders and families (Bates & Borden, 2004). Little is known about the host range of *O. johnsoni*. This species is considered an important egg parasitoid of pentatomid species in North America (Ludwig & Kok, 1998). In general, the genus *Ooencyrtus* comprises polyphagous species, many of which are known to parasitize Lepidoptera or Heteroptera, or even alternate hosts between the two orders (Samra *et al.*, 2015).

In Europe, two native parasitoid species, *Ooencyrtus pityocampae* and *Anastatus bifasciatus* were recovered from field collected egg masses of *L. occidentalis* (Camponogara *et al.*, 2003). Both parasitoid species also parasitize the eggs of the pine processionary moth. This occurrence reinforces the idea that both the native moth and the non-native seed bug may partially share the same natural enemies' complex. Another *Ooencyrtus* species was also recovered in Europe from *L. occidentalis* egg masses (Lesieur and Farinha pers. obs.), but a complete picture of its impact and distribution is still lacking. Nonetheless, these preliminary results open research questions on the impact of native egg parasitoids on the control of *L. occidentalis* and how biological control conservation tactics may help to reduce the bug populations.

Nymphs and adults

Little is known about the predators of the nymphs. Nymph predation by ants was observed in the congeneric species *L. fulvicornis* (Wheeler & Miller, 1990). In life table studies for *L. occidentalis* Bates & Borden (2005) found that only 2.5% to 15% of first instar nymphs exposed in the field survived to adulthood. Yet, protection from predators with cages, resulted only in 14.5% to 17.8% survival. Therefore, the mortality of nymphs by predators is probably minimal. Also, other factors, such as wind or rain could increase mortality on unprotected nymphs, rather than predators. Further, under optimal temperature laboratory conditions the mortality of nymphs is also high (Barta, 2016), with no evident explanatory reason.

Group feeding activity, keeping nymphs close to each other and to the adults, may provide protection against predators. Group feeding behavior of *L. occidentalis* was observed on laboratory experiments (Farinha *et al.*, 2018c) and field observations (Farinha, pers. obs.). Both nymphs and adults of *Leptoglossus* species produce an alarm pheromone (Blatt *et al.*, 1998). In concert with the bug gregarious behavior, alarm pheromone may help protect nymphs and adults from their predators. Again, there is not much information on the natural enemies of the adults. The big size of the adults might make them noticeably for vertebrate predators. Some authors suggest that some predation by birds, bats and frogs may occur (Petrakis, 2011). In laboratory studies, the mantids *Ameles decolor* Charpentier 1825, *Mantis religiosa* Linnaeus 1758 and *Sphodromantis viridis* Forskal 1775 can devour several *L. occidentalis* individuals. However, these predators are not frequently seen in the same habitats of *L. occidentalis* and therefore are not potential predators of this pest (Petrakis, 2011). The generalist parasitoid *Trichopoda pennipes* (Diptera: Tachinidae) attacks adults of *L. occidentalis* in its native range (Ridge-O'Connor, 2001). Yet, the impact of this parasitoid is small.

Barta (2010) tested isolates of three entomopathogenic fungi, *Beauveria bassiana*, *Isaria fumosorosea*, and *Metarhizium anisopliae* in bioassays under laboratory and outdoor conditions. Pathogenicity was highly variable and lower in field conditions in comparison with those in laboratory. Overall best results were achieved with isolates of *Isaria fumosorosea*, suggesting that this could be a potential microbial control agent of the seed bug.

2. Pheromone, trapping and chemical control

Pheromones are frequently used to monitor and control insect pest (e.g. Peso *et al.*, 2015; Walsh *et al.*, 2016; Cokl & Borges, 2017; Rice *et al.*, 2017). Researchers are trying to isolate pheromones efficient for chemical trapping of *L. occidentalis*. So far, only an alarm pheromone has been isolated (Blatt *et al.*, 1998) but with no successful results in controlling this bug populations. There are evidences for the existence of aggregation pheromones (Blatt & Borden, 1996) but the isolation of the chemical(s) compound(s) has never been done so far. Added to the difficulty inherent of the complex chemical communication in insects, little is known on the behavior of this bug that triggers what could be important pheromones to its control. One specific pheromone-mediated aggregation behavior is winter aggregation which results in the population gathering in autumn when adults seek shelter to overwinter. A better knowledge of this behavior may lead to increase the possibilities to

chemically isolating this pheromone and use it to control *L. occidentalis* populations with a mass-trapping tactic.

Multimodal communication systems e.g. the use of acoustic signs in addition to pheromones are described for several insects (Rowe & Guildford, 1996). Takács and team (2009) explored *L. occidentalis* communication. The team tested sonic and vibrational signals as well as cone infra-red radiation in an attempt to develop traps for *L. occidentalis* monitorization or even control (Takács *et al.*, 2009; Takács *et al.*, unpublished) but did not achieve effective results with any of the techniques. Other study explored the possible attractiveness of *L. occidentalis* to more reflective cones of *P. contorta* (Blatt, 1997). Results from field trials showed that preferred cones were neither more nor less reflective than non-preferred cones indicating that this factor might not be relevant for the bug (Blatt, 1997). However, it may be a cue used in long-range attractiveness by distinguishing cone-bearing trees from those not bearing any food resource (Blatt, 1997) as already found for another insect, the larch cone fly, *Strobilomyia melania* Ackl (Roques, 1986).

Visual and spatial cues were also tested in interception traps. Traps with different colors and conformations were tested but all performed negatively to attract *L. occidentalis* (Strong, 2010). The use of heated traps with and without a male bug inside was also tested on field during the period when the bug seeks winter shelter but with no result as well (Farinha, not published). The trapped male was tested based on findings from Blatt (1997) that only males produce an aggregation pheromone that both males and females respond.

Regarding chemical control, broad spectrum insecticides are used in the native region of the bug to control populations (Strong *et al.*, 2001; Strong, 2006). In Europe, only in Turkey we could find indications of a chemical control strategy in *P. pinea* orchards (Mutke & Calama, 2016) but details and results are not known.

In conclusion, to our knowledge there is no effective trapping technique for *L. occidentalis*.

A study using models of climatic niche predicted a low suitability for *L. occidentalis* populations in south Spain and center and south of Portugal (Zhu *et al.*, 2014). Distributions data of the pest combined with climatic data should be explored to understand regions at higher risk of developing outbreaks.

Conclusions and future guidelines

L. occidentalis is currently an invasive species in the whole European territory (EPPO, 2018). However, the Mediterranean countries are the most concerned with this invasive species due to its putative impact on the high valuable edible seeds of *P. pinea*. Preference and

performance studies showed a tendency for this insect to choose and to have higher performance in *P. pinea* (Farinha *et al.*, 2018c; Ponce *et al.*, 2017b). Therefore, the Stone pine large and rich seeds could favor and enhance the bug populations. Further, fertilization and irrigation, currently practiced in intensive Stone pine orchards, seem to favor the bug feeding activity and consequently the damage. Altogether, these results point to a high risk for *P. pinea* producing regions.

The populations of the seed bug are characterized by high inter-annual fluctuations. Several authors mentioned the chaotic population dynamics of the seed bug (Richardson, 2013; Lesieur, 2014). Future research on this behavior is paramount to be capable of predict this species movements and possible fragilities. Being a Mediterranean species well adapted to water scarcity, *P. pinea* is largely distributed along hot and dry regions. Some of these regions might be less suitable for the seed bug. Therefore, the effect of climate on the species distribution and population dynamics requires further studies.

Predators or parasitoids may be partially controlling the seed bug populations. Field prospecting of these agents should be carried on. Regarding *L. occidentalis* impact, it is mandatory to conduct more studies on seasonal variation of its feeding behavior resourcing to field covered branches with different densities of bugs inside. This knowledge is crucial to define economic thresholds in management plans. Joint trials in different climatic regions would be a great asset.

Ultimately, the management plans of *P. pinea* natural forests and seed orchards must integrate the management of *L. occidentalis* populations. Future research should integrate the insect, the pine and the market in a strategy common to all pine nut producer countries.

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CHAPTER 3

How to measure the impact of the bug on Stone pine seeds?



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Chapter 3 - Micro-CT and X- ray suggests cooperative feeding among adult invasive bugs *Leptoglossus occidentalis* on mature seeds of Stone pine *Pinus pinea*

How is *cooperative feeding* related to impact estimations?

When I started this Ph.D. there were no references to *L. occidentalis* capability to feed upon matured seeds of *Pinus pinea*. With a much thicker seed coat compared with the other coniferous in Europe or North America I presumed that it could be an obstacle, or at least an increased effort, for the insect to suck the seed inside. Thus, I start to study the impact of *L. occidentalis* by exposing matured seeds of stone pine to adult bugs and track the damage with x-rays. Not only was the insect able to pierce the seed coat and feed on the kernel inside but also seemed to do so by resorting to an intriguing feeding behavior involving cooperation between bugs. What started as a laboratory experiment to evaluate the impact of the bug ended with a new question. Are *L. occidentalis* adults collaborating to feed on a resource hard to obtain?

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Abstract

1. The consumption of edible pine seeds of Stone pine by the invasive *Leptoglossus occidentalis* represents a major concern for the producers of Mediterranean countries but so far little knowledge is available about its feeding process on these seeds.
2. We tested whether *L. occidentalis* is capable of feeding upon mature pine seeds and estimated the impact they may induce.
3. Sound pine seeds were offered to bugs in laboratory conditions. The seed content was afterward analyzed through a multi-technique approach using stereomicroscope, X-rays and Micro Computed Tomography which was expected to better characterize the damage caused by this bug.
4. Adults of *L. occidentalis* were capable of feeding on mature seeds by piercing the hard and thick coat. Yet, the consumption was low and with a slow start presumably due to the time and effort taken to drill a feeding hole.
5. A collaborative feeding process was suggested since all bugs of the same box seemed to have fed through the same hole in most of the cases.
6. Consumption was estimated to be about a fifth of a seed kernel per bug per month. Consumed kernels looked skunked and wrinkled.

Keywords: Cooperative feeding, *Leptoglossus occidentalis*, *Pinus pinea*, Micro-CT, matured seed

Introduction

Invasive seed feeders can cause serious disturbance to forest ecosystems as they may affect the demography, spatial distribution, diversity and evolution of the plants (Boivin & Auger-Rozenberg, 2016). Furthermore, these insects may have a direct economic impact on seed orchards by reducing the quality and quantity of seeds for reforestation or afforestation purposes and for the production of edible seeds or fruits (Roques, 1983; Auger-Rozenberg & Roques, 2012). An exotic seed feeder bug, *Leptoglossus occidentalis* Heidemann (Hemiptera: Coreidae), native from Western North America, was first introduced in Europe through Italy in 1999 (Taylor *et al.*, 2001). However, the European introductions appeared to originate from a primary invasion in Eastern North America (Lesieur, 2014). The bug quickly spread across most of the continent, probably through additional accidental introductions combined with both natural and man-mediated dispersal, the species being a good hitchhiker (Lesieur, 2014). Indeed, new invasion events have been recently registered in Asian countries such as Japan (Ishikawa & Kikuhara, 2009), China (Zhu, 2010) and South Korea (Ahn *et al.*, 2013) and also in North Africa, in Tunisia (Ben Jamâa *et al.*, 2013). In the Mediterranean basin, this invasive species is of particular concern amongst the producers of the edible pine seeds of Stone pine, *Pinus pinea* L., because of the high commercial and social value of this crop (Roversi, 2009; Bracalini *et al.*, 2013). This issue is particularly relevant to the Iberian Peninsula where Stone pine plantations correspond approximately to 70% of the world range of this pine species distributed by 175 000 ha in Portugal and 490 000 ha in Spain (Mutkle & Calama, 2016). Annual cone productions vary greatly between years and sites. In Portugal, mean production of cones is about 700 kg/ha and in Spain 100 Kg/ha (Mutke *et al.*, 2012). Considering that kernel-per-cone yields are around 2-4%, one hectare may generate 2 - 28 Kg of kernel sold later by the industry at a price between 25 and 45 €/kg (Mutkle & Calama, 2016).

Like other seed-feeding Hemipterans, *L. occidentalis* has sucking mouthparts composed by modified mandibles and maxillae to form a stylet of two channels, the salivary and the food channel, sheathed within a modified labium (Cobben, 1978). First, the salivary channel injects two types of saliva, gelling saliva to line the path of the stylets and watery saliva with enzymes to digest tissues exteriorly. The liquefied food is then sucked through the food channel and ingested (Cobben, 1978). Bugs from the family Coreidae can feed either on vegetative and vascular tissues or on reproductive plant parts (Mitchell, 2000). The hemiptera *L.occidentalis* feeds mainly on reproductive conifer structures but can also,

occasionally, feed on xylem for hydration (personal observations) as previously described in a related species, *Leptoglossus phyllopus* (Linnaeus, 1767) (Mitchell, 2006).

During its active period (May - November in the Mediterranean basin), *L. occidentalis* can feed on cones at different maturation stages, from conelets (first year cones) to developing cones and mature seeds until the onset of the hibernation conditions (Koerber, 1963). In Douglas-fir *Pseudotsuga menziesii* (Mirb.) Franco and in Lodgepole pine *Pinus contorta* var. *latifolia* Engelman, damage caused by *L. occidentalis* was observed to differ among these various cone development stages (Koerber, 1963; Bates *et al.*, 2001; Strong *et al.*, 2001; Bates *et al.*, 2005).

The edible seed of *P. pinea* has an average length of 15-20 x 10 mm (Frankis, 1999) which is about 2-3 times larger than those of most other European conifers. The average seed weight is about 0.92 g and coat thickness is greater than 1.5 mm (pers. observ.). Seeds of Douglas-fir, a native host species of the insect, are 5-6 mm long (Morin, 1993). Other European pines consumed by *L. occidentalis* such as *Pinus halepensis* Miller and *P. sylvestris* L. have seeds with a length varying from 5-6 mm and 3-5 mm, respectively (Frankis, 1999) with the last having around 1.5 mm of thickness (Kaliniewicz *et al.*, 2014). Therefore, the large size of the pine seed in *P. pinea* can be a pull factor to seed-eaters like this bug. On the other hand, the thicker coat of its seeds, when compared to others may represent an obstacle for the piercing mouthparts of *L. occidentalis*.

So far, little is known on the impact of this bug on *P. pinea* seeds whereas the damage to mature seeds has been characterized in a number of other conifer species in both the native and invaded range using X-rays (Bates *et al.*, 2000; Lesieur *et al.*, 2014). This methodology is expeditious and allows for a fast diagnosis of the damage caused by *L. occidentalis*. Although the concern of the impact of this insect on *P. pinea* is currently focused on the first and second year cones or on last year cones still with the seeds in soft coat (from March until August), the impact on mature seeds should not be neglected. With our climate and for this pine species hardening of the seed coat takes place at the end of August (A. Nunes, H. Pereira, M. Tomé, J. Silva and L. Fontes, unpublished data) and thus, hard coat seeds is a resource available to the insect during its active phase which may extent to early November (Bernardinelli *et al.*, 2006; Tamburini *et al.*, 2012; Mas *et al.*, 2013; Pimpão, 2014). In addition, feeding on mature seeds has a further possible important impact on seed germination and natural regeneration (Tamburini *et al.*, 2012; Bracalini *et al.*, 2012; Lesieur *et al.*, 2014).

A multi-technique approach using stereomicroscopy, radiography and Micro X-ray computer tomography (Micro-CT) may allow to get a better characterization of *L. occidentalis* damage on mature seeds of *P. pinea*. Micro-CT is a non-invasive and non-destructive 3D

radiographic microscopy imaging technique, which uses multiple digital two dimension radiography images to produce a three-dimensional (3D) computerized volume of an opaque sample (Kalender, 2006). Although more expensive and time consuming, Micro-CT has micrometric to scale range resolution enabling greater accuracy in 3D volume measurements and visualization. This technique has been used successfully to study insect damage in other seed species (Tarver *et al.*, 2006; Jennings & Austin, 2011; Arkhipov *et al.*, 2015; Suresh & Neethirajan, 2015).

Therefore, our study aimed at using this multi-technique approach in order to test whether the adults of *L. occidentalis* are capable of feeding on mature seeds of *P. pinea* despite the hard coat. If so, we intended to characterize the damage, the seed region consumed by the bug, and estimates the average daily consumption rate of an adult bug.

Methods

Sampling

Twelve mature cones were randomly harvested in January 2015 in three stands of *P. pinea*, two located in Santarém (38°44'N; 8°31'W) and one in Évora (38°39'N; 8°32'W), Portugal. At the laboratory, cones were dried at 45°C for 72 hours in order to open, and their seeds were manually extracted. Seeds from each stand were mixed and a random sample of 100 was taken from each of the three stands. The 300 seeds were X-radiographed in order to separate the sound seeds from damaged ones.

Feeding experiment

A permanent laboratory colony of *L. occidentalis* was set by collecting adult bugs in the Orleans region, France in September of 2009 and then supplemented periodically with more bugs from the same region to avoid consanguinity. Therefore, it consisted of bugs with mixed ages and mixed proveniences. The colony was reared at INRA Orléans, France in climatic chambers under the following controlled conditions: 21°C with 60% RH and 16:8 light/dark cycle. Twigs and cones from *Pinus nigra* were used as food source. Nine plastic boxes of 15x15x5cm, each containing 10 sound seeds of *P. pinea* were used. In six of the boxes, four adults of *L. occidentalis* from the colony were added whereas the other three boxes were free of bugs, to act as controls. The sex of the specimens was not taken into account since previous studies found no significant differences in the consumption of conifer seeds between sexes (Bates *et al.*, 2000, 2002; Lesieur *et al.*, 2014). Bugs were kept

without food with only water 48 hours before the experiments except for the replacement bugs. Individuals who died were replaced immediately, using the stock available from the colony. The experiment was carried out from February 11th to March 11th 2015 at INRA under the controlled climatic conditions mentioned above. Each seed was identified by two numbers separated by a dot in which the first number corresponds to the box and the second to the seed position on the box reading from left to right starting at the top (see results chapter).

The seeds offered to insects and control ones were simultaneously x-rayed weekly using a HP Faxitron-43855® X-raying apparatus and X-ray sensitive films (Kodak® 'Industrex M'), following the procedures described in Roques and Skrzypczyńska (2003) but optimized for larger seeds of *P. pinea*. Through radiography, the size of the damaged area of the kernel was estimated weekly, using the total kernel area at the beginning of the experiment as a baseline. Damage categories were set following a three-level scale starting with sound seeds: (1) no apparent damage (fully filled seed), (2) light to moderate damage (less than 1/2 of the whole seed content consumed), (3) severe damage (more than 1/2 of the seed consumed). Damaged areas visualized on the x-rays were also measured using Image J® software.

Micro-CT study (3D seed processing and measurements)

Before breaking the seed coat to observe the kernel's damage under a stereomicroscope, a sub-sample of seeds was submitted to a Micro-CT analysis. This sub sample consisted of a group of ten seeds, three sound and seven bug-damaged previously characterized with X-rays, that were scanned through computerized micro tomography using Skyscan® 1172. The experimental parameters were optimized by considering the relatively large size of the mature seeds and the best possible pixel resolution (14 to 18 µm) and contrast of the acquisition images. Reconstructed tomographic images were processed using a 3D processing visualization and analysis software from Bruker®. The following variables were measured: seed volume, seed coat volume, kernel volume, thickness of the coat, length and width of the feeding hole made by the insect. The volume measures obtained by Micro-CT combined with the weight measures made in a precision scale allowed to estimate density values for the entire seed (global density), for the coat and for the kernel before and after the feeding experiment (Fig. 1). The entire coat of each seed was carefully examined to look for feeding holes. The dimensions (diameter and length) of each hole were measured, and its position on the seed was recorded. Furthermore, the thickness of the

coat where the feeding hole was drilled was compared with that of the other parts of the seed coat.

In order to investigate if the thickness of the seed coat could be related to the insect's choice to pierce a specific seed and/or a specific region in the seed, the volume of the seed coat in each of the regions, was measured in both bug damaged and sound seeds. The volume of the region(s) with the feeding hole was compared with the average volume of all parts of the same seed. Top and bottom quarters of the seeds were not included.

Diameter of the insect inner stylets (mandibles and maxilla) and the proboscis (labium) were measured in order to look the possible relationships with the hole dimensions. Measurements were made using software CTA_n (v. 1.16) from Bruker® and Imaje J®.

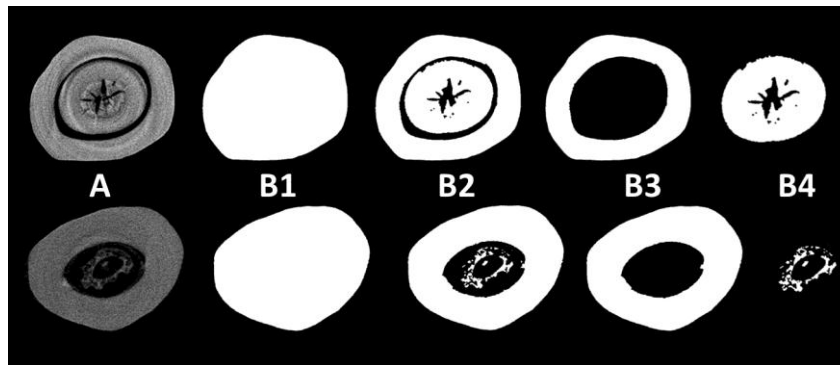


Figure 1. Sequence of images illustrating the imaging methodology for quantitative volumetric measurement of the different components of the pine seed from a reconstructed slice (A) obtained by CT rays X. Sound seed (1.8) on top and consumed seed (1.6) on bottom. (A) 256 grey level images and (Bi) binary images (Seed total volume (B1), volume of the seed coat and kernel (B2), volume of the seed coat (B3) and volume of the kernel (B4)).

Stereomicroscopy observations

At the end of the feeding experiment, and after the radiographic and micro-CT examination, all seeds of the experiment were also externally observed under a stereomicroscope to check for damage in the seed coat caused by insects. Feeding holes, when found, were measured and photographed. Then, all seeds were carefully opened and all components (seed coat, seed tegument and kernel) were weighted and photographed.

Radiographic and Micro Tomography data from the kernels was compared with the visual examinations.

Statistical analysis

Non-parametric statistical test Kruskal-Wallis (χ^2) was used to test for differences in seed densities (global, coat and kernel) between all seeds and between seeds belonging to the same damage category. Differences in thickness between seeds were tested with one-way analysis of variance (ANOVA). Response variables (i.e. seed densities and thickness) were tested for normality (Shapiro–Wilk test) and homogeneity (Levene’s test). Statistical analyses were performed using IBM SPSS Statistics for windows version 23.0 with a statistical significance level of 0.05.

Results

Feeding experiment

Along the four weeks, seed feeding was observed in all the boxes containing insects (Fig. 2). Yet, in each box only one or two of the ten seeds presented signs of feeding damage by *L. occidentalis*, i.e. dark spots in the seed endosperm or, when the consume was higher, deformation or reduction of this part of the seed. Seeds in the control boxes without insects (boxes 7, 8 and 9) remained with their endosperms intact throughout the experiment. During the entire time of the experience 6 bugs died, 4 bugs in box 2 and 2 bugs in box 3. They were replaced immediately. By the end of the experiment, four of the six boxes with bugs included two damaged seeds (boxes 1, 3, 4 and 6) whereas the other two presented only one seed with bug damage (boxes 2 and 5) (Fig. 3). In three of the four boxes where two damaged seeds were observed, the bugs were noticed to start feeding on a single seed, and only after this seed had been significantly depleted they turned to another one. This temporal development of the feeding damage (Fig. 3B) revealed to have a rather general pattern: damage started slowly with consumptions per box up to 22.62%, accelerated on the second or third week when consumption rate doubles, slowed down again, and eventually accelerated again. Box number 5 was an exception with a very high consumption soon in the first week.

Seed kernel damage at the end of the experiment varied between two entire seeds in box 1 to less than half a seed in box 2. Of the ten damaged seeds, five presented light to moderate damage (2.9; 3.4; 3.5; 4.10; 6.3) and five displayed severe damage (1.6; 1.7; 4.8; 5.3; 6.5). The image analyses of the X-rays allowed estimating an average consumption of one third of a seed (0.31) per bug per month.

Kernel weight measurements showed that *L. occidentalis* consumed, in average, 0.08 ± 0.02 g (39.67% of kernels mass) in light-moderately damaged seeds and 0.17 ± 0.03 g (78.76%) in severely damaged seeds. Taking all boxes with bugs together, the total kernel consumption was of 1.15 ± 0.08 g, amount equivalent to approximately 5.15 seeds which gives approximately a fifth of a seed (0.05 ± 0.07 g) per bug per month.

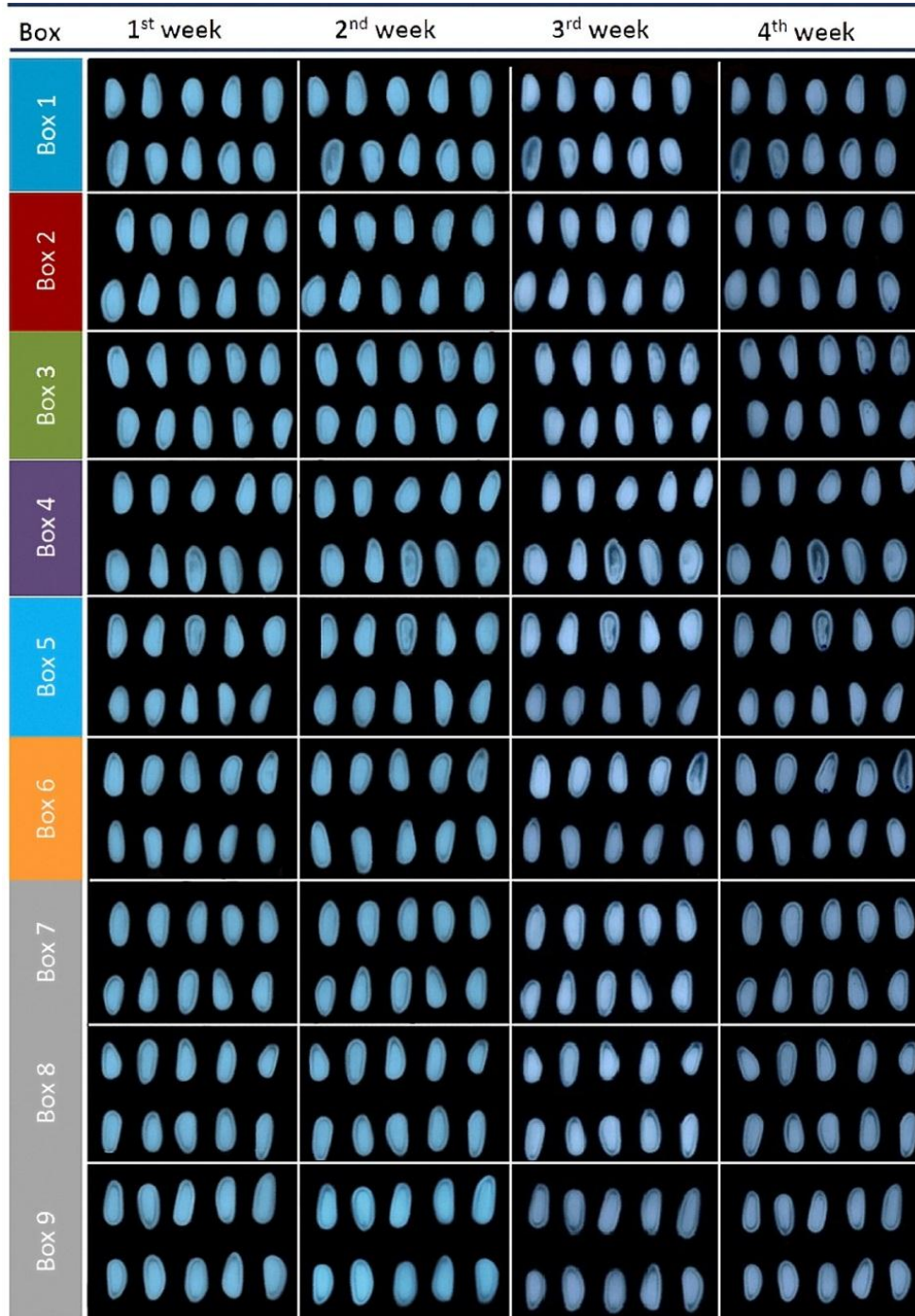


Figure 2. Evolution of the seed kernel exposed to *L. occidentalis* along the four weeks using X-rays technique. Grey boxes correspond to control boxes.

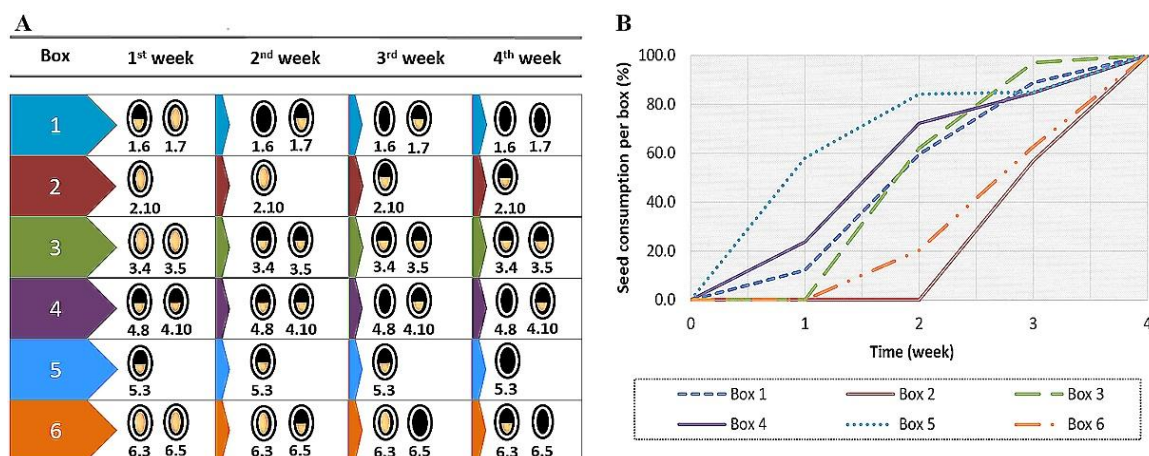


Figure 3. Weekly consumption of the seed kernel by four adults of *L. occidentalis* presented in categories of damage per seed (A) and in percentage per box in relation to the total consumption of the box (B). Leg. No damage, Light/moderate damage (less than ½ of kernel consumption) and severe damage (kernel consumption of ½ and more). Seeds with no damage are not represented.

Micro-CT results

Before exposition to *L. occidentalis*, seed global density did not differ between sound seeds ($p < 0.05$), averaging $1.11 \pm 0.02 \text{ g/cm}^3$. Following the feeding experiment, seed global density decreased to an average of $1.02 \pm 0.04 \text{ g/cm}^3$ in the lightly/moderately damaged seeds and to $0.90 \pm 0.02 \text{ g/cm}^3$ in severely damaged seeds. Seed global density was significantly different between seed categories (KW test: $X^2 = 8.27$, d.f. = 2, $P = 0.016$). Sound and severely damaged seeds appeared significantly different from each other in density (Man-Whitney pairwise comparison test: $p < 0.001$), but densities of lightly/moderately damaged seeds did not differ significantly from those of sound ($p = 0.235$) nor severely damaged ones ($p = 0.142$).

Differences in coat and kernel densities between damage categories were not significant (KW test: $X^2 = 1.169$, d.f. = 2, $p = 0.557$ and $X^2 = 2.927$, d.f. = 2, $p = 0.231$, respectively).

Micro-CT analysis revealed the presence of a single hole per damaged seed (Fig. 4) with the exception of seed 2.10 which presented three holes. Feeding holes were all made into the middle section of the seed and never on the tips. Holes presented an average diameter of $51.60 \pm 10.40 \mu\text{m}$ with an average length of $1.60 \pm 0.20 \text{ mm}$.

Micro-CT measurements on the insect piercing parts showed that inner stylets, which correspond to mandibles and maxilla, are much smaller in diameter than the proboscis

(Labium) (Fig. 5) measuring on average $35.90 \pm 7.40 \mu\text{m}$ and $119.80 \pm 23.70 \mu\text{m}$, respectively.

No significant differences in the average thickness of the seed coat was observed between all seeds, damaged and sound ones (ANOVA test: $F=0.118$, $d.f.=9$, $P=0.890$). The path of the hole was always longer than the thickness of the coat in the same region, meaning that in all damaged seeds, bugs pierced diagonally.

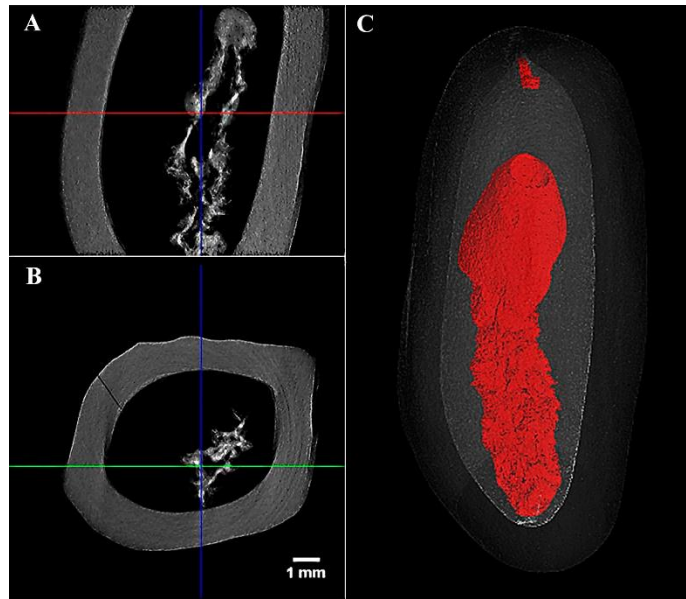


Figure 4. *L. occidentalis* feeding hole in matured seed 1.7. Micro-CT image. (A) Transversal view and (B) coronal view of the seed (C) 3D image of the kernel.

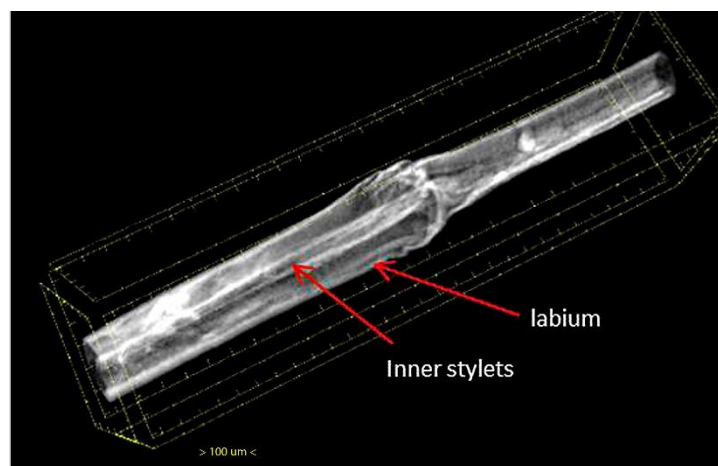


Figure 5. A section of *L. occidentalis* mouthpart using Micro-CT.

Seed results

Examination of the seed's coat under a stereomicroscope confirmed the presence of holes, which were surrounded by a darker zone with some white spots in all the damaged seeds (Fig. 6). The average diameter of holes was $45.70 \pm 4.50 \mu\text{m}$. No holes could be found on seeds that showed no consumption in the x-rays.

A comparative analysis of the kernel of all damaged seeds and one sound, to contrast, using images from the three methodologies (stereomicroscope, X-ray and Micro-CT) revealed a shrunken and wrinkled effect on seeds damaged by *L. occidentalis* (Fig. 7). Even seeds with light damage showed noticeable deformation. When the eating process was complete the entire kernel was shriveled. If damage was low the seed presented a wrinkled area in the kernel just beneath the hole found in the seed coat.

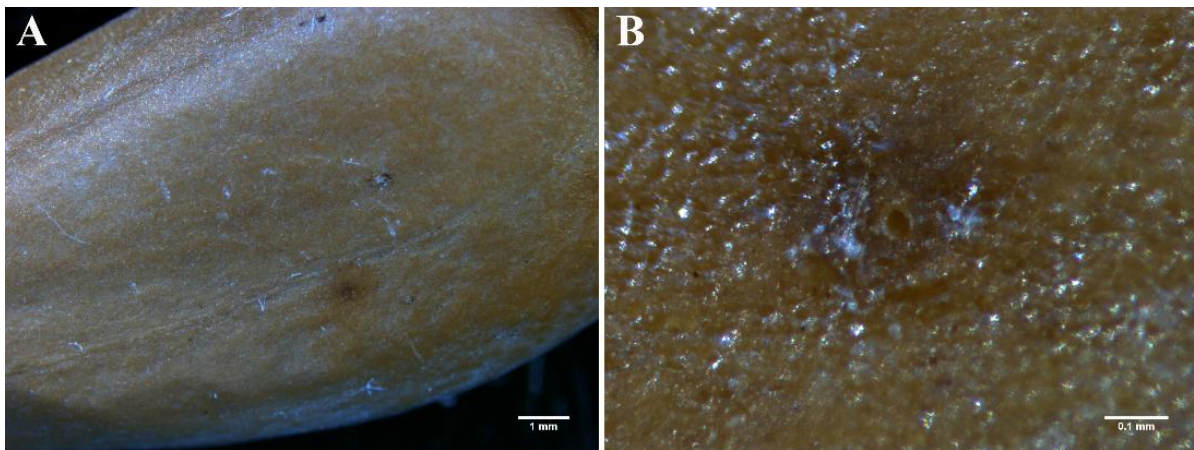


Figure 6. Feeding hole in the coat of the seed 5.3 of *P. pinea* made by *L. occidentalis*. Magnification of 9.6x (A) and 120x (B) Zeiss Stereo Lumar V.12.

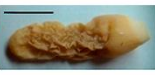
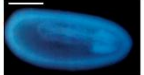


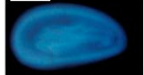


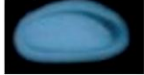


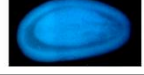

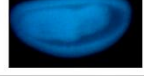

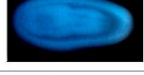
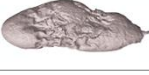




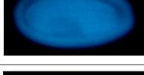

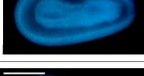





Box	Seed ID	Steromicroscopy	X-ray	Micro-CT
1	1.6			
	1.7			
	1.9			
2	2.10			na
3	3.4			
	3.5	x		
4	4.8			
	4.10			na
5	5.3			
6	6.3			na
	6.5			na

Figure 7. Comparative photos of damaged and sound seeds using binocular microscope, X-rays and Micro-CT. Bar corresponds to 0.5 cm. Seed 3.5 could not be photographed under binocular microscope due to accidental crushing when opening. na – not analyzed.

Discussion

Our results suggest that adults of *L. occidentalis* can overcome competition, benefiting from a group behavior of sharing the same resource. However, we do not know if this hypothesis is a common strategy for this bug or if it is an exclusive behavior in this particular host species, explained by the fact that this seed is too hard to perforate. In fact, *P. pinea* seeds are one of the hardest seeds in Pinaceae family (Perry Jr, 1991). Moreover, these results have been obtained in captivity and may simply correspond to a behavior induced by these

artificial conditions. They have to be confirmed under field conditions. Even so, it was proved here that *L. occidentalis* can feed on mature seeds.

The radiographic weekly follow up in the feeding experiment showed without contest that *L. occidentalis* bugs can perforate the hard coat of mature seeds of Stone pine and consume the inner content. However, the number of seeds damaged per box remained very low, reaching only one or two seeds per box whereas each box contained four adults who were offered ten healthy seeds for one month. Mass consumption was estimated to be about a fifth of a seed kernel per bug per month with a total of ten seeds damaged during the four weeks feeding trial with 24 adults of *L. occidentalis* divided in 6 boxes. Other feeding experiments on other hosts namely, *P. sylvestris*, *P. nigra*, *P. contorta* and *P. menziesii* reported an average feed between 0.7 and 1.7 seeds per bug per day depending on the host species, time of the year and sex of the bug (Hanson 1978, Strong 2006, Lesieur *et al.*, 2014). Knowing that one Stone pine matured seed corresponds grossly to a batch of 100 seeds of the other hosts mentioned, our result (0.014 Stone pine seeds per day per bug corresponding) fits between the average feed reported for the other host species.

When analyzing the feeding rate, a general pattern arises: a slow rate at the beginning of consumption of a given seed, followed by a rapid depletion of its kernel. In one box (n. 5) consumption was already high in the first week maybe due to a more efficient group work in drilling the coat or to a more voracious or dominant bug, when compared to the other boxes. A second slow step in the damage curve was observed in boxes in which two seeds were damaged. This slower rate at the beginning of consumption of a seed tends to indicate that *L. occidentalis* has to make an effort in drill the harden seed coat of *P. pinea* mature seeds before starting feeding on it. Since all bugs were starved previously to the experiments and are proven polyphagous i.e., does not influence the fact that the colony of origin is fed with *P. nigra* twigs and cones, we would expect that they should begin feeding voraciously. Furthermore, the low number of damaged seeds per box, together with the temporal asynchrony in the seed feeding process (deplete a seed and only then move on to another), suggests a cooperative feeding strategy with all the bugs feeding on the same seed presumably because of that initial effort of puncturing the seed coat.

The gregarious behavior in the family Coreidae is already documented for the younger development stages (Mitchell, 2000; Wertheim *et al.*, 2005) but studies reporting adult gregarious behavior, excluding for overwinter purposes, are scarce and concern heteropteran from families other than Coreidae (Ralph, 1976; Aller & Caldwell, 1979). Efficiency in resource exploitation has been pointed out as one of the benefits associated with the gregarious strategy (Ralph, 1976; Aller & Caldwell, 1979; Lockwood & Story, 1986; Cloutier, 1997; Mitchell, 2000; Wertheim *et al.*, 2005; Mitchell, 2006; Fernandes *et al.*,

2015). This efficiency comprises cooperative efforts to overcome host physical barriers, such as waxy layers on leaflets and seed coats (Cloutier, 1997; Prado & Tjallingii, 1997). In the present research, the analysis of the seed coat of the damaged seeds may be in agreement with the concept of communal feeding. In fact, nearly all damaged seeds presented only one perforating hole in the seed coat, which could mean that all four bugs shared the same hole to suck the endosperm. If so, we may further deduce that these bugs do not avoid seeds consumed by others. They may actually profit from using a feeding cooperation strategy.

Thinking that just one of the bugs in each box could be feeding upon the seed is also a possible explanation for the temporal asynchrony although it does not explain why all the other bugs in the box did not feed on the seeds since there were four healthy starved bugs per box. However, the maximum survival time of this insect without food as well as its territorial dynamics and individual ability to drill for feeding has not been tested. Further behavior studies must be carried on verifying the hypothesis of only one insect is feeding while the others are starving.

Koerber (1963) was the first to notice the signs of feeding by *L. occidentalis* on mature seeds of *P. menziesii* in the native Eastern North America range which he described as “punctures with a minute conical fleck of white material around, possibly dried saliva”. Binocular microscope observations of the punctures in *P. pinea* damaged seeds revealed a hole surrounded by a darker area with small white spots. These holes can be undoubtedly assigned to *L. occidentalis* because all seeds without damage showed an intact coat and the measurements of the diameters of these punctures are similar to the ones obtained by DeBarr (1970) for *Leptoglossus corculus* (Say 1832), a very close species, on seeds of slash pine (*Pinus elliotii*) in North America. Furthermore, the holes diameter is also consistent with the diameter of the insect stylets.

Bug mortality along the experiment was negligible (4 out of 36 bugs) and happened only in two boxes, indicating that mature seeds of *P. pinea* are suitable for adult bug feeding. However, and although dead bugs were immediately replaced, these two boxes presented the lowest seed consumption in total. Abiotic factors may be discarded as a reason since all boxes were under the same conditions. The undetermined age of the insects and differences in the ability in drilling the seed coat are possible explanations for this mortality. We can, nevertheless, suspect also of territorial behavior. The sex of the individuals was not controlled following Bates *et al.*, (2000) and Lesieur (2014b) conclusions of no differences in total seed damage with sex. However, boxes in the present study contained four bugs and therefore male-male competition for females and resources, could influence bugs mortality and seed consumption inside the box. In fact, several behavior studies report territorial

defense by males in Coreids (Mitchell, 1980, 2000; Miyatake, 1993, 1995) with the presence of a female being a strong influence factor (Miyatake, 1993). This territorial behavior should therefore be tested as another possible explanation for the presence of just one hole per damaged seed in each box conducting experiments which take sex and number of individuals per box into account.

The use of micro computerized tomography on the *P. pinea* seeds consumed by *L. occidentalis* allowed for the first time to look at the damaged kernel in a 3D perspective without breaking the seed coat. The damaged kernels presented a shrunken and wrinkled appearance corresponding to the sucking of its endosperm. Mass density values of damage coated seeds resulting from this 3D analysis corroborates the efficient use of the expedite method of immersing seeds into water to differentiate sound from damaged seeds. In fact, we found significant differences in mass density between sound and severely damaged seeds, with the last ones revealing values considerably lower than 1 g/cm³ which allow them to float on water. However, seeds with light/moderate damage may be wrongly classified as good by immersing them in water since their mass densities varied between 0.96 and 1.06 g/cm³. It is important to note that these last seeds are no longer viable for the market of *P. pinea* edible seeds. Furthermore, germination tests carried on with mature seeds of other *Pinus* species exposed to adults of *L. occidentalis* had a success rate of less than 30% in seeds with less than 1/3 of the whole seed content consumed (Bates *et al.*, 2001; Lesieur *et al.*, 2014b). Thus, even a low damage to the seed can have a serious impact on seeding programs and in natural regeneration of the plant, because it may reduce seed chances of a successful germination. In addition, *L. occidentalis* perforation in the seed coat may provide an entrance point for pathogens (Mitchell, 2004; Luchi *et al.*, 2012).

A complete analysis of the seed coat in damaged and sound seeds revealed no significant differences in the average thickness between similar sections of all seeds. Thickness of the seed coat region where the bug perforated was not proved to be thinner than the other seed regions (excluding the extremities). In addition, 78% of the measured holes in the seed coat were made diagonally not thereby optimizing the distance that the stylet had to cross. All these results suggest that seed selection as well as choice of the area where to perforate should be determined by variable(s) other(s) than the thickness of the seed coat.

At a tree scale, some host preferences exhibited by *L. occidentalis* were already found. Blatt and Borden (1999) revealed a clonal preference in seed orchards of Lodgepole pine and Douglas-fir and Richardson *et al.* (2017) demonstrated that cone terpenes and cone size may also play an important role in the selection of the host by this bug species. Studies on cues mediating mature seed selection do not exist.

P. pinea seeds gain complete maturation concerning the hardness of the coat from August to late September (A. Nunes, H. Pereira, M. Tomé, J. Silva and L. Fontes, unpublished data) and *L. occidentalis* hibernation dates in Mediterranean countries can extend up to November (Bernardinelli *et al.*, 2006; Tamburini *et al.*, 2012; Mas *et al.*, 2013; Pimpão, 2014). This means that cones containing mature seeds are an available resource to the bug in natural conditions. A deeper insight on *L. occidentalis* adults and nymphs feeding behavior in the field, especially on preferences of cones from different development stages, throughout its active period, is essential before considering any control strategy as already stated by Bates *et al.* (2005). Also, new knowledge on the maturation and development of *P. pinea* cones and seeds must be cross referenced with the biology of this insect. This investigation should provide researchers and producers with a reliable and visual characterization of the damage caused by *L. occidentalis* in mature seeds of *P. pinea*.

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CHAPTER 4

**Does the seed bug prefer the Stone pine
over other Mediterranean pines?**



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Chapter 4 - The Stone pine, *Pinus pinea*, a new highly rewarding host for the invasive *Leptoglossus occidentalis*

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Abstract

The invasive seed bug *Leptoglossus occidentalis*, a species native to Western North America, is of major concern for the producers of stone pine seeds in the Mediterranean countries. The large size of these edible seeds and their nutritive content may represent a pull factor for the seed bug. Cone and seed traits of three main Mediterranean pine species: *P. pinea*, *P. pinaster*, and *P. halepensis*, were evaluated. Preference trials with cone-bearing branches, individual cones and seeds were conducted to test host preference among the three host species.

Considering the kernel size, stone pine seeds provide 4 to 13 times more reward than *P. pinaster* and *P. halepensis* seeds, respectively, but also needed a greater effort to be reached as measured by coat thickness. Still, the benefit/cost ratio was higher on *P. pinea*. Individual seeds and cones of *P. pinea* were 2 to 3 times more consumed than those of the two other pine species. However, branch preference trials did not reveal any difference in bug visits. Moreover, adults manifested strong group behaviour on branches, frequently dissociating into two persisting groups. The implications of these results for *P. pinea* producing areas are discussed.

Keywords: seed feeder, host preference, *P. pinea*, *P. pinaster*, *P. halepensis*

Introduction

The invasive seed bug, *Leptoglossus occidentalis*, Heidemann 1910 (Hemiptera: Coreidae), originating from Western North America, was first recorded in Europe in Italy in 1999 (Taylor *et al.*, 2001). Due to its high dispersal ability, and also probably assisted by more than one introduction event, this bug quickly spread all over Southern and Central European countries and later to the whole continent (Lesieur *et al.*, 2018). Besides of Europe, there has also been new records on other continents in the last decade: Asia (Ishikawa & Kikuhara, 2009; Zhu *et al.*, 2010; Ahn *et al.*, 2013), Africa (Jamâa *et al.*, 2013; Gapon, 2015) and more recently, South America (Faúndez & Rocca, 2017).

With the European invasion, this polyphagous insect, which feeds on cones and seeds of many conifer species in its native range (Koerber, 1963), encountered new potential hosts. In Europe, it has been observed feeding on Mountain pine (*Pinus mugo*), European black pine (*P. nigra*), Scots pine (*P. sylvestris*), Maritime pine (*P. pinaster*), Aleppo pine (*P. halepensis*) and on Stone pine (*P. pinea*) (Villa *et al.*, 2001; Tescari, 2004; Tiberi, 2007; Kment & Baňář, 2008; Lis *et al.*, 2008; Rabbitsh, 2008; Roversi, 2009; Petrakis, 2011; Hizal, 2012). The last three hosts are the predominant pines species in the Mediterranean region of Europe. Maritime pine is native to the Western Mediterranean Basin, but it can be found in other parts of southern Europe and North Africa (EUFORGEN 2009). The Aleppo pine is distributed mainly along the coastal areas in the western Mediterranean regions, being an important forest species in North Africa, France and Italy (EUFORGEN 2009). Finally, the European distribution of *P. pinea* extends across the entire Mediterranean basin, from Portugal to Syria (EUFORGEN 2009). For more than a century, all three have been important species in reforestation and afforestation programs since they can grow in arid and unstable soils (EUFORGEN 2009). Maritime pine is also planted for timber and to extract resin. Aleppo pine, in turn, is also used in the pulp and paper industry (EUFORGEN 2009). Differently, the stone pine *P. pinea* is largely known by its ecological and landscape value but also by its edible seeds, known as Mediterranean pine nuts, of high commercial value, which may reach 100 € per kg in retail (Mutke *et al.*, 2012). In response to this high market value, the plantation area of *P. pinea* has increased in the last decades in several Mediterranean countries, like Spain, Portugal, Italy and Turkey (Mutke & Calama 2016). The high value of this non-wood forest product is precisely the focus of the vast majority of impact studies on the seed feeder *L. occidentalis*, in the Mediterranean countries (Bracalini *et al.*, 2015; Calama *et al.*, 2016, 2017; Farinha *et al.*, 2018a, 2018b). During the last ten years, several countries have reported a strong decrease in cone production and cone yield (i.e., the percentage of commercial kernels per kg of fresh cones) (Mutke *et al.*, 2014), and

L. occidentalis has been suggested as the most plausible cause of such decrease (Roversi, 2009; Bracalini *et al.*, 2013; Mutke & Calama 2016; Parlak, 2017). Like all hemipterans, *L. occidentalis* has sucking mouthparts and, it feeds by inserting its stylets deep into the cone until reaching the seed from which it removes the endosperm (Koerber, 1963).

Although feeding on a wide range of conifers, *L. occidentalis* seems capable of distinguishing between clones of *P. contorta* (Blatt & Borden 1996, 1999; Richardson *et al.*, 2017) and between cones of *P. pinea* of different physiological status (Farinha *et al.*, 2018b). This host selection capability strongly indicates that the bug can probably discriminate between different host conifers.

Evaluating host preference of this seed feeder under natural conditions is not a straightforward task due to the difficulty in detecting the bug and the absence of visible damage on cone surface. The only study on *L. occidentalis* feeding preference was carried on under laboratory conditions using individual mature seeds, extracted from cones (Lesieur *et al.*, 2014). Having offered such seeds to adults in choice tests, Lesieur *et al.* (2014) did not find any preference among several European conifers (*P. sylvestris*, *P. nigra*, *Abies nordmanniana* subsp. *bormuelleriana*, *Larix decidua* and *Picea abies*) compared to the Nearctic Douglas-fir, *Pseudotsuga menziesii* var. *glauca*. However, although quite extensive this study did not include *P. pinea* seeds and to our knowledge, no other studies tested bug preferences for this host species.

Seeds of *P. pinea* are large-sized and highly nutritive which can be a pull factor to a seed-eater like *L. occidentalis*. On the other hand, larger seeds also mean a thicker seed coat which may represent an obstacle for the piercing mouthparts of the bug. We hypothesized that in preference trials using seeds, the bigger individual seeds of *P. pinea* might visually lead to a host preference towards a higher reward whereas the harder seed coat may constitute an additional cost. Seed volume is a proxy to the seed reward and thickness may represent a proxy to the effort. Selective behaviour in the field is known to operate at sequential levels. First, individuals select a tree, then a cone and lastly a seed to feed upon (Blatt, 1997). So, we presumed that host preference may differ regarding the plant component tested; either using seeds, cones, branches or trees. Furthermore, it would be relevant to compare results and discuss the pros and cons when using different methodologies.

In this study, our objectives were to evaluate the host preferences of *L. occidentalis* for branches, cones, and seeds of the three main pine species in the Mediterranean Basin, *P. pinaster*, *P. halepensis* and *P. pinea*. For this purpose, we compared cone volume, seed volume and seed thickness of the three species, and then tested bug preferences in choice tests.

Methods

Three separated choice experiments using different substrates, cone-bearing branches, fresh last year cones and mature seeds, were conducted to evaluate the preference of *L. occidentalis* adults among *Pinus pinea*, *P. pinaster* and *P. halepensis*. All the experiments were carried out under laboratory conditions. Only adults of *L. occidentalis* were used since nymphs are apterous and thus not capable of actively choosing the tree or the host where they will feed. All individuals came from a permanent laboratory colony with adults collected in Santarém region, Portugal during the summer of 2015. The colony was supplemented each summer with more adults from the same region to avoid consanguinity thus consisting of individuals with mixed ages. The colony was reared at Centro de Estudos Florestais, Lisbon, Portugal in a climatic room under the following controlled conditions: 21°C with 60% RH and 14:10 light/dark cycle. Branches and cones from *P. pinea* were used as food source. Trials began by removing experimental adults from the permanent colony and marking them with an individually coloured and numbered label painted in the thorax (as seen in Fig.2). All marked adults were put in a cage with cone-bearing branches and seeds of the three hosts during one week. Individuals were then subjected to a 24-hour starvation period after which the trials began. Adults used to replace dead ones were removed from the permanent colony, marked but were immediately placed in the cages or test boxes.

Cone-bearing branches used in trials were collected in different locations for each of the pine species: stone pine branches were collected in Monsanto, Lisboa (38°43.09'N; 09°12.41'W) in a natural pure stand of adult trees; Maritime pine branches were collected in Sobreda, Almada (38°38.06'N; 09°12.66'W) in an urban patchy stand; finally, branches of Aleppo pine were collected in the university campus, Lisbon (38°72.80'N; 09°12.66'W). Cones / seeds used in preference trials were from branches / cones from the same locations as above with the exception of stone pine seeds which came from a pure, grafted stand in Santarém region, Portugal (39°6.50'N; 08°21.91'W) and Maritime pine seeds in the two-choice trial which came from Setúbal region, Portugal (38°34.82'N; 09°11.09'W).

1. Assessing the differences in size of cones and seeds among the three Mediterranean pine species

The volume of a sample of the cones used in the experiments was measured by displacement of water in a graduated cylinder (n=6 for *P. pinea* and *P. pinaster* and n=12 for *P. halepensis*).

Respecting mature seeds, all that were used in preference trials were weighted at the beginning and at the end of the experiments. At the end of the trials, all seeds from the three pine species were opened, and the volume of the kernels showing no feeding damage were measured by displacement of water in a graduated cylinder with a sensitivity of 0.25 ml. Due to the very small size of *P. pinaster* and *P. halepensis*, these seeds were measured in batches of 20 seeds and then the individual volume was extrapolated. The thickness of the seed coat for each host species was measured on the images collected by the Scanning Electron Microscope (SEM) using Image J® software. The coats of three seeds per host were photographed in SEM with 20 measures taken in each photo.

2. Branch preference trials

Choice experiments were conducted in large cages (100x50x40) cm made up of a wooden frame and mesh walls. Preference among the three host species, *P. pinea*, *P. pinaster* and *P. halepensis* was tested in pairs by offering two potted branches of different plant species per cage to ten adults. All branches used in the trials bear cones at all development stages (1st and 2nd year for *P. pinaster* and *P. halepensis* and 1st, 2nd and 3rd year for *P. pinea*). The number of last-year cones (2nd year for *P. pinaster* and *P. halepensis* and 3rd year for *P. pinea*) in the tested branches was the same for the pair *P. pinaster* - *P. pinea* (ranging from 1 to 2 cones each), but not for the pairs *P. pinaster* - *P. halepensis* and *P. pinea* - *P. halepensis* in which the number of *P. halepensis* cones varied between 2 and 5 due to their smaller size. Young conelets (1st cones for *P. pinaster* and *P. halepensis* and 1st and 2nd cones for *P. pinea*) varied in number in all three species between 1 and 4. The experiment was replicated three times, on 21-22 April, 28-29 April and 5-6 May 2016. Branches for each experiment were collected at the end of the afternoon of the day before the start of the experiment and kept in the refrigerator until then. Ten adult bugs were assigned to each of the three big cages. Cage number 1 had three males and 7 females and cages number 2 and 3 had four males and 6 females. All adults were individually marked in the thorax with a colour and number. The group of insects of each cage remained constant in all three trials varying only the host pairs to be tested. Between trials all individuals were kept together in a single big cage in the laboratory under controlled conditions and with branches from all the three hosts. For each trial, the ten adults were introduced into the cages by placing them one by one, within a two minutes interval, at the centre of the cage, between the two potted branches. Individual bug's behaviour and localization was recorded after that, at 1-h intervals from 8 a.m. until 6 p.m. for two days.

3. Cone preference trials

Three separate laboratory trials, with 3 to 5 days length each, were conducted from April to September 2017, using a video camera BRINNO® TLC200 Pro. In each trial, two video cameras each videotaping two plastic boxes (23x20x19) cm simultaneously, were set. Each box contained a small branch of *P. pinea*, a petri dish with wet cotton to keep moisture and small aluminium cases filled with sand to place the cone. In this way, the insect was not allowed to hide underneath the cones. Similarly as in the branch preference trials, the bugs were individually marked with a coloured label (Fig. 2). One cone of *P. pinea*, one of *P. pinaster*, two of *P. halepensis* and three adults of *L. occidentalis* were then added to each box. All cones were from last year of development which corresponds to the 3rd year in *P. pinea* and 2nd year in *P. pinaster* and *P. halepensis*. Experiments were carried on in a room under control conditions (26°C, 60 % RH, 16:8 L:D) from 20-24 April, 2-6 May and 12-14 September of 2017. The videotaping was done using the time lapse function with one picture taken every two minutes, and played back at a speed of one frame per second. Videos were analysed with the program VLC media player 2.2.6 Umbrella for windows (<https://www.videolan.org/vlc/index.html>). Both the number of times each bug started feeding on a cone (frequencies), and the duration of the feeding was recorded. Feeding was assumed to have occurred whenever stylet insertion was observed.

4. Testing bug preference for seeds among the three pine species

Two laboratory trials were carried out to evaluate bug preferences for individual seeds of the Mediterranean pines. A two-choice test compared *P. pinea* and *P. pinaster* whereas a three-choice test included the three species. The first trial lasted three weeks whilst the second one lasted four weeks. The experiments were carried out using small plastic boxes (20 x 15 x 10) cm with a perforated lid for gas exchange in a climatic chamber under controlled conditions (21°C, 60 % RH, 16:8 L:D).

The two-choice trials were carried out at INRA Orléans, France, and the three-choice one at CEF Lisbon, Portugal.

In the two-choice experiments boxes containing both *P. pinea* and *P. pinaster* seeds ($n_{\text{seeds}}=5$ and $n_{\text{seeds}}=12$, respectively) were set ($n_{\text{box}}=2$). No-choice, control experiments were conducted using boxes with only *P. pinea* seeds ($n_{\text{seeds}}=10$ per box; $n_{\text{box}}=6$) and boxes only with *P. pinaster* seeds ($n_{\text{seeds}}=24$ per box; $n_{\text{box}}=2$). The experiments were carried out in February and March of 2015 with four adults per box.

The three-choice experiment was performed during January and February of 2017. Twelve boxes, each with ten seeds per pine species, acted as replicates. Each box had three adults. All boxes included for water supply and as a substrate for resting and laying eggs, a twig of *P. nigra* in the two-choice and of *P. pinea* in the three-choice trial. Previous trials using boxes have shown that insect mortality increases greatly when there is no fresh twig inside (personal observation). The use of different pine species was dependent on conifer availability near the laboratory where the experiments took place. In addition to the twig, a petri dish with wet cotton to keep moisture and foam to support the seeds were also added to each box. Control boxes with no bugs were present in both trials. The sex of the adult specimens was not considered since previous studies found no significant differences in the consumption of conifer seeds between sexes (Bates *et al.*, 2000, 2002; Lesieur *et al.*, 2014). Bug mortality was checked every working day, and dead individuals were replaced immediately, using the stock available from the permanent colony. All seeds from the two-choice trial were radiographed before the trial using the HP Faxitron-43855® X-raying apparatus and X-ray sensitive films (Kodak® 'Industrex M'), following the procedures described in Roques & Skrzypczyńska (2003) but optimized for the seeds of the pine species tested. Seeds from the three-choice trial were X-rayed at the University of Lisbon, Faculty of Veterinary using the Philips Practix 300 machine and the constants 45Kv / 25mAs⁻¹. Only seeds showing no damage on the X-rays were used in the trials. Each seed was followed individually. Kernel consumption was estimated by subtracting the final seed weight from the initial. During the trial, seeds were radiographed every week for four weeks to track the damage by *L. occidentalis*. In the end, all seeds were opened manually, and kernels were separated into damaged and not damaged. Damaged kernels were photographed using a camera Canon 1100 D and their seed coat was carefully analysed under a stereomicroscope and a Scanning Electron Microscope (SEM) TM3030Plus Tabletop microscope Hitachi.

5. Statistical analysis

The size of the mature seeds, the thickness of the seed coat and the volume of the cones all had a normal distribution. The analysis was made using a one-way ANOVA, with three levels corresponding to the three-host species (*P. pinea*, *P. pinaster* and *P. halepensis*). *Post-hoc* pairwise comparisons were made using the Student-Newman-Keuls (SNK) test. In both the cone and branch preference trials we used Generalized Linear Models (GLM) with repeated measures (each bug was an individual with repeated observations). To compare frequencies among cones and branches, we used GLM with a Poisson distribution, log link function, and Wald Chi-Square test. In the branch preference trials, we performed the analysis for the

overall data for each pine species combination, pulling the three cages, as well as per cage. Finally, we used GLM with normal distribution and log link function to analyse differences in the feeding duration time in the cone trials. Again, each bug was considered an individual with repeated measures. For the seed preference trials, to compare frequencies among seeds, we used GLM with a Poisson distribution, log link function, and Wald Chi-Square test. In the two-choice seed trial, we compared: i) the mean number of seeds consumed between choice and non-choice tests for each pine species; ii) the mean number of seeds consumed between pine species on both choice and iii) on non-choice tests. In the three-choice trial, we analysed the differences in the mean number of seeds consumed between the three pines species with boxes considered as repeated measures. We further compared the seed weight consumed and the percentage of kernel consumed per host and box in both seed trials using GLM with normal distribution and log link function. Boxes with no consumption were removed from the analysis.

All statistical analyses were performed using SPSS, version 24.0 (IBM Corp., Armonk, New York) with a statistical significance level of 0.05.

Results

1. Size of seeds and cones and coat thickness

The volume of last-year cones differed significantly among pine species ($F=92.38$; $df=2$; $p<0.001$), with the volume of *P. pinea* cones being two and three times larger than those of *P. pinaster* and *P. halepensis*, respectively. The weight of mature coated seeds also differed significantly among species ($F=10387.92$; $df=2$; $p<0.001$) as well as the kernel volume ($F=1526.33$; $df=2$; $p<0.001$), and coat thickness ($F=4681.251$; $df=2$; $p<0.001$: Table 1). Seed kernel was four times larger in *P. pinea* than in *P. pinaster* and 13 times larger than in *P. halepensis*. Seed coat thickness was more than three times greater in *P. pinea* than in *P. pinaster* and 12 times greater than in *P. halepensis*. The ratio Kernel volume/coat thickness (KV:CT) that could be used as a proxy of benefit/cost for the bug showed a higher value in *P. pinea* (Table 1).

Table1. Seed and cone average measures (\pm SE) of the three host species. Different letters within a column indicate significant differences between the values per host species after ANOVA tests (p -value=0.05) followed by SNK test.

Host species	Cone volume (cm ³)	Mature seed weight (g)	Kernel volume (KV) (mm ³)	Coat thickness (CT) (mm)	KV: CT
<i>P. pinea</i>	90.0 \pm 7.6 ^a	0.867 \pm 0.008 ^a	202 \pm 17.0 ^a	1.438 \pm 0.030 ^a	140.5
<i>P. pinaster</i>	43.0 \pm 1.7 ^b	0.062 \pm 0.001 ^b	50 \pm 11.0 ^b	0.380 \pm 0.009 ^b	131.6
<i>P. halepensis</i>	27.3 \pm 7.7 ^c	0.022 \pm 0.000 ^c	15 \pm 1.0 ^c	0.117 \pm 0.003 ^c	128.2

2. Branch preference

No host preference was detected in either of the three host pine choice combinations on the frequencies of visits per bug (*P. halepensis* x *P. pinea*: Wald $\chi^2=2.485$, $p=0.115$; *P. pinaster* x *P. pinea*: Wald $\chi^2=0.005$, $p=0.943$; *P. halepensis* x *P. pinaster*: Wald $\chi^2=0.008$, $p=0.927$). Overall, 70% of the individuals remained on the same host species during the trial period (48h) with the few changes happening on the first day. When each cage was considered separately, a significant preference was observed for one branch or the other, whereas preferred host species may differ from one trial to the other for the same host species combination (Figure 1).

As a general trend, we observed that the ten individuals from each cage dissociated into one or two fix groups in the three trials (Figure 1). The record of the specific place in the plant where the adults were revealed that in more than 80% of the observations the insects were resting between the needles or on the last year cones, regardless the host.

3. Cone preference

We found no differences between the three trials and so results were analysed together. The adults were observed visiting and feeding more frequently on *P. pinea* cones in comparison with cones from the other two species (visiting: Wald $\chi^2 =17.42$; $p<0.001$; feeding: Wald $\chi^2 =15.31$; $p<0.001$). Per feeding meal, the adults also spent more time feeding on *P. pinea* cones in comparison with other cones (Wald $\chi^2 =12.05$; $p=0.002$) (Table 2).

Chapter 4 – Seed bug host preferences

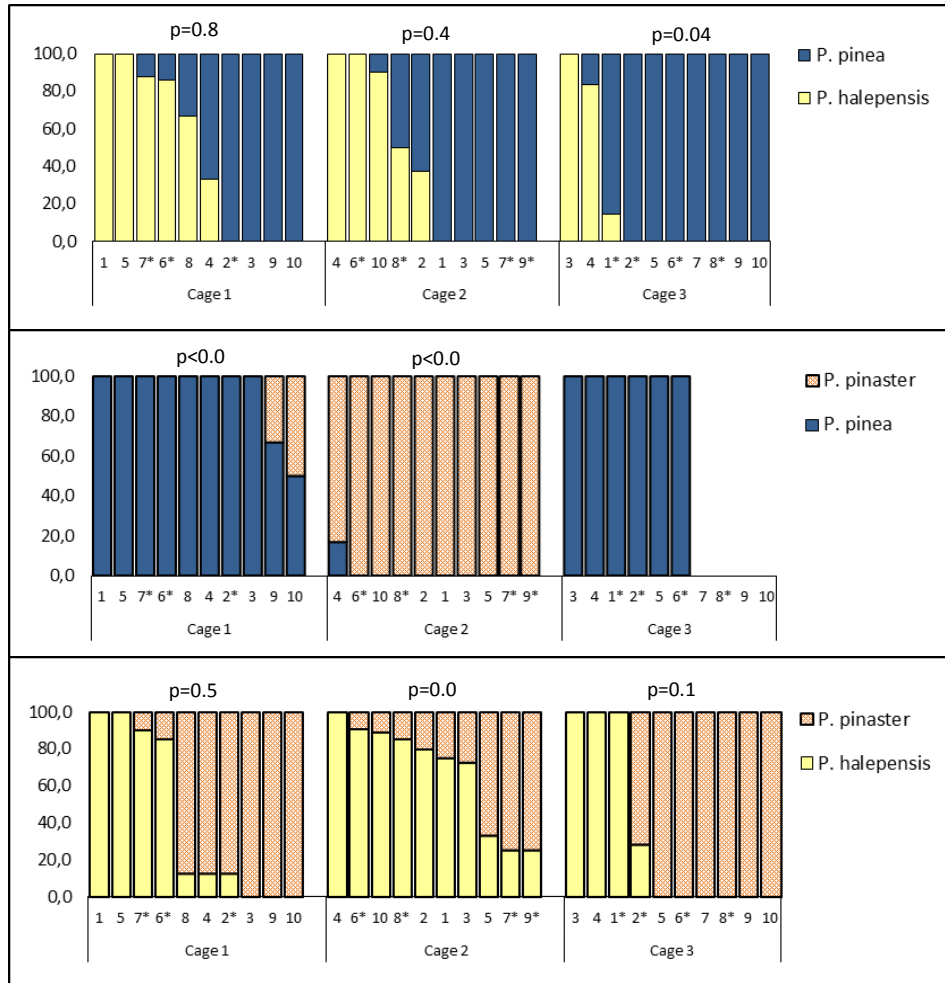


Figure 1. Branch preference trials. Bugs distribution per host in each cage and for each host pair comparison in the preference trials using potted branches. Numbers with asterisk on the x axis correspond to male bugs. The absence of bars means that individuals were never observed on the branches during the trial, but remained on the floor or on the walls of the cage.

Table 2. Bug behaviour averages (\pm SE) in the cone preference trials. Different letters within a column indicate significant differences between the values per host species after GLM tests (p -value=0.05).

Host species	Visiting frequency	Feeding frequency	Time spent per feeding meal (minutes)
<i>P. pinea</i>	13.4 \pm 4.2 ^a	5.5 \pm 1.2 ^a	131.9 \pm 25.9 ^a
<i>P. pinaster</i>	5.1 \pm 1.2 ^b	2.0 \pm 0.6 ^b	53.2 \pm 12.0 ^b
<i>P. halepensis</i>	3.7 \pm 1.5 ^b	1.1 \pm 0.5 ^b	62.5 \pm 13.3 ^b

4. Seed preference trials

4.1 Bug preference between seeds of *P. pinea* and *P. pinaster*

For four weeks, the four individuals always ate two, out of ten, seeds of *P. pinea* per box, either if it was offered alone (non-choice tests) or mixed with *P. pinaster* seeds (choice tests) (Table 3). Conversely, when adults fed on *P. pinaster* alone, the number of seeds consumed was on average 5.5 ± 1.7 , which was higher than the *P. pinaster* seeds consumed in choice tests, 0.5 ± 0.5 (Wald $\chi^2=5.271$, $df=1$, $p=0.022$). Considering non-choice tests alone, the number of *P. pinaster* seeds was significantly higher than those of *P. pinea* (Wald $\chi^2=4.74$, $df=1$, $p=0.029$). Nevertheless, the consumption of seeds expressed on kernel weight consumed was higher on *P. pinea* than *P. pinaster* both on the choice test (Wald $\chi^2=6.800$, $df=1$, $p=0.009$) and non-choice test (Wald $\chi^2=25.450$, $df=1$, $p<0.001$).

4.2 Testing bug preference among seeds of *P. pinea*, *P. pinaster*, and *P. halepensis*

Seed consumption was observed on eight out of the 12 boxes (Figure 2). On these boxes overall, there were no significant differences in the number of seeds consumed between species (Wald $\chi^2=0.011$, $df=2$, $p=0.995$) (Table 3). The total number of seeds consumed was 15, 16 and 15, respectively for *P. pinea*, *P. pinaster*, and *P. halepensis*. However, when considered the weight of the seed kernel eaten by the adults, we found significant differences among pine seeds (Wald $\chi^2=117.632$, $df=2$, $p<0.001$). On average the bugs ate more *P. pinea* seed kernel than the two other species ($p<0.001$) which, in turn, had no difference between them ($p=0.726$). When analysing the consumption in terms of the percentage of consumed kernel per host in each box and trial differences were obtained in both two and three-choice trial (Wald $\chi^2=1002.485$ $df=1$, $p<0.001$, Wald $\chi^2=15.625$, $df=2$, $p<0.001$, respectively). The adults consumed a higher percentage of *P. pinea* kernels than the other two hosts ($p<0.001$ for both comparisons) (Table 3).

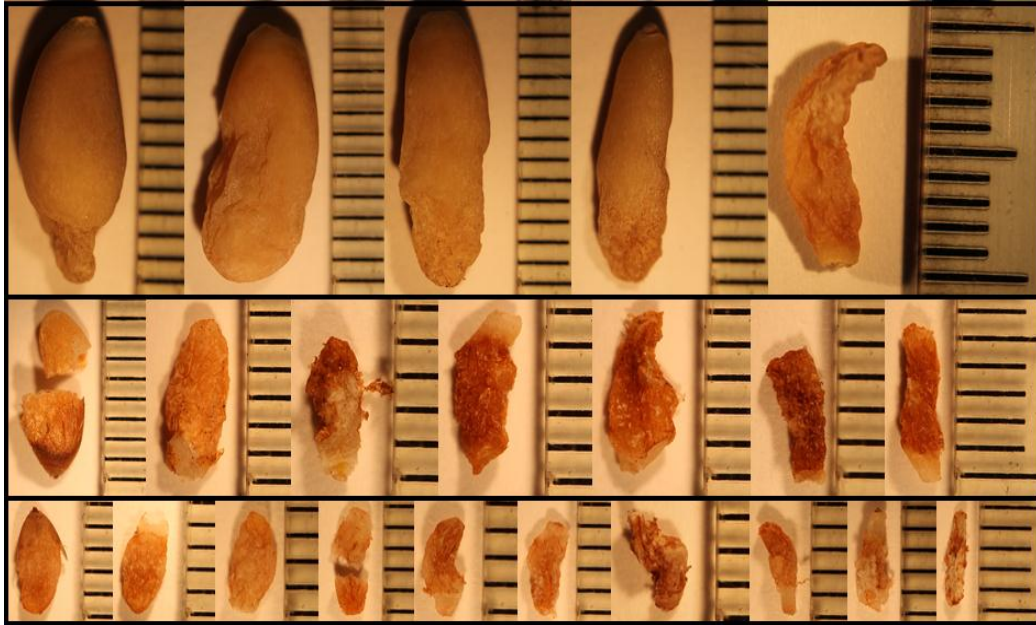


Figure 2. Seed three-choice trial. A sample of seeds consumed by *L. occidentalis* on the three-choice trial. Each row corresponds to a different host pine: (from top to bottom) *P. pinea*, *P. pinaster* and *P. halepensis*, with seeds arranged in each row from the less (left) to the more damaged (right). Photographs taken by Canon 1100 D. The marks on the scale correspond to 1mm. (Photos were taken by Charlene Durpoix).

Table 3. Seed consumption in choice and non-choice trials. Average number (\pm SE) of consumed seeds per box, kernel weight consumed per box and bug and percentage of the kernel that was consumed in each of the seed preference trials. Different letters within a trial indicate significant differences between the values per host species after GLM tests (p -value=0.05).

Type of trial	Host (s)	Seeds (total)	number of seeds consumed	kernel consumed (mg)	kernel consumed (%)
two-choice	<i>P. pinea</i>	5	2.0 \pm 0.0	11.71 \pm 2.53 ^a	97.12 \pm 15.01 ^a
	<i>P. pinaster</i>	12	0.5 \pm 0.5	1.28 \pm 3.58 ^b	5.77 \pm 21.23 ^b
non-choice	<i>P. pinea</i>	10	2.5 \pm 0.7	21.03 \pm 2.21	-
	<i>P. pinaster</i>	24	5.5 \pm 1.7	4.13 \pm 2.12	-
three-choice	<i>P. pinea</i>	10	2.7 \pm 1.2	23.91 \pm 1.40 ^a	92.30 \pm 10.62 ^a
	<i>P. pinaster</i>	10	3.0 \pm 1.8	3.69 \pm 1.76 ^b	29.14 \pm 10.61 ^b
	<i>P. halepensis</i>	10	5.3 \pm 2.7	4.51 \pm 1.55 ^b	39.73 \pm 12.26 ^b

Discussion

The impact of an invasive species must be assessed at different levels from the individual to the ecosystem processes level (Parker *et al.*, 1999). The seed feeder, *L. occidentalis* is classified as having a negative impact to the native biodiversity at the individual level (herbivory, predation, competition, disease transmission) and, in addition, a negative economic impact (Rabitsch, 2008). Indeed, the presence of the bug in the Mediterranean Basin is a severe threat to the Mediterranean pine nut production as very relevant non wood forest product (Roversi, 2009; Calama *et al.*, 2016, 2017; Mutke *et al.*, 2017) by causing direct damage to cones and seeds and as putative vector of the fungus *Diplodia pinea* (Luchi *et al.*, 2012; Tamburini *et al.*, 2012). Here we tested the preference of the bug on the three main lowland conifer species in the Mediterranean Basin region, *P. pinea*, *P. pinaster* and *P. halepensis* at three levels of selection: branch, cone, and mature seed. These three pine species significantly differ in the shape of the tree silhouette and also in the cone size, seed size, and seed coat thickness. Cues behind tree selection by cone feeders may be related to these morphology traits (Turgeon *et al.*, 1994), but also to chemical factors which are indicators of the tree nutritional quality or of its level of chemical defence (Schultz, 1988). In fact, there are two predominant hypotheses for the process of host selection by insects: first, that an increased abundance of insects is explained by increased plant vigor (*Plant Vigor Hypotheses*: Price, 1991), or secondly, that it is explained by increased plant stress (*Plant Stress Hypotheses*: White, 1969). Studies on how herbivores select the host plant reported responses according to the theory of plant vigor (Moran & Whitham, 1988; Waring & Price, 1988; Kimberling *et al.*, 1990; Mopper & Whitham, 1992; Hull-Sanders & Eubanks, 2005; Mitchell, 2006), plant stress (Bjorkman *et al.*, 1991, Rappaport & Wood, 1994; Cobb *et al.*, 1997; Virtanen & Neuvonen, 1999) or even both simultaneously (Fernandes, 1992; Pérez-Contreras *et al.*, 2008) depending on the insect species, its trophic sub-guild (e.g. herbivores that feed on growing plant parts seems more likely to attack vigorous plants (Price, 1991; White, 1993) or even on the type of experiment (Waring & Cobb (1992), in a review found that on observational studies, White's theory was prevalent whilst on experimental studies was the vigor hypothesis). Under natural conditions, *L. occidentalis* has been shown to select a host in a multi-level process. At first, the bug selects a tree, then a cone and finally a seed where to feed upon (Blatt, 1997). Regarding the selection of the tree, the bug tends to select vigorous trees, i.e. denser canopies and longer needles, (Farinha *et al.*, 2018b) bearing moderate crops

because it is usually a sign of having larger cones and therefore larger seeds (Blatt, 1997; Blatt & Borden, 1999; Richardson *et al.*, 2017).

With all that has been said in mind, we hypothesized that branch selection by this bug, a polyphagous insect that feeds on growing plant parts, should rely mostly on vigour (e.g., increased resources, higher food quality, and lack of induced defensive compounds) and cone size and not so much on plant species and their chemical profiles.

In our experimental trials using potted branches with cones, the seed bug showed no clear preference between the three host species but rather a preference for one of the two branches on each trial. The plant vigour hypothesis (Price, 1991) for the selection of the host may explain this result. The vigour of the chosen branch (e.g. nutritional state, morphology) and the size of the cones on the branch may have been more critical for a polyphagous insect like *L. occidentalis* than the species of the host plant. Although, we choose branches with similar size, with no signs of diseases and always bearing cones of all ages, yet differences on the number of cones and on its physiological status (e.g. nutritional quality, allelochemicals) might have caused differences between host species and between trials. Furthermore, since branches came from different locations and trees, there could be both a tree and site effects.

Preference studies on a related species, *L. phyllopus* (L.), which is also polyphagous, have evidenced that nutritional and host quality issues (wild vs cultivated plants) are more determinant in host plant selection rather than plant species (Mitchell, 2006). A study of *L. occidentalis* impact in a *P. pinea* plantation, also revealed higher bug damage on irrigated and fertilized trees than on control ones, showing a bug preference for high vigour trees (denser canopies, greater needles) (Farinha *et al.*, 2018b).

The branch preference trial also revealed an overall trend for *L. occidentalis* to form two groups of individuals per cage, one in each plant. In general, the composition of the two groups remained similar in each cage in all three trials. During the time between trials, all insects (n=30, 10 from each cage) were placed together in a single cage but when they were replaced one by one again in the test cage they regrouped in the same way as in the very first trial. Furthermore, once the individuals had chosen one of the plants, they remained there, in 70% of the cases, throughout the experiment. Group dynamics and not an individual host selection is, thus, a more suitable explanation for the results obtained. The gregarious behaviour of this insect has already been proposed by other authors (Koerber, 1963; Mitchell, 2006).

Preference for a host species was further tested exposing cones to adults in trials using video cameras. The use of video recording can be very informative on the insect preference because it allows capturing the behaviour of the insect continuously. Moreover, the use of cameras with time lapse mode made it possible to process all data since it condenses several days of filming in movies of few minutes. So, by tracking the feeding behaviour, a clear bug preference could be observed for visiting and feeding on *P. pinea* cones. Furthermore, the individuals spent twice more time feeding, by each feeding meal, on this host species than on the other two species. A higher reward per seed would probably keep insects feeding longer times. It has also to be noted that the seed coats were not yet totally hardened inside cones because we used last year cones collected in the spring. Under natural conditions, the larger cones of *P. pinea* could be more attractive since visual stimuli are important to this bug in the process of host selection (Blatt & Borden 1999; Richardson *et al.*, 2017). The higher reward value may further benefit the performance of *L. occidentalis*, when feeding on *P. pinea* seeds, namely through a higher survival rate and faster development, as indicated by Ponce *et al.* (2017). Bernays & Minkenberg (1997) in an experimental study with seven polyphagous insects (four Lepidoptera and two Hemiptera) came to the conclusion that it is the greater resource availability rather than the nutritional enhancement or differences in allelochemicals among host species that resulted in a higher performance (survivorship, gain in mass and fecundity). We assume that in our cone preference experiments, the larger cones of stone pine represent a greater resource availability comparing to the other hosts.

In respect to seed trials, no clear preference between mature seeds of *P. pinea*, *P. pinaster* and *P. halepensis* was observed if we compare the number of seeds consumed. However, *P. pinea* kernels are much larger than the others. Furthermore, it has been shown that the same insect feeds several times on the same seed and that different insects may also feed on that same seed by sharing the feeding hole (from video recording observations, data not showed) (Farinha *et al.*, 2018a). Therefore, the mass or the percentage of kernel consumed is a better indicator of the real consumption by the individuals. When considering kernel consumption, *P. pinea* emerges as the most consumed host species in all trials. When expressed in percentage of consumed kernel per host species and per box, *L. occidentalis* consumed about 97% and 92% of *P. pinea* kernels in two-choice and three-choice tests, respectively.

Other preference study at the seed level showed that *L. occidentalis* appeared capable of differentiating a viable seed from one infested by chalcid, *Megastigmus spermotrophus*

(Hymenoptera: Torymidae), discarding the latter (Blatt, 1997). However, since all the seeds used in our trial were first radiographed and only the healthy ones were used, we hypothesize that feeding onset in a given seed might be random or visually determinate. After first opening a hole on the seed, feeding activity would be concentrated on that seed, which in the case of *P. pinea* seeds would satiate longer, further requiring less number of consumed seeds per bug. Also, bigger seeds may result in higher nutrients income in shorter periods which optimize the feeding. This result becomes evident when we compare *P. pinea* and *P. pinaster* kernel consumption in non-choice tests (over 5 times more mass consumed in boxes with *P. pinea* seeds) (Table 2). In another preference trial using mature seeds, Lesieur *et al.* (2014) found no difference between host species. Still, in that study, the size of the seeds did not differ so much among tested host species.

Despite the larger size of *P. pinea* seeds, which constitute a more significant reward, the seed coat implies a higher cost, being three and twelve times thicker than that of *P. pinaster* and *P. halepensis*, respectively. Even so, a benefit/cost analysis pointed to *P. pinea* seeds as being more advantageous. Feeding behaviour videos show that drilling a hole in *P. pinea* seeds can take more than 8 hours to complete (unpublished data) but then the benefit is high and, most importantly, it is shared by the remaining insects of the box as other bugs use the same hole to feed. We should note that, although easy to replicate under laboratory conditions, host selection trials resorting to mature seeds have a limited ecological significance because the seeds, enclosed within the cones, are not subjected to selection in natural conditions.

Conclusions

In spring or early summer, depending on the climatic conditions, this insect becomes active and begins to search for a site with coniferous where to feed and reproduce. No data can be found on the bug behaviour when leaving its winter shelter except for an inconclusive study by Richardson (2013) in Lodgepole pine seed orchards for two consecutive years. Does it return to the same place as the year before or disperse elsewhere? How does it select the site to colonize? Is there any host preference at this moment?

Among the three main pine species in the Mediterranean Basin, *P. pinea*, *P. pinaster* and *P. halepensis*, our results support evidence that the cones and seeds of *P. pinea* are highly rewarding for *L. occidentalis*. From a nutritional point of view, we may then expect that *P. pinea* trees and plantations may favour *L. occidentalis* population growth. Whereas in seeds

and cones there was always a preference trend for *P. pinea*, no clear preference for host species was detected when we used potted branches.

It must be highlighted that the quality of the host plant rather than the species, and the aggregation behaviour of this bug are important factors to take into account when designing the methodology of future host preference studies. Furthermore, larger scale trials are required. The population dynamics of this insect must be a priority research topic. No management plan will succeed without understanding which factors influence the distribution and abundance of this pest, including the availability of, and its performance on, different hosts.

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CHAPTER 5

Does Stone pine management influence this bug impact?



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Chapter 5 -Is *Leptoglossus occidentalis* entirely responsible for the high damage observed on cones and seeds of *Pinus pinea*? Results from a fertirrigation trial in Portugal

Brief introduction to the chapter

This chapter corresponds to part of the field experiments that I conducted during this thesis. By working at a seed orchard of Stone pine trees under different management regimes I could evaluate two important aspects: the effect of different tree management regimes on the damage by biotic agents and by *L. occidentalis* and also the impact of *L. occidentalis* on young and matured cones instead of matured seeds (chapter 3). Thus, this chapter is not only about tree management vs insect damage but it also presents an estimate of *L. occidentalis* damage in field conditions. Results from chapter 3 respecting the characterization of damage on seeds that can be assigned to this bug were crucial to further estimate the damage on this field trial.

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Abstract

The uncertainty surrounding the part played by an invasive North American seed bug, *Leptoglossus occidentalis*, in the observed decrease in seed production of the Mediterranean pine, *Pinus pinea*, is a limiting factor for its management. Furthermore, the possibility of increasing cone production through irrigation and fertilization regimes is gaining interest among landowners, but its effects on insect pests are still unknown.

Using bagged branches in the field, we aimed at evaluating the impact of *L. occidentalis* on young and mature cones of Stone pine trees submitted to fertirrigation (FR) compared to trees with no treatment (C). For two consecutive years (2015 and 2016), we carried out both an insect-exclusion trial and an insect-bagged trial. In the first one, polyester-covered branches, excluding insects, were compared to branches exposed to natural insect infestation. In the second trial, bags included either two adult bugs or 3-4 third-instar nymphs, placed there for one month during mid-summer, or were kept without insects. Branch protection resulted in a significant decrease in the mortality of second-year conelets which dropped to 6% compared to 30% in exposed branches. Seed damage also decreased from 60% on exposed branches to 10% on protected ones. The presence of nymphs in the bags resulted in a mortality of second-year conelets 63% higher than in exclusion bags, whereas adults had no effect. In contrast, bags with adults presented the highest seed

damage. The partial damage of the kernel can be a signature of *L. occidentalis* feeding since such damage was not observed in exclusion bags. Additionally, another type of seed damage, showing a wholly shrunken and dry embryo without remaining endosperm, and a reduction in the number of extractable seeds may also be attributed to this seed bug. Overall, seed damage per mature cone reached up to 12% in bags with two adult bugs enclosed for a month, i.e., twice the seed damage in protected cones. Overall, FR trees were more susceptible to both conelet mortality and seed damage. In the particular case of *L. occidentalis*, FR regime influenced the consumption positively by the nymphs but not by adults.

Keywords: seed damage, conelet abortion, orchard management, seed feeder

Introduction

Since its introduction in Europe in 1999, the invasive insect pest Western Conifer Seed Bug, *Leptoglossus occidentalis* Heidemann, (Hemiptera: Coreidae), has been gaining increasing attention by the Mediterranean Basin countries. This interest is mainly due to the putative impact of *L. occidentalis* on the edible, and of high commercial value seed of the Stone Pine, *Pinus pinea* L. with current retail prices that can exceed 100 euros/Kg (Mutke *et al.*, 2014). The tree species, *P. pinea*, commonly known as Stone pine or Umbrella pine, is native to the Mediterranean region (Costa *et al.*, 2008). Cones of this species have a 3-yr reproductive development cycle, which begins with the burst of the female strobili (conelet) in the spring of the first year, with wind pollination occurring a few weeks after that. Strobili fertilization, however, takes place only in the spring/summer of the third and last year of cone maturation when the nutritious edible seeds are thus formed (Valdivieso *et al.*, 2017). The total area of *P. pinea* represents now nearly 1 million hectares in the Mediterranean Basin resulting from new plantations during the last decades (Mutke & Calama, 2016). Great investigation efforts have been made to disentangle the drivers of cone production. Irrigation and fertilization, for example, are becoming a common practice in young plantations aiming to increase the number and weight of the harvested cones (Calama *et al.*, 2007; Montero *et al.*, 2004).

Several countries recently report a significant decrease in cone production per hectare and substantial cone yield losses (percentage of commercial kernels per kg of the fresh cone) (Mutke *et al.*, 2005, 2014; Mutke & Calama, 2016). Biotic factors like the invasive *L. occidentalis* have been pointed out as most plausible cause (Bracalini *et al.*, 2013; Mutke & Calama, 2016; Parlak, 2017; Roversi *et al.*, 2009) although climate change is also often

referred (Calama *et al.*, 2011; Mutke *et al.*, 2005). This bug can damage the seeds of *P. pinea* last-year cones (Bracalini *et al.*, 2015; Calama *et al.*, 2017; Elvira-Recuenco *et al.*, 2016; Farinha *et al.*, 2018). First-year and second-year conelets although without developed seeds are also susceptible to be fed upon by *L. occidentalis* (Mutke *et al.*, 2014; Parlak, 2017).

Assigning damage to this bug is not as easy as no visible external signs of feeding can be detected in cones where the bug pierced. Antibody (Lait *et al.*, 2001) and DNA techniques (Bracalini *et al.*, 2015) were able to track seeds fed by *L. occidentalis* although limited by the time that has elapsed since the feeding and also by the high costs associated which makes these techniques not expeditious.

The slow maturation process of the cones means that the same cone may be exposed to the insects for two to three years, resulting in an accumulation of damage which is difficult to track. This fact, along with the asymptomatic damage, makes the task of assessing the impact of the bug extremely difficult. One alternative to characterize and quantify the damage by *L. occidentalis* is force-feeding mature seeds under controlled laboratory conditions (Bates *et al.*, 2000; Farinha *et al.*, 2018; Koerber, 1963; Lesieur *et al.*, 2014). However, extrapolating these results to the natural environment where live conelets and cones are the primary food source becomes a questionable exercise. Another relevant setback is the absence of specific traps able to capture the insect and estimate its density in the field. This methodological limitation makes it difficult to correlate the damage found on cones with the natural insect density and distribution. In fact, up to the present, it has never been demonstrated that the current increase in damage to *P. pinea* cones and seeds reported by several Mediterranean countries is directly attributed to an increase in the population of *L. occidentalis*.

In an attempt to overcome these limitations, and to obtain a field assessment of bug damage, several studies used branches bearing cones covered by bags, into which insects were then either added (Bates *et al.*, 2001, 2002a, 2002b; Connelly & Schowalter, 1991; Lesieur *et al.*, 2014; Schowalter & Sexton, 1990; Strong *et al.*, 2001; Strong, 2006;) or excluded (Blatt & Borden, 1996; Elvira-Recuenco *et al.*, 2016; Strong, 2016). Variables such as the host species, the period and duration of the experiments as well as the density of the insect inside the bags vary significantly between studies. *Pinus contorta* Douglas and *Pseudotsuga menziesii* (Mirb.) Franco are the most referenced hosts which comes from most publications being from the North American continent where these species are of relevant importance, especially in seed orchards (Bates *et al.*, 2001, 2002a, 2002b; Blatt & Borden, 1996; Lesieur *et al.*, 2014; Schowalter & Sexton, 1990; Strong *et al.*, 2001; Strong, 2006, 2016). Regrettably, the reported damages caused by *L. occidentalis* on these

two hosts vary greatly depending on the experimental and local conditions (Bates *et al.*, 2001, 2002a, 2002b; Blatt & Borden, 1996; Lesieur *et al.*, 2014; Schowalter & Sexton, 1990; Strong *et al.*, 2001; Strong, 2006, 2016;) making it difficult to compare impact estimates or even extrapolate damages between different hosts.

In Europe, publications on *L. occidentalis* are mostly from the Mediterranean Basin countries and are essentially concerning *P. pinea* (Bracalini *et al.*, 2015; Calama *et al.*, 2017; Farinha *et al.*, 2018; Mutke & Calama, 2016). Lesieur *et al.* (2014) however, provided a typology of seed bug damage on many European conifers, but the study did not include *P. pinea*. Moreover, accurate quantification of seed bug damage on *P. pinea* is not yet available (Kenis *et al.*, 2017).

There is also a lack of knowledge on how tree growing conditions will influence tree susceptibility to the bug. In fact, site productivity is one of the critical factors influencing the colonization and establishment of insect pests in a specific area (Liebhold & Tobin, 2008). It is known, for example, that changes in water and nutrient availability affect not only the plant productivity but also its susceptibility to herbivores (Ayres, 1993; Moon & Stiling, 2000; Netherer & Schopf, 2010; Rusch *et al.*, 2010). For many invertebrate herbivores it has been demonstrated they could discriminate, for the same host species, plants of high nutritional quality from those of low quality (Awmack & Leather, 2002; Firempong & Zalucki, 1989; Kareiva, 1982; Santiago Lastra *et al.*, 2006).

The pine, *P. pinea*, is known to be quite resistant to drought. Nonetheless, trees growing in arid sites tend to show a lower leaf area index and lower cone production than trees growing with irrigation (Bono & Aletà, 2013; Calama *et al.*, 2007, 2011; Correia *et al.*, 2017; Loewe-Muñoz *et al.*, 2016; Mutke *et al.*, 2005).

Concerning fertilization, nitrogen (N) is the most widely used fertilizer (Chen *et al.*, 2010). The increase in N availability can enhance host attractiveness to an herbivore-insect by altering herbivore-induced plant volatiles (Holopainen & Gershenson, 2010; Saha *et al.*, 2012) or by modifying the plant structure (Chen *et al.*, 2010).

The expansion of *P. pinea* orchards for cone production using irrigation and fertilization (Calama *et al.*, 2007; Loewe *et al.*, 2017; Mutke, 2017b) are excellent opportunities to study plant-insect interactions related to the tree vulnerability status.

Thus, in this work we had two main objectives: i) characterization and assessment of *L. occidentalis* impact on cone and seeds of *P. pinea* under natural conditions, using branches covered with mesh insect-exclusion bags and ii) analyze the effect of tree fertirrigation in *L. occidentalis* feeding activity.

This study allows us to isolate the type of damage caused by *L. occidentalis* feeding on mature seeds of *P. pinea* during part of its active period, which is essential to the future development of impact quantification methodologies related with this bug.

Methods

1. Study site and management protocols

The study site is located in Coruche, in center Portugal, in a ten years old pure *P. pinea* stand grafted at the age of four. The climate is Mediterranean with a cold and moist winter and dry and hot summers, with annual precipitation of 641 mm and a mean annual temperature of 15.1°C. Within site, an area of 3 ha was selected and eight randomized plots, corresponding to 2 treatments with four repetitions each, were installed during the summer of 2014. The two plot treatments were Control (C) and Fertirrigation (FR). Each plot comprehends 15 to 21 trees spaced by 3 x 8 m. FR trees were irrigated with diluted fertilizer (40 kg N*ha⁻¹) every day from March until June. The trees were also irrigated for 3.5 hours during October of both years. Besides the natural precipitation occurring each year, this irrigation regime corresponded to an additional water input to the system of approximately 400 mm ha⁻¹ year⁻¹.

Climatic data were obtained from a local meteorological station on site. The year of 2015 was a dry year, totaling 315 mm of precipitation from January to December and with an unusually dry spring. In 2016, total precipitation was above the annual average (529 mm). Regarding air temperature, August 2016 was particularly warm with 17 days with maximum temperatures above 35°C contrasting with only four days observed in 2015. No rain events were recorded during August 2016 and in 2015 only rained 3mm. August corresponds to the insect trial establishment in the field.

2. Tree physiological traits

Trees from the C and FR treatments were characterized by their ecophysiological traits regarding leaf and crown structure during the two years of the experiment. For that, we selected harvested trees from both treatments for component biomass separation and weighing. All the aboveground components were then separated and weight according to the methodology described in Correia *et al.* (2010). The total tree needle area, used as an indicator of the primary photosynthetic production, was calculated using the tree needle

biomass and an average of the specific needle area by age cohort. For the needles' morphological characterization, we randomly selected one branch from each node. A sample of current year needles (2015 and 2016) was collected in each branch, scanned in a flatbed scanner and analyzed with Winseedle software (Regent Instruments Inc.) for the needles' length and projected area calculations. The needles were then oven dried and, together with the needles' projected area, used for specific needle area calculations.

3. Cone and seed production

All mature cones (third-year cones) in the sampled branches of the field trials were harvested in the winter of the sampled year (November/December), dried in the laboratory (at 45°C until opening) and weighted. We then evaluated the average weight of cones and number of seeds per cone. The average number of cones per tree in each year and plot treatment was estimated using all trees from the site (n=246).

4. Field trials

The experiment occurred during two consecutive years: 2015 and 2016. Ten trees from each treatment, C, and FR, bearing at least six branches with second and third-year cones simultaneously, were randomly selected each year. Two trials were conducted on the same trees: i) an exclusion trial and ii) a bagged insect trial.

Branches bearing second year and third-year cones were selected for both trials. Bagged branches were enclosed from the base of the fork to the tip within a mesh of 0.5 mm, about 50-90 cm long depending on the branch length, leaving room for it to grow. All bags had an inner wire structure to keep the cones away from the bag walls thus preventing insects from outside from feeding on the cones, and also to provide more space for the insects in the case of the bagged insect trial. In both trials, bags were set up in April, before the emergence of the overwintering adult bugs, and removed in December of the same year, when the third-year cones were collected.

All second-year conelets and third-year cones present on the selected branches were counted in spring and their mortality assessed by December of the same year. Conelets of the second-year were considered dead when they appeared dried or if they broke easily when touched. Last year cones were classified as dead when more than 1/3 of the scales were damaged thereby making them non-marketable. Conelets of the first-year were not followed because by the time the bags were set up they were not formed yet.

Mature cones (third-year cones) were opened after drying and the number of seeds counted. Seed health status was then evaluated using X-ray (Philips Practix 300) and by a manual opening. Seed kernels were counted and separated into the four following categories: i) apparently sound (kernel intact); ii) kernel partially damaged /with a different colour /serous appearance (named as “damage type I”); iii) completely shrunken and dry embryo with no endosperm (named as “damage type II”); and, iv) empty shell without embryo (named as “damage type III”). This classification follows Calama *et al.* (2017), but we changed the description of type I damage by type II, and vice versa, because it seemed to be more evident to consider an increasing level of damage (Figure 1).

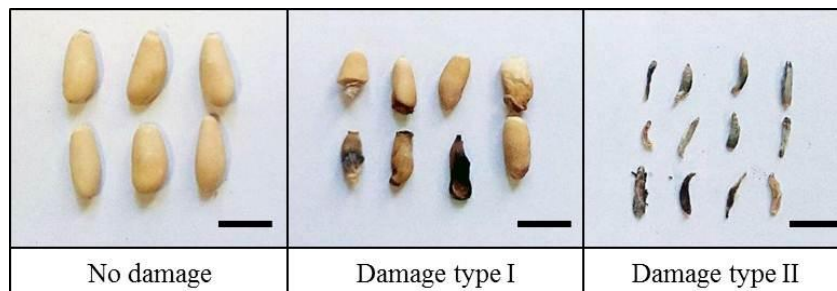


Figure 1. Damage categories of *P. pinea* kernels. Bar corresponds to 1 cm.

4.1 Exclusion trial

Branches enclosed by bags were set up in April, in two consecutive years, 2015 and 2016 (Figure 2). Simultaneously 2-3 branches on the same trees were marked with a rubber ring and left exposed throughout the year. Chosen branches were preferably from the same and most recent fork of the tree to minimize variations of abiotic factors such as solar exposition, wind or humidity. The exclusion trial tested the effect of the protection of the cones of *P. pinea* from April until December in comparison with exposed ones.

Two bags were removed from the analysis, one from each treatment, FR, and C, respectively, because they were found unsealed at the time of removal. Also, three exposed cones from FR were removed from the analysis for presenting symptoms of *Diploidia pinea*.

4.2 Bagged insect trial

To determine the number of insects to use inside bags we conducted a pilot study in 2014 aiming to estimate the density of *L. occidentalis* in the experimental site. Monthly observations were carried out spending, at least, 3 minutes in each tree (n=20), observing all cones and tree crown. This pilot study showed a maximum average density of 1.6

bugs/tree during July-August. Taking into account that visual monitoring techniques may underestimate real insect density (Richardson, 2013) we rounded the ratio obtained by excess, and we use it by branch rather than by tree. Therefore, in each bagged branch with insects, either two adults, one male and one female, or 3 to 4 nymphs, were added to simulate a high natural impact.

Three types of bags were randomly assigned to three cone-bearing branches: Adults (bag with one couple), Nymphs (bag with 3-4 nymphs of third-instar) and Exclusion bag (a branch with a bag but without insects).

Insects were placed inside the bags at the beginning of August and removed after four weeks (Figure 2). All bugs used in one year were from the F1 generation of wild adults captured in May /June of that same year and kept in the laboratory under natural temperature and photoperiod. Bags were monitored twice a month. Any dead or missing individuals were replaced whenever possible with other F1 individuals from the lab colony.

The bagged insect trial tested the effect of the bugs on *P. pinea* for one month (August). From March till December, these branches remained covered by the exclusion bags. Data were compared with exclusion bags with no bugs.

One bag with nymphs from a C tree was removed from the analysis because it was found with a hole at the time of removal.

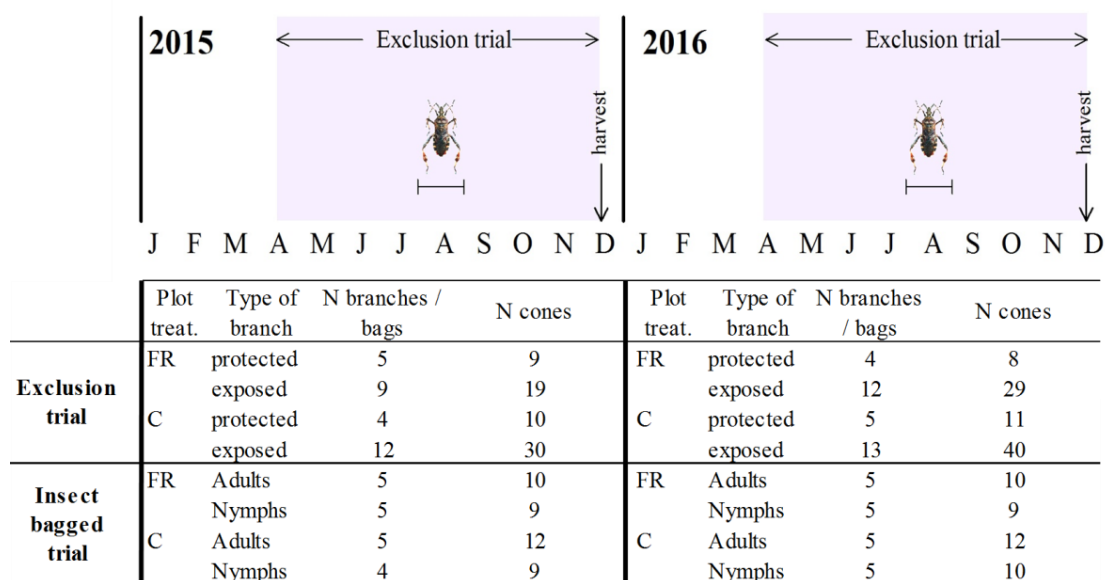


Figure 2. Field trials. The top diagram concerns field trials methodology: shade corresponds to the time during which the sampled branches were covered with bags (*Exclusion trial*), and the insect represents the time when the adults and nymphs of *L. occidentalis* were placed inside sampled bags (*Bagged insect trial*). The bottom table shows the number of branches and cones sampled by treatment and year.

5. *Statistical analysis*

To examine the differences among the ecophysiological traits, we used a two-way ANOVA: factors were tree treatment (2 levels, FR and C) and year (2 levels, 2015 and 2016). Tested variables were the needle's maximum length and the total leaf area following Normal Distribution. Post-hoc pairwise comparisons were performed using the Student-Newman-Keuls Method.

For the differences on the cone and seed production between the two plot treatments, C and FR, and years, the t-student test or the Mann-Whitney test was used whenever normality of the data was not accomplished. Tested variables were the number of cones per tree, the cone weight and the number of seeds per cone.

For both the exclusion trial and the bagged insect trial, statistical analysis was performed using generalized linear models (GLM) with a Binomial distribution or Negative Binomial distribution, according to the variable distribution and a link model binary probit. The following variables were analyzed: i) mortality of second-year conelets; ii) proportion of seeds from mature cones with total damage and proportion of seeds with damage type I and II, and iii) number of extractable seeds per cone. Type III damage were rare and analyzed with Man-Whitney test. Tested factors were the plot treatment and type of bag and its interaction. A separate analysis was done to test the factor year. The Wald Chi-Square ($W \text{ Chi}^2$) was used to test the effect of the factors in the model. Post-hoc pairwise comparisons were performed with the Least Significant Difference (LSD) test between all pairs of groups.

All statistical analyses were performed using SPSS, version 24.0 (IBM Corp., Armonk, New York) with a statistical significance level of 0.05.

Results

1. Tree physiological traits

Trees from FR plots presented longer needles and a higher total leaf area in both years. FR trees showed better vegetative growth conditions when compared with C trees (Table 1). Trees from control group also exhibited better vegetative physiological parameters in 2016 than in 2015 (Table 1).

Year	Tree treatment	Needles maximum length (cm)	Total leaf area (m ²)
2015	C	10.4 ± 0.6 a	31.6 ± 2.9 a
	FR	15.6 ± 0.6 b	55.2 ± 5.5 b
2016	C	14.2 ± 0.6 b	40.3 ± 3.7 ab
	FR	15.5 ± 0.4 b	57.3 ± 5.7 b

Table 1 - Leaf and canopy characteristics in the Control (C) and Fertirrigated plots (FR) by treatment and year (average ± standard error). Differences between average values for each tree characteristic are indicated by different letters.

2. Cone and seed production

A total of 237 last year cones were harvested in the two years with all seeds inside (20 849) being checked and categorized. In 2015, the overall average cone fresh weight was lower than in 2016, and no significant differences were found between C and FR trees in this first sampled year. By contrast, in 2016 cones from C trees were significantly heavier than cones from FR trees (Table 2). The number of seeds per cone did not differ between the two plot treatments in each year, but differences were found between the years. The heavier cones in 2016 lead to a significantly higher number of seeds per cone in that year (Table 2). In respect to the number of cones per tree, it did not differ significantly between treatments or years although in 2016 a higher number of cones was observed in FR trees (Table 2).

Year	Tree treatment	Fresh weight per cone (g)	Number of cones per tree	Number of seeds per cone
2015	C	249.4 ± 11.1 a	3.6 ± 2.7	68.1 ± 4.3 a
	FR	254.48 ± 13.2 a	2.6 ± 1.2	76.5 ± 3.7 a
2016	C	361.2 ± 11.7 b	6.2 ± 3.2	99.9 ± 4.1 b
	FR	310.0 ± 8.7 c	12.4 ± 6.1	95.9 ± 6.1 b

Table 2 - Characterization of the site trees and cones regarding production (average ± standard error) in control (C) and in fertirrigated (FR) plots. Differences between averages in each column are indicated by different letters.

3. Field trials

3.1 Exclusion trial

Cone mortality

In both years of experiment, the branches exposed to natural infestation presented mortality of second-year conelets almost six times higher than the protected ones ($W \text{ Chi}^2= 51.246$; $df =1$, $p<0.001$) (Figure 3A). Furthermore, mortality was higher in 2015 compared to 2016, regardless of plot treatment ($W \text{ Chi}^2= 13.730$; $df =3$, $p=0.003$). Overall mortality of young conelets was higher in FR trees than in C ($W \text{ Chi}^2= 4.531$; $df =1$, $p=0.033$). However, in pairwise comparisons, this difference was only significant in the exposed branches ($p=0.047$) (Figure 3B).

Mortality of mature cones (third-year cones) happened only in 2015 on exposed branches, still corresponding uniquely to 4.3% ($n=6$) of all exposed cones collected ($n=137$). Accordingly, the year and the type of bag influenced the mortality of last year cones ($W \text{ Chi}^2=36.736$, $df =1$, $p<0.001$ and $W \text{ Chi}^2=5.089$, $df=1$, $p=0.024$, respectively).

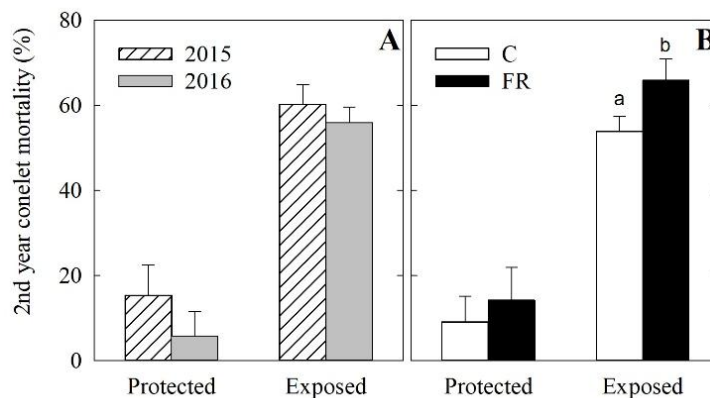


Figure 3. Mortality (\pm SE) of second-year conelets per year (A) and per plot treatment (B) in the protected and exposed branches of the exclusion trial. C- Control trees; FR – fertirrigated trees. Whenever there are significant differences ($P<0.05$) between years (A) or between treatments (B) they are indicated by different letters.

Seed damage

Exposed cones presented on average ca. 30% damaged seeds while on protected ones it was only 6% (Figure 4). Neither the protected nor the exposed cones showed differences in

the proportion of damage per cone between years. In respect to the plot treatment, FR trees always had a higher proportion of damaged seeds than C trees whether they were exposed or protected ($p < 0.001$).

Most of the damage found on cones was of type II with 20.6% and 4.6% seeds damaged in exposed and protected cones, respectively. A statistically significantly higher proportion of damage in FR trees was found when compared to C ($p < 0.001$) (Figure 5).

Nearly all the remaining damaged seeds per cone were of type I (7.1% and 0.8% of total seeds, respectively for exposed and protected cones). In respect to the plot treatment, the proportion of type I damaged seeds was similar on FR and C cones from exposed cones ($p = 0.396$) but differed in the protected ones ($p = 0.039$).

Type III damaged seeds, also known as empty seeds, were residual, corresponding to less than 2% of the total number of seeds per cone and did not differ between treatments, FR and C trees, or between protected and exposed cones (Figure 4).

In respect to the average number of extractable seeds per cone, significant differences were noted between exposed (80.4 ± 1.3) and protected cones (88.8 ± 2.3) ($p = 0.005$). When comparing the FR and C cones, although the former presented slightly lower mean values of extractable seeds, these were not significant, regardless of the type of branch.

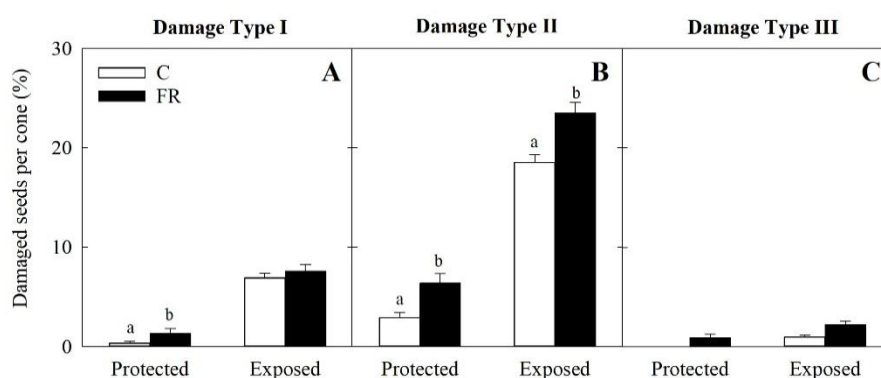


Figure 4. Mean percentage (\pm SE) seeds per cone per damage category and type of bag in each of the plot treatment in the exclusion trial. C - control trees; FR - fertirrigated. Whenever there are significant differences ($P < 0.05$) between treatments, they are indicated by different letters.

3.2 Bagged insect trial

Cone mortality

Overall mortality of second-year conelets was in average 18.4% in the bags with adults, 31.6% in the bags with nymphs and 11.7% in the exclusion bags, with only the last two

being significantly different between them ($p < 0.001$). However, we observed high variability in the conelet mortality (Figure 5).

None of the three types of bags presented different second-year conelet mortality between the two sampled years. Likewise, no significant differences were noted between FR and C cones in each type of bag (Figure 5).

We did not observe mortality of third-year cones, except two cones which died with *Diplodia pinea* and were removed from the analysis.

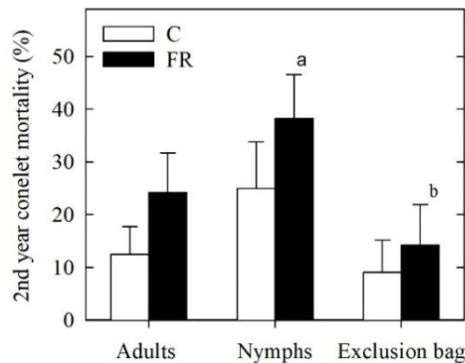


Figure 5. Average mortality (\pm SE) of second-year conelets in the two years, per plot treatment in the bagged insect trial. C- control trees; FR – fertirrigated trees.

Seed damage

The mean percentage of seeds damaged per cone was highest in the bags with adults (11.9%), followed by those containing nymphs (10.8%) and finally by exclusion bags with no bugs (5.7%). These proportions were significantly different between the exclusion bags and both types of bags with insects ($p < 0.001$) but not between these last two ones ($p = 0.374$).

Total damage per cone in the bags with adults and with nymphs was significantly higher in 2016 than in 2015 ($p < 0.001$). However, total damage in the exclusion bags was similar in both years (Figure 6).

Regarding the two plot treatments, the damage was higher on FR compared to C on bags with nymphs ($p < 0.001$) and exclusion bags ($p < 0.001$), but not on bags with adults ($p = 0.551$).

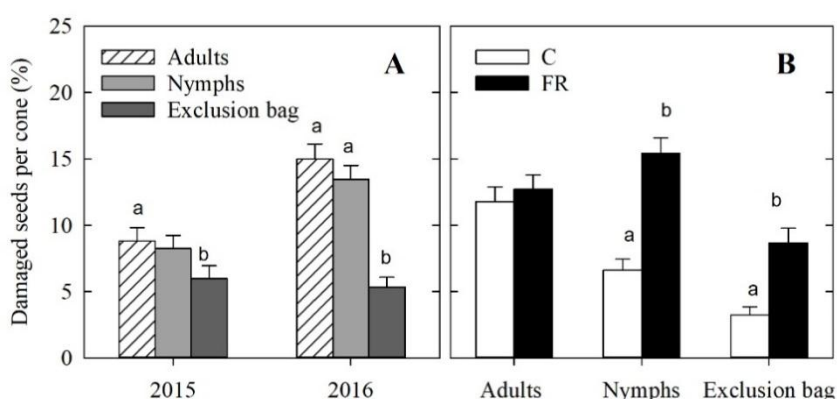


Figure 6. Mean percentage (\pm SE) of seeds damaged per cone in each year (A) and plot treatment (B) in the bagged insect trial. C - control trees; FR - fertirrigated trees. Whenever there are significant differences ($P < 0.05$) between types of branches in each sampled year or between treatments, they are indicated by different letters.

We found no differences on the number of extractable seeds per cone from the three types of bags (Adults: 89.0 ± 2.1 ; Nymphs: 88.5 ± 2.1 ; Exclusion bag: 88.8 ± 2.3). Regarding the plot treatment, none of the types of bags had different values between FR or C.

Analyzing damage seed by category, the overall average proportion of type I damaged seeds per cone was significantly different between the three types of bags ($p < 0.001$). Bags with adults had the highest proportion (6.9%), followed by bags with nymphs (4.1%) and lastly by the exclusion bags with only 0.7%. On the contrary, the average proportion of type II damaged seeds was similar between cones from all types of bags with exclusion bags presenting damage as well (Adults - 5.2%; nymphs - 5.7%; exclusion bags - 4.3%) ($p > 0.05$). Also, the proportion of type III damaged seeds in cones was not different between the three types of bags and was always below 1.5%.

Differences between damaged seed in cones from FR and C trees were observed in bags with nymphs and in exclusion bags for both type I (nymphs' bag: $p < 0.001$; exclusion bag: $p = 0.039$) and type II (nymphs' bag: $p < 0.001$; exclusion bag: $p = 0.002$) damaged seeds by cone (Figure 7). FR cones always presented greater damage (Figure 7). The average percentage of seeds with damage type III was not different between the two plot treatments in the bags with insects. The cones from FR were the only ones showing type III damages (Figure 7C).

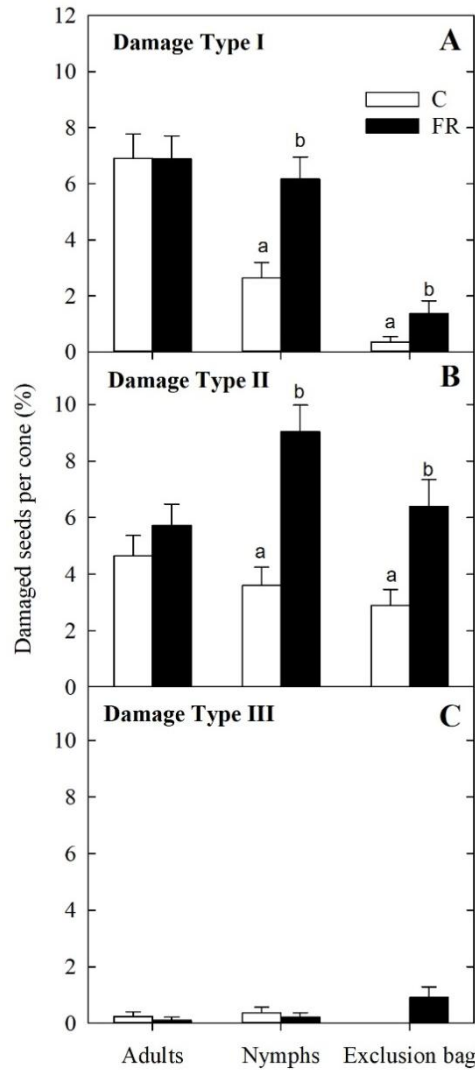


Figure 7. Percentage (\pm SE) of type I (A), type II (B), and type III (C) seeds damaged per cone in each type of bag and plot treatments in the bagged insect trial. C - control trees; FR - fertirrigated trees. Whenever there are significant differences ($P < 0.05$) between years or between treatments are indicated by different letters.

Discussion

During the two years of the experiment, we found differences in the ecophysiological performance of the trees from the FR and C plots, with fertirrigated trees showing denser canopies and a higher cone production average. However, with only two years of data and having in mind that cone production shows a very high interannual fluctuation (Calama *et al.*, 2008, 2011; Mutkle *et al.*, 2005), the conclusions regarding cone production should be

carefully analyzed. What has already been demonstrated in other studies on *P. pinea* is that water can be a limiting factor for *P. pinea* growth in some arid Iberian Peninsula regions (Natalini *et al.*, 2015) and that artificial watering can increase the induction of first-year conelets, the survival of second-year conelets, the yield in ripening cones and the area and number of leaflets (Bono & Aletà, 2013; Correia *et al.*, 2017; Loewe *et al.*, 2017; Mutke *et al.*, 2005, 2014). Our study confirms a high vigor in FR trees, but a lower survival of second-year conelets, apparently contradicting the results from other studies.

Second-year conelet mortality

Overall, the mortality rate of second-year conelets on site was very high, with each tree losing on average more than half of the cones (ca. 60%) between spring and the harvest of matured cones in December. Protecting branches with a bag reduced second-year conelets mortality rate by 83%, leading to the conclusion that biotic agents such as the insects *L. occidentalis* (Hemiptera), *Dioryctria mendacella* (Lepidoptera), the fungus *Diplodia pinea* and other unknown agents are, most certainly, involved in the considerable mortality observed in young cones of *P. pinea*.

The highest mortality of young cones was recorded in 2015. The spring of 2015, with only 84 mm of rain from February to May (tree times lower than the recorded in 2016), may have compromised the trees' physiological performance. The observed decrease in total needle length expansion and overall leaf area affected photosynthetic activity (data not shown) and therefore available carbon resources for growth. Concomitantly, it is likely that the spring drought influenced the insect's activity, along with other biotic agents, probably making them more active in a context of exacerbated tree vulnerability.

It must be stressed that the high number of aborted conelets of *P. pinea* reported here does not come as a surprise. Several Mediterranean countries have revealed alarming data on an unusually high abortion rate of unripen conelets in the last decade in a phenomenon known as the Dry Cone Syndrome (DCS) for which the cause is not yet completely understood (Mutke *et al.*, 2017a). Although *L. occidentalis* is pointed out as one of the most plausible causes, we were not able to support this hypothesis straightforwardly in this study. More research is needed to fully understand this phenomenon. Bagged branches with bugs inside showed that both adults and nymphs of *L. occidentalis* could feed on second-year conelets, but only nymphs caused significant damage in one of the sampled years. The impact of the bagged bugs on these young cones was always much lower than on the exposed ones. We must note that the second-year conelets were exposed to the insects for

only one month, whereas exposed cones were vulnerable to the seed bug for more than six months. We thus hypothesize that nymphs of *L. occidentalis* might be, in part, responsible for the mortality of conelets. These findings are following field observations where nymphs were very often spotted feeding on conelets, unlike the adults which were almost always found feeding on last year cones. The impact of adults on second-year conelets was negligible in our trials with these insects showing a preference for mature cones, at least during August, when the trial was set up. It is important to note, however, that the variability in the mortality of second-year conelets found inside the bags, including in the insect exclusion bag, was very high (around 10% in the exclusion bags) which might be due to physiological or reproductive causes which we were not able to track properly. Effectively, our data suggest that *L. occidentalis* is most certainly not the only responsible for the high mortality rate of young conelets in *P. pinea*. A multiple cause scenario combining climatic effect, tree physiological responses as well as biotic agents other than *L. occidentalis* are more plausible hypothesis according to our findings.

Other studies showed that differences in preference between young and mature cones of adults and nymphs of *L. occidentalis* are not consistent between hosts. In *Pinus contorta*, nymphs only feed on conelets if cones are not available and even then, fail to develop to the next instar (Bates *et al.*, 2002a). In *Pinus monticola*, both nymphs and adults cause considerable damage on conelets (Bates *et al.*, 2002a) and in *P. sylvestris* no difference in young cones mortality was found between bags with adults and with nymphs and control ones (Lesieur *et al.*, 2014). In addition, *L. occidentalis* also has different feeding behavior according to the season (Bates *et al.*, 2001, 2002a, 2002b; Calama *et al.*, 2017; Connelly & Schowalter, 1991; Schowalter & Sexton, 1990; Schowalter, 1994; Strong, 2006). All reveal great adaptability of *L. occidentalis* to different hosts and climates.

In opposition, mortality of all last year cones (mature cones) analyzed summing the two sampled years was negligible (less than 5%) with these cones being much less vulnerable than second-year cones.

Seed damage

This study shows nearly one-third of damaged seeds in mature cones that were exposed. The protection of the cones inside a bag from early spring until harvest resulted in an 80% reduction (from 30% on exposed cones to 6% on protected ones) of damage per cone. Thus, suggesting that biotic agents and not physiological or environmental factors are involved in this ratio. Still, damaged seeds were also found in protected cones (5-7% of all

seeds) which we attribute to both physiological causes and biotic factors affecting the cones in the previous years of the cone development when a bag did not protect it. Studies in *P. pinea* seed orchards in Central Spain where cones protected by mesh bags were compared to unprotected cones shows very similar ratios of damage per cone in exposed (36%) and protected cones (6%) (Elvira-Recuenco *et al.*, 2016).

In addition to the higher damage in matured seeds of the exposed cones, the number of extractable seeds per cone was 10% lower in these cones when compared to the protected ones. The reduction in the total number of extractable seeds per cone has been related to *L. occidentalis* feeding of seeds not yet fully formed, of several different conifers: *Pinus monticola* (Connelly & Schowalter, 1991), *P. contorta* (Bates *et al.*, 2002b; Strong *et al.*, 2001, 2006), *Pseudotsuga menziesii* (Bates *et al.*, 2000; Schowalter & Sexton, 1990) and *P. sylvestris* and *P. nigra* (Lesieur *et al.*, 2014). Our results reveal that, most probably, the same fused seeds phenomenon happens for pine cones of *P. pinea*. Cones from the bags that had insects inside had an average of extractable seeds similar to cones that were always protected, but we only bagged insects during one month in August. Feeding by *L. occidentalis* in August, when the seed is already fully formed, does not cause fused seeds. The lower number of extractable seeds on the exposed cones presumably resulted from the feeding of the bug before the end of July, when the seed is not yet fully formed (Calama *et al.*, 2016).

When seeds were separated by damage categories, we note that type II damage (totally shrunken embryo) was the most represented, corresponding to around 80% of all the damaged seeds per cone inside the protected bags and 70% of all the damaged seeds in exposed cones. The type II damaged seeds on protected cones suggest it can be either climatic, physiological or caused by biotic agents before the settlement of the bag, that is, in the previous years of cone development. Results from the bagged insect trial support, in part, this hypothesis since cones from this trial presented a similar overall proportion of type II damaged seeds per cone whether they came from a bag with *L. occidentalis* adults or nymphs during one month or from an exclusion bag that had no insect at all. However, in 2016 when the total damage per cone was higher than in 2015, there were some differences. Cones from bags with insects, especially with nymphs, had two times more type II damaged seeds per cone than cones from the exclusion bags indicating that this bug may also be responsible for part of the seeds with this type of damage in the tested period. We admit that if the insects were allowed to feed during a more extended period, the type II damage could have been higher. In fact, this utterly shrunken seed, classified as damage type II, was observed in laboratory forced feeding studies when one or more adults of *L. occidentalis* feed over an extended period on the same mature seed (Farinha *et al.*, 2018).

However, we still do not know precisely how the seeds of *P. pinea* that have *not* yet been fertilized, that is, in the years before harvest, develop after *L. occidentalis* feed on it. Studies on the impact of *L. occidentalis* on other hosts, particularly on *P. menziesii*, have shown differentiated damage accordingly to the period when the feeding occurs (Bates *et al.*, 2001; Schowalter & Sexton, 1990).

Concerning damaged type I seeds (partially damaged kernel), the almost nil percentage observed in the protected cones, which contrasts with the consistent percentage of 7% in the exposed cones, indicates that this type of damage is due to biotic agents. The results from the bagged insect trial strongly support the hypothesis that *L. occidentalis* adults are responsible for type I damage. A study using exclusion bags hypothesized that also damage type I could be a signature of *L. occidentalis* feeding since no seeds with this damage were recorded on the cones protected from the bug (Elvira-Recuenco *et al.*, 2016). In practical terms, this means that we may use type I damage to assess damages caused by *L. occidentalis* among sites and years, being an accurate indicator of the seed bug activity.

Lastly, the proportion of seeds with damage type III (empty seeds) per cone was constantly negligible regardless if the cone was protected, fully exposed or exposed uniquely to *L. occidentalis* for one month. These results showed unambiguously that *L. occidentalis* or other biotic agents do not cause this type of damage. In fact, studies on other pines have shown that the lack of fertilization leads to an empty seed (Owens, 2006).

Host management

From a plant protection point of view, several studies showed that insects perform nonrandom foraging, being able to distinguish plant quality in a heterogeneous patch of the host (Awmack & Leather, 2002; Firempong & Zalucki, 1989; Kareiva, 1982; Santiago Lastra *et al.*, 2006). A higher total leaf area together with a higher average production of pine cones in the FR plots leaves little doubt for this study site to be considered heterogeneous about the quality of the host in the period that the field experiments occurred. It is expected that seed feeders like *L. occidentalis* choose the tree to colonize by the number and quality of the available cones. Nevertheless, the canopy should also be a critical factor as it serves as a refuge to weather conditions, to natural enemies (Chen *et al.*, 2010) and is also a source of water. In fact, whereas second-year conelets protected by a bag revealed no differences in mortality between C and FR trees, exposed ones had higher mortality rates in the FR trees. This result may indicate that a preference for FR conelets or trees by the biotic agents may be occurring. However, other factors should be considered. Bagging *L.*

occidentalis for one month in mid-summer revealed differences only for the nymphs in FR trees with a mortality of second-year conelets 63% higher in nymphs' bags compared to the insect exclusion bags. Mortality in the bags with adults was not significantly different from the exclusion bags whether it was FR or C trees suggesting that adults had a similar feeding rate in both types of trees.

In the matured cones, the number of extractable seeds per cone did not vary between the two plot treatments in any of the trials. However, looking at the proportion of damaged seeds per cone, we could see that the influence of the plot treatment was noteworthy. Both exposed and protected cones from FR trees had a higher damage percentage (more 22% and 67%, respectively) than cones from C trees. This result suggests that the cause for such increase should be related with the management regime although we cannot identify which particular factors or processes are involved in it.

This same pattern of higher damage on FR cones was observed in the bags with nymphs of *L. occidentalis* but not on adults. We hypothesize that for the nymphs, the cone and seed wall tissue on FR trees are more suitable for piercing and feeding, with nymphs being more demanding for nutritional and water food requirements for growth, whereas an adult would not be so sensitive to food quality at this level. However, for detailed and proven knowledge of such an effect, more experimental work is needed.

Furthermore, cones from the nymphs' bags had a much higher proportion of type II and type I seeds than the exclusion cones which indicates that nymphs were responsible for part of these damage types.

Finally, type III damaged seeds (empty seeds) appear in similar proportions in cones from C and FR, which reinforces that causes are probably physiologic and ecologic and not biotic.

Conclusions

The production of the edible seed of *P. pinea* is now facing a challenging reality with the rate of production falling. Researchers are struggling to understand the impacts of *L. occidentalis* and its relation to the Dry Cone Syndrome. Our study shows that type I damage is a signature of *L. occidentalis* and therefore can be used in future studies to assess its impact on seed damage. However, seed type II damage must also be partly attributed to *L. occidentalis* feeding in mid-summer. Moreover, this bug may also have a significant impact on the number of extractable seeds per cone when feeding of seeds not yet fully formed (before late July) causing them to fuse to the seed coat. Hence, inferring the impact of this insect using just type I damage is underestimating its real impact.

The increasing market demand for this seed has been leading producers to invest in management programs using artificial watering and fertilization in an attempt to increase production. The present study is a first attempt to evaluate the impact of *L. occidentalis* on young and ripen cones in trees that are fertirrigated compared with trees growing in natural conditions. In general, fertirrigated trees were more susceptible to seed damage and cone mortality, which appear to be driven by biotic agents. It is possible that these trees are more attractive to the seed bug *L. occidentalis*, as well as to other seed and cone pests, like *D. mendacella*. In the particular case of the seed bug, nymphs demonstrate a higher feeding activity on fertirrigated cones compared to control ones, when forced to feed inside a bag.

Overall, we found that the activity of two adult insects in one month in mid-summer could contribute to as much as 6% of seed damage. Still, efficient field monitoring techniques are needed to determine an economic threshold density to *L. occidentalis* in this host. The high price of *P. pinea* seed implies that this threshold will be low, thus justifying prophylactic management.

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CHAPTER 6

How did the seed bug get here?



In preparation

Chapter 6 - Another chapter in the history of the European invasion by the western conifer seed bug, *Leptoglossus occidentalis*: The Iberian Peninsula

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Abstract

The seed bug, *Leptoglossus occidentalis*, a native from North America is considered a significant pest in several European countries since its first record in Italy in 1999. In Spain and Portugal, it was first observed in 2003 and 2010, respectively, and its impact on seeds (nuts) of Stone Pine (*Pinus pinea*) is of major concern. Before trying to develop control measures for this insect pest, it is paramount to clarify its invasion dynamics. Therefore, our aims were to (a) characterize the genetic structure, diversity and (b) invasion pathways of *L. occidentalis* populations in the Iberian Peninsula.

Specimens of *L. occidentalis* were collected in 13 regions covering most of the Iberian Peninsula. The genetic analysis followed a multi-marker strategy using both mtDNA (Cytb) and microsatellites (11 loci).

Our genetic results combined with the dates of first records strongly supported a stratified expansion for the invasion process of *L. occidentalis* in the Iberian Peninsula. At least three independent introductions have probably occurred: one in the Barcelona area, one in the Valencia area or further South (possibly in Almeria) and another one in the North of the peninsula.

Keywords: stratified dispersal, Iberian Peninsula, Stone pine, pine nut, seed feeder, Hemiptera

Introduction

Invasive insect species are often cited as drivers of biodiversity loss (Ricciardi, 2003; Clavero & García-Berthou, 2005; Che *et al.*, 2006; Didham *et al.*, 2007; Kenis *et al.*, 2009), as well as responsible for significant economic losses (Schaefer & Panizzi 2000), and they can largely change the dynamics and functions of the invaded ecosystems.

An invasive hemipteran, *Leptoglossus occidentalis* Heidemann, 1910 (Hemiptera: Coreidae), is originating from western North America. This polyphagous seed feeder is preying seeds in developing and mature cones of several species of conifers in genus *Picea*, *Pinus*, *Cedrus*, and *Abies* (Lesieur *et al.*, 2014). It was first spotted in Europe in Italy in 1999 (Bernardinelli & Zandigiacomo 2001; Taylor *et al.*, 2001), and then it invaded quite the entire continent during the following decade (Lesieur *et al.*, 2018).

Historical and observational data on the spread of invasive populations are often sparse but complementation with genetic data can result in important insights into the sources, routes and mechanisms of the invasion process.

When entering Europe, *L. occidentalis* faced new conifer species with regard to those of its native Eastern North America range. Thus, Stone pine, *Pinus pinea*, is a rather recent host of *L. occidentalis* but several observational records already exist over the Mediterranean basin where this pine is distributed (Roversi *et al.*, 2009; Hizal, 2012; Bracalini *et al.*, 2015; Calama *et al.*, 2016, 2017, Farinha *et al.*, 2018b). Besides its ecological and landscape value, stone pine has an important economic value due to its edible seeds (pine nuts) which can reach retail prices around 100 € per Kg (Mutke *et al.*, 2014). Almost 70% of the world *P. pinea* plantations are located within the Iberian Peninsula (Portugal and Spain), making this region one of the major producers of this valuable seed (EUFORGEN 2009). It is not surprising, therefore that almost all the impact and ecological studies of this bug in the Iberian peninsula are focused on the host pine *P. pinea* (Bracalini *et al.*, 2015, Mutke & Calama, 2016; Calama *et al.*, 2017; Farinha *et al.*, 2018a, 2018b).

Inferring invasion routes and source populations of invasive alien species is a first key stage to decipher its population dynamics and ecological characteristic in the new environment with obvious practical applications for designing and implementing control measures (Handley *et al.*, 2011), and for better prevention. However, tracing the invasion routes of this bug is a rather complex task primarily due to the large flight capacities of the insect, which allows a rapid dispersal (Lesieur, 2014), and to its autumnal aggregation behavior during which populations are mixed. This aggregation usually takes place in man-made structures or under the bark of coniferous trees, which can be translocated far away with wood trade between countries (Lesieur, 2014). This may lead to a genetic homogenization of the populations, making it difficult reconstructing the history of the invasion (Boubou *et al.*, 2012). In addition, the difficulties of sampling small populations typical from recent introductions further complicate the historical reconstructions. Indirect methods such as molecular markers have proven to be relevant in helping to decipher invasion routes in many species (Estoup & Guillemaud 2010; Fitzpatrick *et al.*, 2012).

Using such molecular markers, Lesieur *et al.* (2018) showed that the bug invasion to Europe included two successive phases. The insect first moved from its native range in western North America to invade Eastern North America, resulting in a strong decrease in genetic diversity. Then, the insect invaded Europe, and the European invasive populations were more genetically related to those of the primary invaded region, Eastern North America, rather than to those of the native range in a phenomenon known as 'bridgehead effect' (Figure 1). Lesieur *et al.* (2018) results also suggested that the European invasion proceeded from several different introductions originating from the Eastern part of the Eastern North America continent. Moreover, translocations of individuals within the European continent were also suggested in a global scenario of bug invasions in Europe (Lesieur *et al.*, 2018) (Figure 2). Biological traits of the bug such as a strong flight capacity and its polyphagous habits may have significantly accelerated the expansion. Regarding the Iberian Peninsula, Lesieur *et al.* (2018) presented evidence for two additional introductions in Spain, one in Barcelona most probably originating from Eastern North America, and one in Valencia possibly corresponding to an admixture between invasive populations from Europe and Eastern North America (Figure 2). Nevertheless, the invasion scenario in the entire Iberian

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Peninsula still needs to be largely precised with regard to the high economic value of Stone pine nuts, the main attacked host in this region.

Deciphering the origin of the Iberian populations from the first Spanish records in Barcelona and Valencia as well as the invasion routes in the Iberian Peninsula may contribute not only to find susceptible points of entry of insect in the Peninsula but also to understand the insect dispersal dynamics, leading this way to a more efficient monitoring and control strategy in the future.

This study aimed at understanding the spatial and temporal invasion patterns of *L. occidentalis* in the Iberian Peninsula, relying on the genetic study of Lesieur *et al.* (2018) which raised important issues concerning this region that needs to be answered. The following questions were thus addressed: (i) does the genetic and observational evidence suggest more than two introductions events in Iberian Peninsula? (ii) Does it support natural and continuous expansion? Or (iii) does it indicate stratified dispersal, which involves both local diffusion and long-distance translocations?

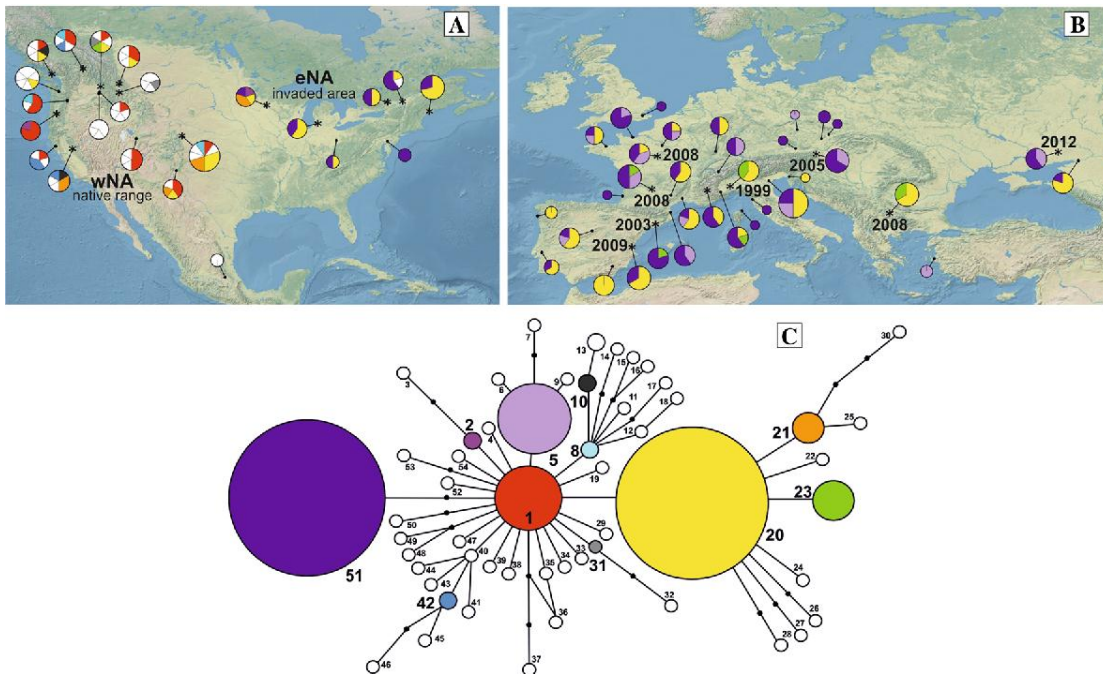


Figure 1. Geographic distribution of *L. occidentalis* mitochondrial haplotypes in (A) North America, (B) Europe and (C) haplotype network in Lesieur *et al.*, 2018. Single haplotype or haplotype found in a single site are represented in white.

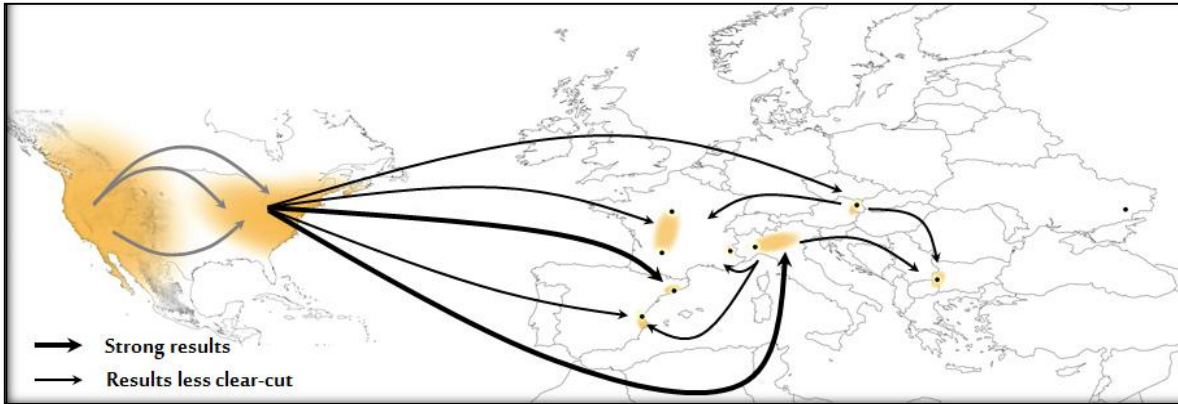


Figure 2. Most probable scenario of the European invasions by *L. occidentalis* in Lesieur *et al.*, 2018.

Methods

Sampling and DNA extractions

Samples were collected between 2011 and 2015 from 14 sites distributed all over the Iberian Peninsula (Table 1). The different dates of collection were due to the insect population dynamics, in which population densities are highly fluctuating from one year to the next. Whenever possible, the insects were collected on different trees and sites within a region in order to minimize consanguinity. However, it has been possible to capture only a few individuals in southern Portugal (Faro), but none were obtained from the far south of Spain (Huelva, Cadiz), where we faced very low population densities during the years of collection. Insects were collected manually and immediately put into individual vials with absolute ethanol. A total of 239 bugs were thus obtained (233 adults, 6 nymphs).

DNA was extracted from one or two legs of adults following procedures from the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and eluted in 200 µL of AE buffer. The numbers of individuals per population used for sequencing (mtDNA) and genotyping (microsatellites) are presented in Table 1.

Molecular analysis (mit DNA)

In 60 individuals from 14 populations, a segment of cytochrome b was amplified by polymerase chain reaction (PCR) using the PCR reaction mix and procedure described in Lesieur *et al.*, (2018). All PCR products were purified with QIA - quick PCR purification kit (Qiagen) and sequenced with the amplification primers. Sequencing was performed using the BigDye terminator sequencing kit (Applied Biosystems) and carried out with an ABI Prism 3500 Genetic Analyzer (Applied Biosystems). All sequences were obtained in the forward and reverse directions, assembled using CodonCode Aligner V.3.7.1 (www.codoncode.com) and then aligned using CLUSTAL W (Thompson *et al.*, 1994) as implemented in BioEdit 7.05 (<http://www.mbio.ncsu.edu/bioEdit/bioedit.html>). All sequences were truncated to the same length (662 bp) not having observed any insertion, deletion or stop codons. Stop codon were checked with MEGA v.6 (Kumar *et al.*, 2008).

To infer the genetic diversity and invasion routes of *L. occidentalis* populations in the Iberian Peninsula sequences from Lesieur *et al.*, (2018) (n=48) were downloaded from GenBank and added to the dataset (n=60) for a joint analysis (n_{total}=108) (Table 1 - populations in bold typeface). Data from Lesieur *et al.* (2018) included two populations from Eastern North America (Montreal and Pittston): the source of the European invasion was downloaded to check if there were additional introductions from Eastern North America to the Iberian Peninsula. We also added data from four populations representative of invaded areas in Western Europe (Yvoy-le-Marron in north Central France, Lavercaillère in Central France, Serre-Ponçon in the Southern French Alps, and Alessandria in Northern Italy) that could be secondary insect sources for new introductions in the Iberian Peninsula due to geographic proximity and also based on the history of invasion of other insects (Lopez *et al.*, 2011; Grosso-Silva & Maia, 2012; Salvatore, 2013). Lastly, data of the populations suggested as new introductions in the

Iberian Peninsula (Barcelona and Valencia) were also added to the dataset. For mtDNA analysis three extra bugs from Coruche were added from the Lesieur's samples.

The number of haplotypes, haplotype diversity, and nucleotide diversity was obtained using *DNASP* 5.0 (Librado & Rozas, 2009). Statistical parsimony network was computed with TCS v. 1.21 (Clement *et al.*, 2000). To evaluate the variation in genetic diversity at different hierarchical levels and between different groups of populations, we used the analysis of molecular variation (AMOVA; Excoffier *et al.*, 1992). We tested several groups of populations suggested by F_{ST} values, Principal Component Analysis and Structure analysis to find the group configuration that maximize the global value of F_{CT} (variation among groups) and is significantly different from random distributions. Populations with very few sequenced individuals were not used in the analysis (Murcia, Islas Cies, and Huelva in Spain and Faro in Portugal). All AMOVAs were performed with Arlequin v 3.5 (Excoffier *et al.*, 2010) and tested with 1000 permutations. The program Arlequin was also used to estimate F_{st} values between all pairs of populations in Europe.

Molecular analysis (microsatellites)

Data analysis: The whole dataset included 374 individuals from 17 populations: 195 individuals from 8 populations from Lesieur *et al.* (2018) (Table 1 - populations in bold typeface) and 179 individuals from 9 populations corresponding to new Iberian samples (Table 1). Data from Lesieur *et al.* (2018) included sequences of individuals from 2 populations of Eastern North America, and 3 populations from France, 1 from Italy and 2 from the Iberian Peninsula. To be able to compare the two datasets, we used samples already analyzed by Lesieur *et al.* (2018) as controls in our genotyping procedure.

All individuals were genotyped at 11 microsatellites loci (Lep04, Lep05, Lep07, Lep16, Lep17, Lep25, Lep31, Lep36, Lep43, MSLO07 and MSLO15) previously developed for *L. occidentalis* (Lesieur *et al.*, 2014). PCR amplifications were conducted following the protocol described by Lesieur *et al.*, (2014) and PCR products were run in an ABI 3500 Genetic Analyzer using the size standard GeneScan-600 LIZ (Applied Biosystems). The program GeneMapper v. 4.1 (Applied Biosystems) was then used to score the alleles.

Allelic richness (AR) and frequencies, as well as observed and expected heterozygosity (H_o and H_e), was calculated for each locus and population using GENALEX 6.41 (Peakall & Smouse, 2006). The richness of private allele (PAR) was calculated in HP-RARE using the rarefaction procedure (Kalinowski, 2005) which compensates for the increase in the likelihood of sampling rare alleles as sample size increases. Hardy-Weinberg equilibrium was tested using GENALEX 6.41 (Peakall & Smouse, 2006) for each locus and population, with 1000 permutation steps and 100 000 steps in the Markov chain. Linkage disequilibrium was tested in each population for all pairs of loci with 10 000 permutations using ARLEQUIN 3.1 (Excoffier *et al.*, 2005). Sequential Bonferroni corrections (Rice, 1989) for multiple comparisons were applied for both tests. FREENA package (Chapuis & Estoup, 2007) was used to estimate the null allele (NA) for each locus in each population using the expectation-maximization algorithm.

Population genetic structure was first analyzed by calculating pairwise F_{st} estimates as described by Weir & Cockerham (1984) with and without the null alleles (ENA) correction implemented in FREENA. The 95% confidence intervals were obtained by bootstrapping 1000 times over loci. A population-based neighbor-joining (NJ) tree was reconstructed using Populations 1.2.30 software (Olivier Langella, <http://bioinformatics.org/~tryphon/populations/>) using Cavalli-Sforza and Edwards chord distance on the genotype dataset corrected for null alleles. Bootstrap values were computed by resampling loci and are given as a percentage of 2000 replicates. Both the pairwise F_{ST} and the phylogenetic trees of populations were assessed after exclusion of the two populations for which the sampling size was insufficient, namely those from "Caceres" and "Faro." After, we used the program STRUCTURE 2.3.1 (Pritchard *et al.*, 2000) to estimate the number of genetic clusters represented in the data set and, in this way, try to disentangle the genetic structure over the sampled area. We used 200 000 burn-in steps followed by 500 000 MCMC simulation steps with a model allowing admixture in all runs. To ensure the consistency of results, 20 independent runs for each value of K were performed. This analysis was first run on the whole dataset (17 populations) with the number of clusters (K) varying from 1 to 10. It was then run on subsets of the data containing (1) all the European populations (15 populations) and (2) only the Iberian populations (11 populations), with K ranging from 1 to 8 in both runs (see Results). The optimal number of clusters (K) represented by the data was determined with the method described in Evanno *et al.* (2005),

implemented in STRUCTURE HARVESTER (Earl & vonHoldt, 2012). We also performed assignment tests using all genotyped individuals, i.e., also including the populations with low sample size.

Results

Molecular analysis (mit DNA)

In total, sequences from 108 individuals (60 sampled in the field and 48 downloaded from GeneBank) distributed in 22 sites were analyzed. Only four haplotypes were detected in these European populations (Figure 2). The most frequent haplotypes were H20 and H51 which were observed in 51% and 35% of the individuals, respectively (Table 1). However, different relative haplotype frequencies were found, the haplotype H20 being more frequent in Iberia whilst H51 was more frequent in the nearby French populations. H05 and H23 presented frequencies below 10% with H23, being only observed in Iberian individuals. One haplotype, H27, was found only in one individual from Eastern North America (Montreal, Canada). The overall haplotype and nucleotide (π) diversity were estimated at 0.613 and 0.0026, respectively, with the three regions, Eastern North America, France+Italy and Iberia presenting similar mean values. Five populations from the Iberian Peninsula had a haplotype diversity of 0 (table 1).

To estimate population differentiation, several groups of populations were tested with AMOVA analysis based on the geographic distance between populations, dates of occurrence and the results from F_{st} values and Structure analysis of microsatellites data. When considering the whole dataset, the group configuration that maximized the FCT value was separating the dataset into three groups: [Eastern North America, Barcelona, Lleida], [France, Italy, Northern Iberia] and [Southern and Central Iberia] (Table x). Considering only Iberia, none of the group configurations result in a significant structure ($p < 0.05$). Interestingly, the configuration that explained the highest percentage of variation among groups was defined by occurrence dates (13.09%).

In all groups tested, most of the genetic variation was explained by differences between individuals within populations (always $> 86\%$).

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Table 1. Sample information including: Region, site, host species, sample size, haplotype frequency, nucleotide frequency (n), mean expected heterozygosity (He), mean observed heterozygosity (Ho), allelic richness adjusted to a minimum sample size of 6 (AR) and private allelic richness (PAR) estimated after a rarefaction procedure. Bold correspond to samples downloaded from internet databases (Lesieur *et al.*, 2018).

Origin	Region	Latitude	Longitude	Year of collection	Host	mtDNA				Microsatellites				
						N	Haplotype	Hd	π	N	He	Ho	AR	PAR
Eastern America	Montreal, Canada	45,562	-73,563	2011		5	H20 (1); H51 (3); H27 (1)	0,700	0,004	14	0,540	0,519	2,88	0,636
	Pittston, USA	44,222	-69,756	2011		7	H20 (5); H51 (2)	0,536	0,002	25	0,568	0,524	2,88	0,909
Europe	Yvoy-le-Marron, France	47,632	1,854	2012		5	H5(2); H20 (1); H51 (2)	0,800	0,003	31	0,623	0,508	2,96	0,091
	Lavercantière, France	44,637	1,318	2011		7	H5(2); H20 (1); H51 (4)	0,667	0,003	30	0,606	0,547	2,91	0,182
	Serre-Ponçon, France	44,523	6,332	2011		5	H20 (2); H51 (3)	0,600	0,003	29	0,571	0,468	2,89	0,000
	Alessandria, Italy	44,897	8,406	2011		5	H20 (3); H51 (2)	0,600	0,003	26	0,603	0,531	2,91	0,182
Iberian Peninsula	Barcelona, Spain*	41,520	1,687	2012		5	H23 (1); H51 (4)	0,400	0,002	20	0,491	0,486	2,48	0,091
	Valencia, Spain*	39,447	-0,463	2012		6	H20 (4); H51 (2)	0,533	0,002	20	0,487	0,385	2,51	0,000
	Almeria, Spain	37,712	-2,172	2012		8	H20 (8)			14	0,386	0,392	2,08	0,000
	Burgos, Spain	42,672	-3,417	2015	<i>P. pinaster</i>	3	H20 (1); H51 (2)	0,667	0,003	25	0,576	0,475	2,88	0,000
	Galicia, Spain	42,364	-8,622	2015	<i>P. pinaster</i>	4	H5 (1); H23 (1); H51 (2)	0,833	0,004	30	0,607	0,528	2,96	0,000
	Lleida, Spain	42,637	1,247	2013		5	H20 (2); H23 (1); H51 (2)	0,800	0,003	20	0,603	0,468	2,97	0,000
	Cáceres, Spain	40,451	-6,19	2015	<i>P. pinea</i>	2	H5 (1); H20 (1)	1,000	0,003					
	Segovia, Spain	40,902	-4,007	2012		11	H5 (2); H20 (6); H51 (3)	0,733	0,003	14	0,564	0,410	2,84	0,000
	Soria, Spain	41,536	0,023	2013		3	H20 (2); H51 (1)	0,667	0,003	12	0,564	0,410	2,55	0,182
	Valladolid, Spain	41,304	-5,233	2015	<i>P. pinea</i>	6	H20 (5); H23 (1)	0,333	0,001	14	0,555	0,413	2,77	0,000
	Murcia, Spain	37,826	0,016	2013		3	H20 (3)							
	Islas Cies	44,897	8,406	2012		2	H20 (2)							
	Huelva, Spain	37,211	-6,563	2012	<i>P. pinea</i>	1	H20 (1)							
	Aveiro, Portugal	40,674	-8,727	2015	<i>P. pinaster</i>	3	H20 (2); H51 (1)	0,667	0,003	23	0,596	0,437	2,96	0,000
	Coruche, Portugal	38,961	-8,527	2015	<i>P. pinea</i>	5+3	H5 (1); H20 (3+2); H51 (1+1)	0,700	0,003	27	0,615	0,506	3,02	0,182
	Faro, Portugal	37,236	-7,922	2015	<i>P. pinaster</i>	4	H51 (4)							

Molecular analysis (microsatellites)

In total, we analyzed microsatellites data of 374 individuals from 17 populations (Table 1). The analysis on linkage disequilibrium revealed five cases of significant disequilibrium in the 605 pairwise tests carried out, but a given pair of loci was never in significant linkage disequilibrium more than two times. Thus, the eleven microsatellite markers were considered independent. The average proportion of null allele for each locus was below 5% except for four loci (Lep04, Lep05, Lep31 and Lep36) which showed a mean estimated proportion of null alleles above 8 %. All populations were in Hardy-Weinberg equilibrium for all loci after the Bonferroni correction for multiple comparisons except in 8 out of 99 combinations of population x locus (Lep04 in Lleida, Galiza, Aveiro and Coruche; Lep36 in Segovia and Aveiro; MSLO07 in Lleida and MSLO15 in Coruche). However, in global tests across all loci, no population deviated significantly from HWE. Some of the high rates in null allele's frequencies namely in Lep04 and Lep36 loci, corresponded to deviations from Hardy-Weinberg equilibrium. To ensure more robust and unbiased results, the following analysis was performed with and without the loci Lep04 and Lep36. As the results were similar in both cases, only the results with the entire dataset are presented.

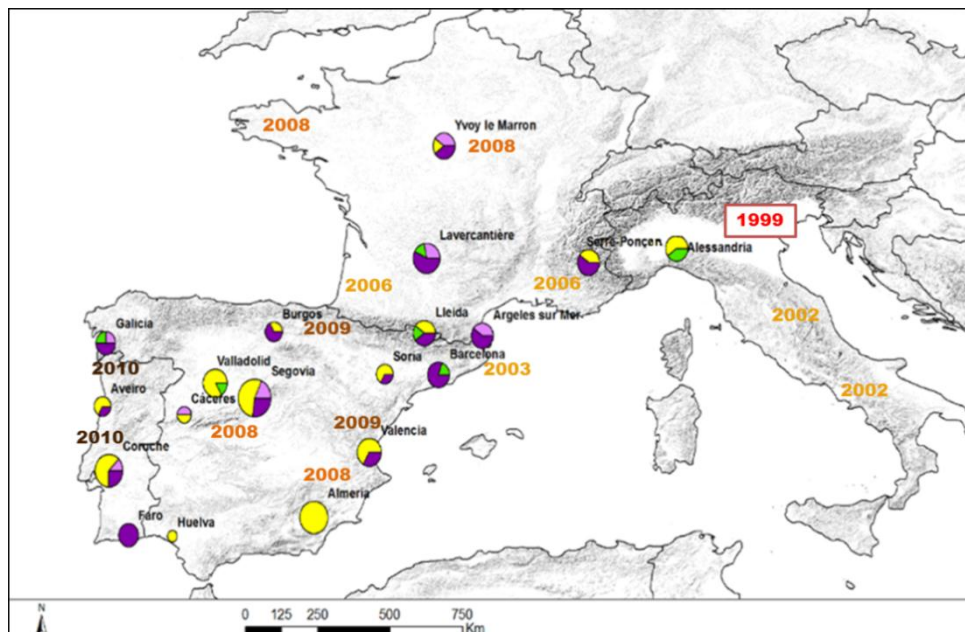


Figure 2. Geographic distribution of *Leptoglossus occidentalis* mitochondrial haplotypes and Iberia, France and Italy first record dates.

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Table 2. Analysis of molecular variance (AMOVA) of *Leptoglossus occidentalis* populations from Eastern North America, France+Italy and Iberian Peninsula groups according to different criteria.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	p-value
AMOVA - Global dataset grouped by Structure results					
Among groups	2	8.172	0.10898	12.28*	0.01466
Among populations within groups	14	10.542	0.00575	0.65	0.52395
Within populations	77	60.361	0.78390	88.36	0.07234
AMOVA - European populations grouped by Structure results					
Among groups	3	9.742	0.13644	15.49*	0.01075
Among populations within groups	11	7.480	0.01496	1.70	0.54252
Within populations	67	50.875	0.75933	86.21	0.05474
AMOVA - Iberian populations grouped by geographic proximity					
Among groups	2	4.185	0.08423	9.96	0.07136
Among populations within groups	9	6.243	0.02079	2.46	0.58553
Within populations	40	31.283	0.78208	92.50	0.27273
AMOVA - Iberian populations grouped by date of first detection					
Among groups	4	6.484	0.10968	13.09	0.06647
Among populations within groups	7	3.944	0.05353	6.39	0.71750
Within populations	40	31.283	0.78208	93.30	0.26491
AMOVA - Iberian populations grouped by Structure results					
Among groups	2	4.571	0.10379	12.14	0.06354
Among populations within groups	9	5.857	0.03103	3.63	0.60704
Within populations	40	31.283	0.78208	91.49	0.28152

The estimated genetic variability across the 11 loci and for each population is summarized in Table 1. The observed and the expected heterozygosity (H_o and H_e) ranged from 0.478 to 0.623 in the Eastern American and French populations and from 0.385 to 0.615 in the Iberian populations. A total of 97 alleles were detected across all eleven loci, with 4 alleles at the Lep04, Lep05 and Lep07 loci to 20 alleles at locus Lep43. The parameter allelic richness (AR) corrected for 6 individuals per sample was employed to compare among populations. Allele richness was slightly higher in the invading European populations from France (2.92 alleles per locus) than in those of Eastern North America (2.88). Populations from the Iberian Peninsula presented the lowest and the highest allele richness in all dataset, in Almeria (2.08) in Coruche (3.02), respectively.

The matrix of F_{st} values estimated using the ENA correction is given in Table 2. Pairwise genetic differentiation between all the European populations and Pittston in Eastern North America was lower than with Montreal also from Eastern North America, with no European population presenting significant differences with Pittston except for Almeria. In fact, Almeria was the most differentiated population, presenting high and significant F_{st} values with all populations in Eastern North America, France, Italy and with the two Spanish populations of Barcelona and Lleida.

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Considering only the Iberian Peninsula samples, Barcelona and Almeria presented the highest F_{st} indices (0.164 and 0.99, respectively) with all other population's pairwise comparisons obtaining no significant F_{st} values that ranged from 0.013 to 0.079. Overall, the highest F_{ST} value was observed between the sample Montreal of Eastern North America and the Spanish sample of Almeria (0.194). The phylogenetic tree of populations showed a differentiation of Almeria from all other populations, which is consistent with high F_{st} values between these sample site and all others. The french populations formed a monophyletic clade whereas those of Eastern North America and Barcelona formed another one.

Table 2. Pairwise F_{st} divergence between populations of *L. occidentalis*. Populations with less than 12 individuals were excluded.

Populations	E America		France			Italy	Spain							Portugal		
	Mont	Pitt	Yvoy	Lave	SePo	Ales	Barc	Llei	Vale	Alme	Sego	Vall	Burg	Gali	Avei	Coru
Mont	0,000															
Pitt	0,037	0,000														
Yvoy	0,066	0,030	0,000													
Lave	0,067	0,031	0,009	0,000												
SePo	0,060	0,029	0,018	0,021	0,000											
Ales	0,058	0,028	0,025	0,024	0,030	0,000										
Barc	0,055	0,041	0,071	0,066	0,049	0,067	0,000									
Llei	0,087	0,036	0,026	0,036	0,034	0,045	0,056	0,000								
Vale	0,081	0,036	0,067	0,071	0,064	0,056	0,086	0,064	0,000							
Alme	0,194	0,125	0,128	0,151	0,121	0,146	0,164	0,098	0,121	0,000						
Sego	0,094	0,041	0,031	0,040	0,032	0,055	0,080	0,025	0,053	0,079	0,000					
Vall	0,126	0,078	0,052	0,068	0,054	0,078	0,111	0,045	0,079	0,060	0,031	0,000				
Burg	0,088	0,040	0,023	0,030	0,026	0,040	0,077	0,028	0,044	0,095	0,014	0,036	0,000			
Gali	0,095	0,042	0,020	0,026	0,022	0,042	0,079	0,025	0,059	0,099	0,020	0,036	0,013	0,000		
Avei	0,083	0,036	0,024	0,031	0,024	0,033	0,077	0,028	0,050	0,091	0,020	0,045	0,017	0,014	0,000	
Coru	0,097	0,043	0,021	0,029	0,031	0,036	0,088	0,024	0,064	0,095	0,026	0,038	0,024	0,021	0,016	0,000

Principal components analysis (PCA) gave similar results except for the population of Valencia which appeared closer to the Eastern North America/Barcelona clade than to all the other samples (Figure 3).

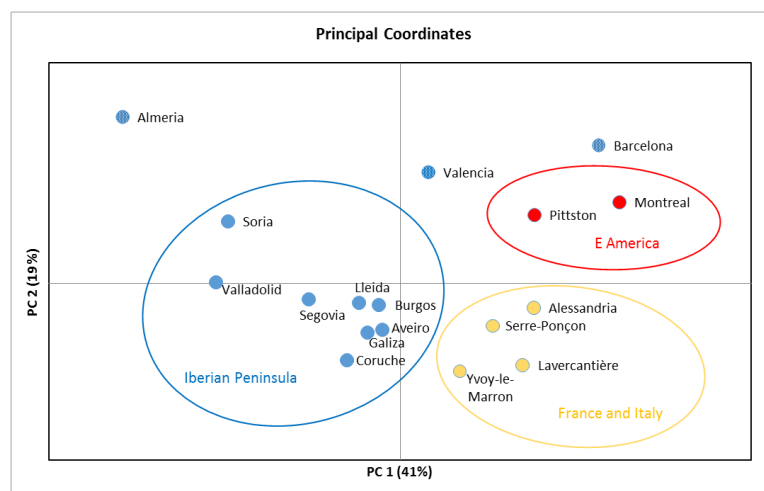


Figure 3. Principal component analysis diagram.

The STRUCTURE analysis using the Evanno’s method showed a distinctive peak at $K=3$ and a smaller one at $K=7$ for the global dataset (Figure 4).

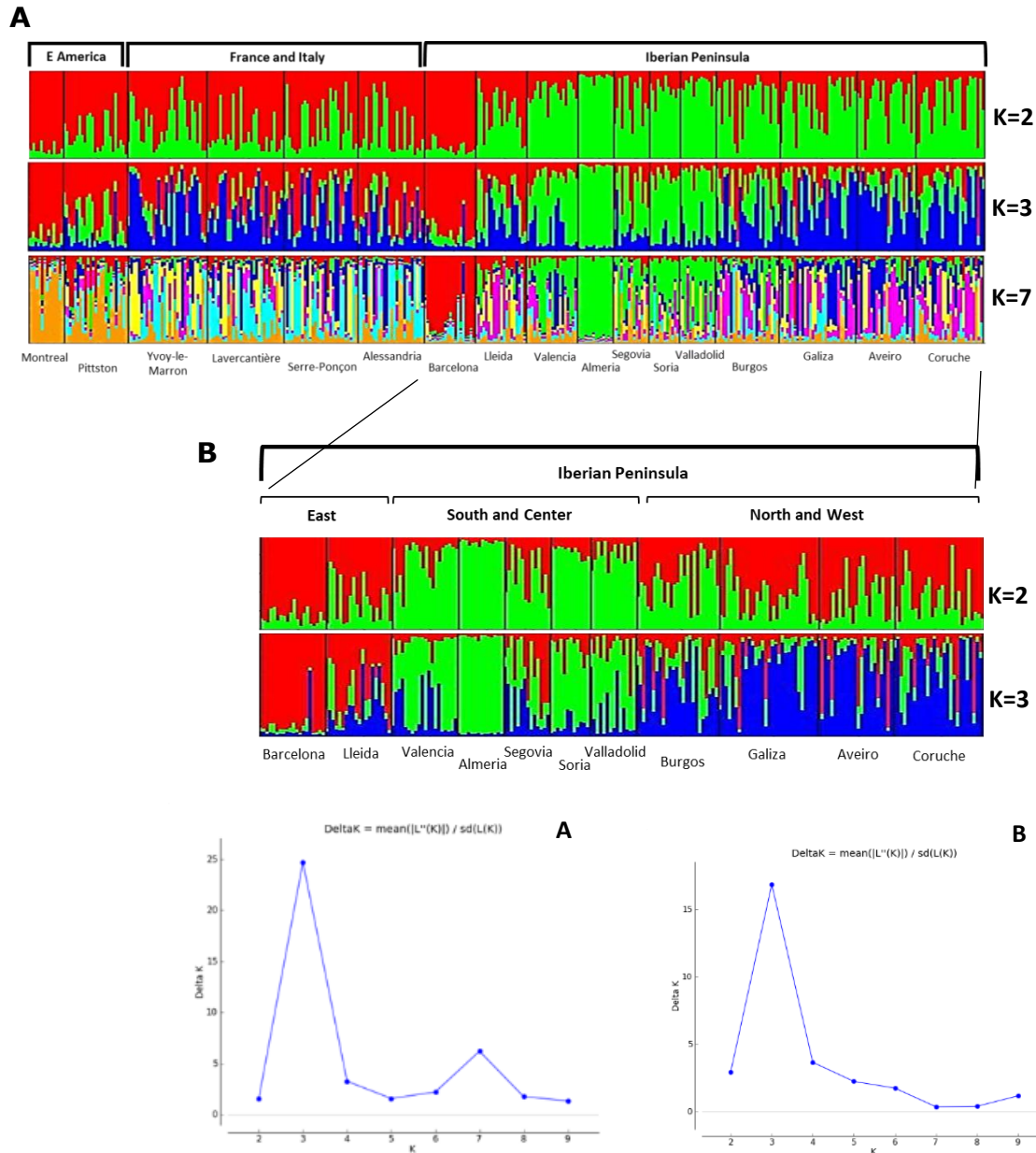


Figure 4. Graphical representations of genetic clusters for samples from (A) the global dataset and (B) the Iberian Peninsula inferred from STRUCTURE simulations, with the corresponding Evanno’s DeltaK. Each vertical line represents an individual, and each color represents a cluster. Individuals are grouped by sample location.

For $K=3$, two of the clusters were more defined than the third one. The two clusters were [Eastern North America, Barcelona and Alessandria] and [southern and central parts of the

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Iberian Peninsula]. The third cluster was formed by the French populations and those of northern and western Iberia. Lavercaitière (south-central France) and Lleida (north-eastern Spain) were intermediate between clusters. For $K=7$, southern and central Iberia remained a separated group.

The same method was used to analyze only the individuals from the Iberian Peninsula. The highest ΔK was $K=3$. The three clusters corresponded to populations from (1) the Eastern, Southern and Central parts, (2) the Northern region, and (3) the Western region of the Peninsula.

Restriction of the Bayesian clustering analysis to the nine loci with low proportion of null alleles had no qualitative effect on the results obtained and the use of other Structure models (with or without correlated allele frequencies or sampling location information) gave similar results (data not shown).

Discussion

By combining occurrence records with mtDNA and microsatellites data we characterized the invasion dynamics of *L. occidentalis* in the Iberian Peninsula. The former genetic study by Lesieur *et al.* (2018) presented evidence for the occurrence of two independent introductions in the Iberian Peninsula (Barcelona and Valencia) corresponding to the only two Iberian populations analyzed by them. We found evidence for, at least, a third introduction in the northern or western part of the peninsula.

Diversity and Structure of Iberian populations

The observation of the same haplotypes in Iberia as in the rest of Europe suggests that the bug invasion in the Iberian Peninsula results from populations having already invaded Europe, and not from Western North America, the native region of *L. occidentalis*. However, a similar or even slightly higher haplotype and nucleotide diversity observed in Iberia with regard to those of Eastern America and European populations indicates no loss of diversity in the Peninsula. Populations of invasive alien species are traditionally thought to have reduced genetic diversity relative to their source populations due to founder effects related to small population sizes during the stage of introduction of the invasion (Dlugosch & Parker, 2008). However, several other studies of different invasion processes did not find significant loss in the genetic diversity of the invasive populations using neutral molecular markers (for e.g. Facon *et al.*, 2003; Kolbe *et al.*, 2004; Bossdorf *et al.*, 2005; Kang *et al.* 2007; Ciosi *et al.*, 2008, 2010). We should note that redoing the same studies using

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molecular markers other than neutral ones, which are irrelevant for selection and adaptation of the individuals to the new environment, could have told a different story (Handley *et al.*, 2011).

The Almeria population in southern Spain was an exception to the average genetic diversity of the Peninsula presenting very low values. All individuals had the same haplotype (H20) and the lowest values of heterozygosity and allele richness in microsatellite loci. One possible explanation is the occurrence of a strong demographic bottleneck associated with a genetic bottleneck due to environmental conditions that reduced the population to a few number of individuals. Southern Iberia regions were very difficult to sample because of low insect densities. Indeed, climatic niche models reveal that Southern Iberia is presenting a low suitability to *L. occidentalis* survival due to high temperatures (Zhu *et al.*, 2013).

Regarding the presence of genetic structure among all studied populations, AMOVA results point to structured populations between the three invaded areas: Eastern North America, France+Italy, and Iberia after grouping the populations in accordance to the results obtained for microsatellite data. However, no structure was detected within the Peninsula using mtDNA. In contrast, the analysis of microsatellite data revealed the presence of three clusters in the Iberian Peninsula, two quite well-defined (the Barcelona cluster and the one grouping populations from Southern and Central Spain) and another constituted by populations with a great level of mixture (Northern Iberia and Central Portugal). Because of the very low diversity of the Almeria population, the analysis was repeated without this population. Still, a strong southern and central Iberian group including populations from Valencia, Valladolid, Segovia and Soria was defined. Barcelona and Lleida, which are only a little over 150 km apart, grouped together in most of the analysis despite Lleida having a great admixture population. The strong differentiation of the Barcelona population from the other European populations was tentatively explained by Lesieur *et al.* (2018) as a result of a new independent introduction from Eastern North America populations. In the Iberian peninsula, Barcelona still forms a strongly differentiated cluster, occasionally grouped with Lleida, which indicates a possible spatial isolation of this population as suggested by the lower suitability of the Ebro Valley located at its left (Zhu *et al.*, 2013) and the Pyrenees mountains at its right. The fact that the first record of *L. occidentalis* in Barcelona was in 2003, long before all the other Iberian regions (between 2008 and 2010) suggests that this isolation has probably been maintained over time.

Routes of invasion in the Iberian Peninsula

If we consider only the dates of observation, the primary record of the bug was in Barcelona in 2003 (Ribes & Escolà, 2005), while all the first records in other regions of the Iberian Peninsula began 5 years later (2008) and extended over 3 years until 2010 (Ribes *et al.*, 2008; Valcárcel & Portillo, 2009; Vázquez *et al.*, 2009; Grosso-Silva, 2010; Pérez Valcárcel & Prieto Piloña, 2010). Such a large time difference between the first Iberian record and most of the other detections without intermediate occurrences raises doubts on the hypothesis of a continuous geographic dispersal from the source population of Barcelona.

Genetic data also refute this hypothesis. Lesieur *et al.* (2018) already found that Barcelona and Valencia were two independent introductions in Iberia with divergent source populations. In our study, high F_{st} values (from 0.077 to 0.164) between Barcelona and all the other Iberian populations except Lleida and the constant separate cluster for all tested K further proved that the mechanism of invasion in Iberia cannot be a natural expansion from Barcelona. A natural expansion would also mean a loss in the genetic diversity (Dlugosch & Parker, 2008; Handley *et al.*, 2011) in the other Iberian populations which was not detected in both the mtDNA and microsatellites data.

Microsatellites analysis detected three well-defined clusters in the Iberian Peninsula. One cluster formed by the populations in the eastern part (Barcelona and Lleida), another one by those of south and center of the peninsula (Valencia, Almeria, Valladolid, Segovia, Soria) and a third one by those from the northern and western part (Burgos, Galiza, Aveiro, Coruche).

Putting together first observational dates, the absence of a decrease in genetic diversity, former genetic analysis and cluster analysis on the whole Peninsula, is suggesting the occurrence of multiple introductions that could be transatlantic, from Eastern America, intracontinental from the rest of the invaded Europe, intra-Peninsular or combining all of these processes. The occurrence of multiple introductions is a common phenomenon to the history of invasion of many alien species (Fonseca *et al.*, 2000; Facon *et al.*, 2003; Kolbe *et al.*, 2004; Bossdorf *et al.*, 2005; Kang *et al.*, 2007; Roman & Darling, 2007; Ciosi *et al.*, 2008, 2010), and was already proved for the seed bug in Europe as shown by Lesieur *et al.* (2018). Stratified dispersal, i.e. a combination of short- and long-distance dispersal during geographic expansion has also been widely reported (Davies *et al.*, 2004; Bialozyt *et al.*, 2006; Facon *et al.*, 2008; Ciosi *et al.*, 2010). Note that long-distance dispersal includes primarily human-mediated transportation of insects (Handley *et al.*, 2011). This type of dispersal mechanism accelerates the invasion and may maintain genetic diversity in

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expanding populations (Davies *et al.*, 2004; Bialozyt *et al.*, 2006; Facon *et al.*, 2008; Ciosi *et al.*, 2010).

Our results showed that the population from Valencia is genetically similar to other populations of southern and central Spain (Almeria, Valladolid, Segovia, Soria; grouped together for all K), suggesting a common source population to the invasion of this part of the Peninsula. Historical data shows that the bug was first detected at the same time in the south and center of the peninsula (Almeria and Segovia) in 2008 despite the separation of more than 400 km between occurrence points. Which population corresponds to the source population is difficult to assess. However, relying on observational dates, there had to be human-mediated transportations within the region (south and center). Even with a good flight capacity (Koerber, 1963), estimated at 20 Km / day maximum in laboratory studies (Lesieur, 2014), it would probably be a too narrow period for *L. occidentalis* populations to invade all Iberia (approximately 1 000 x 800 Km) by natural and continuous dispersal. This insect flies great distances at specific periods such as when searching for winter shelter in the end of summer.

Microsatellites results also indicate a high probability for an independent introduction in the north and west of the Peninsula. The populations of Coruche, Aveiro, Galiza and Burgos had low F_{st} between them and always clustered together in the PCA and Structure analysis. The source population of north and west populations is, this way, genetically different from the source population of the south and center clade. Moreover, given that the first occurrence dates in Northern Iberia are only one year after the southern ones, a natural expansion from the South to the North would be very unlikely. Very low values of F_{st} and the joint clade of north and west Iberia with France for K=3 in the whole dataset, could suggest France to be the origin of the source population for the invasion of northern and western Iberia. In fact, pairwise F_{st} comparisons revealed very low values between the French population of north-central France, Yvoy-le-Marron, and north and west Iberian populations (0.020 to 0.024).

Cone trade market and the invasion

Leptoglossus occidentalis has been frequently spotted on Stone pine, *Pinus pinea* cones (Bracalini *et al.*, 2015; Calama *et al.*, 2016, 2017; Farinha *et al.*, 2018b). World market demand for *P. pinea* nut kernel moves several hundred million euros annually (Awan & Pettenella, 2017; Mutke *et al.*, 2017). The Iberian Peninsula produces nearly 70% of the world traded nuts (Mutke *et al.*, 2014). Cones are collected from December to April of the year after and then sold to processing plants to be open and commercialized. A high volume

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of cones is transported within and between the main producer's countries, Portugal, Spain, Italy and Turkey to be processed and sold (Mutke *et al.*, 2012). These intensive translocations of cones may easily serve as ride to *L. occidentalis* individuals from one place to another justifying in part both the rapid rate of invasion in the Peninsula and the high genetic diversity of Iberian invasive populations. In the north part of the Peninsula, where Stone pine is not distributed, the trade of timber may play an important role in dispersing the bug. Above all, *L. occidentalis* is considered a very good hitchhiker, which can come in cars, trucks or containers even not related to the cone or timber market.

Multiple introductions of individuals from genetically divergent populations results in high levels of intraspecific hybridization (i.e. "admixture") which can confer survival advantages (Facon *et al.*, 2010) and greater adaptation of the admixed individuals.

Human activities, namely the intensive cone trade, timber trade and other commercial exchanges between European countries, may therefore be the prominent responsible for long-distance *L. occidentalis* dispersal in the Iberian Peninsula. Stratified dispersal is probably affecting this pest population dynamics increasing its capacity to disperse and ultimately leading to high levels of admixture which, in turn, are likely to increase the probability of adaptation to new environments potentially increasing the invasiveness and economic impact of this pest.

Conclusion

Genetic evidence combined with first record dates suggest that stratified dispersal mechanism characterized by short-distance dispersal and long-distance primarily commanded by human-mediated is what better explain the history of *L. occidentalis* invasion in the Iberian Peninsula. The intensive trade of cones of *P. pinea* in Iberia and the high capacity of this bug to highjack is probably leading to a constant gene flow between populations.

Besides the two independent introductions already suggested by Lesieur *et al.* (2018) we showed evidence for at least a third introduction in the north or west of the Peninsula.

These multiple introductions and translocations are the main obstacle to pest control and future management strategies

In addition, ecological studies of *L. occidentalis* populations, namely the climatic suitability, host preferences, natural barriers are crucial to understand what can determine the range expansion of this bug. An accurate prediction and consequent management of the invasion dynamics of this bug is only possible after a strong knowledge of its population dynamics

The use of ABC analysis (Estoup & Guillemaud 2010; Handley *et al.*, 2011) may help to precise the invasion scenarios in Iberian peninsula and the number of introduction events.

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CHAPTER 7

Conclusions and Guidelines for future research



Chapter 7 – Conclusions and guidelines for future research

The establishment of non-native insects in Europe has increased exponentially since the 1950s, triggered by globalization of trade (Roques, 2010). Added to this, forests are also under pressure from climate change (Allen *et al.*, 2010; Lindner *et al.*, 2010). *L. occidentalis* is currently an invasive species in the whole European territory (EPPO, 2018). However, Mediterranean countries are the most concerned with this invasive species due to its putative impact on the high valuable edible seeds of *P. pinea*

Is this seed feeder entirely responsible for the observed decrease in Stone pine seed crop?

The impact quantification of *L. occidentalis* on Stone pine still needs precision to allow the definition of economic thresholds in management's strategies.

This impact represents a major concern for the countries that are producers of the edible seeds. The records of a decrease in seed production during the last years (Roversi, 2009; Bracalini *et al.*, 2013; Mutke *et al.*, 2014) led to speculate a lot about the possible causes: climate change, phenological shifts (Mutke *et al.*, 2005; Calama *et al.*, 2011) and biotic agents. *L. occidentalis* has often been cited as one of the main causes for the cone decline (Bracalini *et al.*, 2013; Mutke *et al.*, 2014). However, doubts about the amount of damage that can be attributed to the bug remain present.

In this Ph.D. thesis I started the impact study by fully characterizing this bug damage on mature seeds of Stone pine. Following force-feeding trials and using a multi-technique strategy with stereomicroscopy, X-rays, Micro Computed Tomography and direct observation, I obtained a better and more useful characterization of the bug damage.

The damaged kernels presented a shrunken and wrinkled appearance corresponding to the sucking of the endosperm. I observed two types of damaged kernels in these trials that were later named as Type I and Type II (see chapter 6). Type I corresponded to a kernel partially damaged / with a different colour / serous appearance and Type II corresponds to a completely shrunken and dry embryo with no endosperm. In this trial these two typologies of damage varied according to the duration and frequency of feeding. Type II corresponded to a longer or more frequent feeding. Consumption was estimated to be about a fifth of a seed kernel per bug per month with only one to two seeds being damaged by box. The low number of seeds consumed was an unexpected result that led me to explore

the reasons for it. Firstly, I hypothesized that this concentration of feeding on 1-2 seeds per box out of 10 was due to a possible “collaborative” feeding process between all bugs in a box. The first cue for this collaborative feeding behaviour was the existence of only one feeding hole per damaged seed. Another question arose: Are insects sharing the hole or are some feeding on while others are starving? Videotaping trials later confirmed the collaborative behaviour with several bugs using the same hole for feeding. Opening the hole on the thick and woody coat represents a great effort for the bug. It can take more than 8 hours to do it (personal observations). Once it is open, all bugs may use it for feeding, although they fight for it. Sharing resources may lead to a concentration of damage in trees or sites which has implications in impact estimations.

Field trials (Chapter 5) revealed a very high overall mortality rate (ca. 60%) of second-year young conelets. Caging *L. occidentalis* adults and nymphs in cone bearing branches of Stone pine showed that only nymphs caused a significant mortality of conelets. I thus hypothesized that, in mid-summer, nymphs of *L. occidentalis* might be, in part, responsible for the mortality of conelets but adults prefer ripening cones. This hypothesis is in accordance with field observations where nymphs were very often spotted feeding on conelets, unlike the adults which were almost always found feeding on ripening cones (last year cones).

Several Mediterranean countries have revealed alarming data on an unusually high abortion rate of unripen conelets in the last decade in a phenomenon known as the Dry Cone Syndrome (DCS) for which the cause is not yet completely understood (Mutke *et al.*, 2017). Although *L. occidentalis* is pointed out as one of the most plausible causes, I was not able to support this hypothesis straightforwardly with the experiments that I conducted during this thesis. The coinciding dates of the first observations of DCS phenomenon in Stone pine in Europe with the first records of *L. occidentalis* strongly suggested that this bug is the main responsible for DCS (Mutke *et al.*, 2017). However, field studies that explore the temporal and spatial variation of conelets mortality combined with an assessment of insect density are needed. My field records on conelet mortality presented a high variance between trees and years which suggests that other causes, such as climate, may also be related to DCS. A multiple cause scenario combining climate effects, tree physiological responses as well as the impact of biotic agents is the more plausible hypothesis for explaining DCS according to my findings.

Concerning seed damage, field trials showed that mature cones exposed to biotic agents had an average of 30% damaged seeds whereas cones that were protected during the entire spring and summer had only 6% of their seeds damaged. Thus, biotic agents are

damaging nearly one quarter of the seeds and this must be ascribed mostly to the feeding activity of *L. occidentalis* but in laboratory experiments feeding of two *L. occidentalis* adults inside a bagged branch during August only damaged 6% of the seeds. Damage type I was demonstrated to be a signature of *L. occidentalis* feeding in mid-Summer. Additionally, the seed damage type II may also be attributed to this seed bug although to a lesser extent. Other causes such as physiological ones may also contribute in large part to type II damage. Furthermore, these trials indicated that the reduction in the number of extractable seeds, often called fused seeds, could be caused by *L. occidentalis* feeding in an early stage of seed development (between March and June) as it has been reported in other pine species (Schowalter & Sexton, 1990; Connelly & Schowalter, 1991; Bates *et al.*, 2000, 2002b; Strong *et al.*, 2001, 2006; Lesieur *et al.*, 2014).

This Ph.D. provided a reliable and visual characterization of the damage caused by *L. occidentalis* in mature seeds of *P. pinea*. Moreover, it also provides cues for *L. occidentalis* damage signature in mid-summer, when field populations reach high densities. Still, efficient field monitoring techniques as well as seasonal feeding studies comparing different insect densities are needed to determine an economic threshold density to *L. occidentalis* in this host.

Plant-insect interaction: the seed feeder and the Stone pine

I explored both the insect host preference and the insect host selection.

Laboratory trials that tested the preference of the bug among the three main conifer species in the Mediterranean Basin region, Stone pine, Maritime pine and Aleppo pine obtained different outcomes depending on the level of selection that was used: branch, cone, or mature seed. No clear preference for host species was detected when I compared branches indicating that individual branch quality (i.e. higher nutritional content and high vigour) rather than plant taxonomy seemed more important for a polyphagous insect like *L. occidentalis*. Moreover, bugs manifested strong group behaviour in branches, frequently splitting into two persisting groups. The gregarious behaviour of this insect has already been demonstrated (Koerber, 1963; Mitchell, 2006). In addition, results from chapter 4 which suggested group feeding further support this behaviour.

In contrast, in both the cone and seed preference trials, *L. occidentalis* showed a preference for Stone pine. The large differences in size of cones and seeds among the three pine

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species, with Stone pine presenting much larger seeds and cones, may be the reason for the preference. Stone pine represents a higher reward which for a polyphagous insect may be the cue to the preference behaviour. In fact, visual stimuli are important in the process of host selection by *L. occidentalis* (Blatt & Borden, 1999; Richardson *et al.*, 2017). From a nutritional and visual point of view, we may then expect that Stone pine trees and plantations may favour *L. occidentalis* population growth.

Field studies in forest sites mixing *P.pinea* with other pines, e.g. *P. pinaster*, must now be carried out to test if any bug preferences are effectively occurring under natural conditions. I highlight though, that to be able to design efficient field methodologies to test preferences it is critical to control the quality of the host plants and consider the aggregation behaviour of the bug. Such trials may also contribute to clarify the population dynamics of this bug.

Host selection was tested in field trials in relation to tree physiological status. Trials were carried on in a Stone pine seed orchard with plots under two different management regimes: plots with fertilization and irrigation and plots with no treatment (control). Trees from the two groups differed on physiological parameters and cone production. Regarding second-year conelets, results showed a significantly higher mortality rate in trees that were fertilized and irrigated compared with trees with no treatment. This result may indicate that a preference for conelets or trees under fertilization and irrigation regimes by the biotic agents may be occurring. My results using caged adults and nymphs in branches for one month in mid-summer showed that only nymphs are influenced by the tree treatment causing higher mortality of conelets in fertilized and irrigated trees compared to control ones. Adults had a similar feeding rate in both types of trees.

Regarding damage on seeds from ripe cones; the cones from fertilized and irrigated trees had a higher damage than cones from control trees, with no treatment. However, this outcome was observed similarly in exposed cones and protected cones during spring and summer. Therefore, an endogenous treatment related cause might justify the results.

Once more, like noticed for second-year conelets in terms of seed damage only nymphs presented a differentiated damage between fertilized and irrigated and the control trees with the first having higher values. Thus, I hypothesized that for the nymphs, the conelets and the cone and seeds on fertilized and irrigated trees would be more suitable for piercing and feeding and more nutritious. Differences might be explained by the fact that nymphs are more demanding for nutritional and water food requirements for growth and survival, whereas adults are not so sensitive to food quality.

In general, trees that were fertilized and irrigated were more susceptible to seed damage and cone mortality, which appear to be driven by biotic agents. It is possible that these trees are more attractive to the seed bug *L. occidentalis*, as well as to other seed and cone.

Bug invasion history in the Iberian Peninsula still to be confirmed

By combining the occurrence records with mtDNA and microsatellites data I characterized the invasion dynamics of *L. occidentalis* in the Iberian Peninsula and analyzed together with the characteristics of the pine nut market. The former genetic study by Lesieur *et al.* (2018) presented evidence for the occurrence of two independent introductions in the Iberian Peninsula (Barcelona and Valencia). I found evidence for a third introduction in the north or west of the peninsula.

Cluster analysis using microsatellite revealed three clusters in the Peninsula: [Barcelona, Lleida], [Southern and Central Iberia – Valencia, Almeria, Valladolid, Segovia, Soria] and [Northern Iberia and central Portugal: Burgos, Galiza, Aveiro, Coruche]. A former study already showed that the Barcelona population originated from an independent introduction from Eastern America (Lesieur *et al.*, 2018). By analyzing new genetic data from the rest of the Peninsula, Barcelona still forms a strong differentiated cluster which indicates a possible spatial isolation of this population as suggested by the lower suitability of the Ebro Valley on the left (Zhu *et al.*, 2013) and the Pyrenees Mountains on the right. It also suggests that the other Iberian populations are not a result of a natural expansion from the Barcelona population, the first one to be detected in the Peninsula.

if we remove the record of Barcelona in 2003, record dates of the first field observations of the bug in the Peninsula differ only by three years going from 2008 to 2010 (Ribes *et al.*, 2008; Pagola Carte, 2009; Valcárcel & Portillo, 2009; Vázquez *et al.*, 2009; Grosso-Silva, 2010; Pérez Valcárcel & Prieto Piloña, 2010).

The very close first observational dates, the absence of a decrease in genetic diversity and the cluster analysis on the whole Peninsula, points to the occurrence of multiple introductions that could be transatlantic, from Eastern America, intracontinental from the rest of the invaded Europe, intra-Peninsular or all of them. The occurrence of multiple introductions is a common phenomenon in the invasion history of many alien species (Fonseca *et al.*, 2000; Facon *et al.*, 2003; Kolbe *et al.*, 2004; Bossdorf *et al.*, 2005; Chen *et al.*, 2006; Kang *et al.*, 2007; Roman & Darling, 2007; Ciosi *et al.*, 2008, 2010), and was already proved for the seed bug in Europe (Lesieur *et al.*, 2018). Besides multiple introductions, *L. occidentalis* is also known to be a strong flyer with high dispersal capacities (Lesieur, 2014).

Altogether, our results and the dispersal capacities of the bug strongly suggest that the rapid invasion of the Iberian Peninsula was due to stratified dispersal, i.e. a combination of short- and long-distance dispersal. Short due to the high dispersal capacities of the pest and long due to human-mediated transportations possibly related to the intensive pine nut market in this Peninsula. A high volume of cones is transported within and between the main producer's countries, Portugal, Spain, Italy and Turkey to be processed and sold (Mutke *et al.*, 2012). These intensive translocations of cones may easily serve as ride to *L. occidentalis* individuals from one place to another justifying both the rapid rate of invasion in the Peninsula and the high genetic diversity of Iberian invasive populations. Multiple introductions lead to high levels of intraspecific hybridization (i.e. "admixture") which can confer survival advantages and greater adaptation of the admixed populations (Ellstrand & Schierenbeck, 2000; Ryan *et al.*, 2009; Facon *et al.*, 2010). One test of the effect of admixture on invasive success was carried out in the invasive harlequin ladybug, *H. axyridis* (Facon *et al.*, 2010) combining genetic analysis and laboratory experiments. Admixed individuals developed quickly, had a larger size and a higher genetic variance for survival in starvation conditions.

All these features potentially increase the invasiveness and the economic impact of *L. occidentalis* and greatly constrain the pest control and future management strategies.

The use of ABC analysis (Estoup & Guillemaud 2010; Handley *et al.*, 2011) may help (or will be required) to precise the invasion scenarios in Iberian peninsula and the number of introduction events. However, *L. occidentalis* invasion history of the Iberian Peninsula is probably a complex process difficult to reconstruct such as in some other case studies insect invasions to Europe; e.g., the harlequin ladybug, *H. axyridis* (Lombaert *et al.*, 2014), the Asian long-horned beetle, *Anoplophora glabripennis* (Javal *et al.*, 2017), and even *L. occidentalis* (Lesieur *et al.*, 2018). Multiple introductions from different sources, human-mediated spread and natural dispersal are arguments put forward by these authors to justify this complexity.

Guidelines for future research

With an economically and socially valuable market behind, research focus on this pest and on its interaction with Stone pine is an urgent matter and should be in all producer countries agenda.

Research on Stone pine physiological mechanisms and ecology is as pertinent as studying the insect. Although being a native pine species we still have to go a long way to decipher its physiological processes and ecological interactions.

My results and of others, suggest that *L. occidentalis* tend to choose and to have a higher survival rate in Stone pine (Farinha *et al.*, 2018a, 2018b; Ponce *et al.*, 2017). However, climatic models predict a low suitability of *L. occidentalis* for a large part of the distribution area of Stone pine (Zhu *et al.*, 2014). This apparent mismatch must be checked in the field. In 2014, the density of *L. occidentalis* field populations was very high in Stone pine seed orchard in the regions of Setúbal, Santarém and Évora in Portugal (correspond to the main productive region of pine nuts in Portugal). Yet, since 2015 population densities have been decreasing. In the last two years the density of this bug populations in the same regions was very low being hard to see any bug (Ana Farinha, personal observations). What led to this decrease? Several authors already mentioned this chaotic population dynamics (Lesieur, 2014; Richardson, 2013) but it is not known what set it. It is thus crucial to answer the question: Are climatic conditions determining the highly variable population dynamics of this bug in the Mediterranean Basin? And if not, what is it? Future research on this behavior is paramount to be capable of predict this species movements and possible fragilities. Studies should be large-scale and encompassing different biotypes and climatic conditions.

Other important question concerns the natural control of this invasive insect. Both, predators or parasitoids are important agents for the control of insect populations. However, *L. occidentalis* main native parasitoids do not exist in the European continent and little is known about its predators. Exploratory field trials in Spain found that parasitoids of native pests such as *Thaumetopoea pityocampa* (pine processionary moth) can lay eggs and develop on *L. occidentalis* eggs in natural conditions. It is now necessary to carry on intensive and systematic field studies aiming at assess the composition of *L. occidentalis* native parasitoid communities and its efficiency in controlling these bug populations. However, before thinking of control measures it is mandatory to better characterize this bug impact. Studies on seasonal variation of its feeding behavior using field covered branches with different densities of bugs inside are crucial and it should be the next step. Only then we define economic thresholds for these bug populations. In addition, survey of the bug-associated pathogenic fungi like *Diplodia pinea* is equally necessary since it is possible that *L. occidentalis* is an important vector of this pathogen fungi (Luchi *et al.*, 2011).

Most importantly, future control strategies of *L. occidentalis* populations must integrate Stone pine forests management plans in a three-player strategy: the insect, the pine and the pine nut sector, in a Mediterranean scenario.

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About the Author

Insects are my passion!

I have been studying them in a very wide range of perspectives from medical vectors to agricultural pests and as forensic indicators. More recently, I've been focusing on forest insect pests. After studying insects in so many perspectives, it became clear to me the path I want for my future as a researcher. Apart from the fascinating physiology of insects, their spatial and temporal organization, along with all the multitrophic interactions, opens a world even more impressive.


It started with a degree in Biology. During the degree I occupied my free time with voluntary work in research projects related to entomology. These experiences gave me a will to pursue a research career. Right after my graduation I won a summer internship on medical parasitology in the Faculty of Agriculture of Novi Sad, Serbia. I went! On the next September I was applying for masters in Medical Parasitology at the Universidade Nova de Lisboa. After the masters, I worked in two projects, a project of forensic entomology and a project of endemic insects from Madeira Island. Both were fascinating and kept my passion for insects high.


In the beginning of 2015 I started this Ph.D project. It was my first time working with forest pests. I quickly fell in love with the forest, and my enthusiasm for my doctorate was even greater. With this project I was sure that my future would be intertwined with the forest.

In short, I can say that I'm very lucky. I get to discover not only one but two passions and get to work with both of them daily; the tranquility and splendor of the forest and the restless insects.

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