European invasion of the Western conifer seed bug: so what happened?

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The invasive *Leptoglossus* seed bug, a threat for commercial seed crops, but for conifer diversity?

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Concise title: Is conifer diversity at risk?

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ABSTRACT:

Among the recent introductions of alien insects in Europe, the polyphagous western conifer seed bug, *Leptoglossus occidentalis* Heidemann (Heteroptera; Coreidae) can seriously be regarded as a major threat for all the European conifer forests. In the current study combining laboratory and field experimentations, we characterized first bug damage by developing specific damage categories on seeds of different conifer species by the use of X-ray. Secondly, we investigated the impact of the invasive bug on key conifer species used for afforestation in Western and Central Europe. For this purpose, we performed germination tests on predated seeds which revealed that even light damage (consumption of less than ⅓ of the whole seed content) strongly reduced the germination capability of the seed. We also compared the impact of feeding on the proportion of filled seeds. Second year cones of *Pinus sylvestris* and *P. nigra* have been enclosed and offered to different life stages (nymphs and adults) and the results showed a significant reduction of filled seeds whatever the life stage. In field, we annually surveyed the bug seed damage for six different conifer species planted in southwestern French seed orchards. Taking into account the economic value of improved seeds in seed orchards, economic impact of bug damage was important although never exceeded 25%. Two natural or semi-natural alpine pine stands were also surveyed and appeared to be highly affected by the bug (up to 70% of damaged seeds). Therefore, bug damage could also be considered as a serious threat for seed production in natural stands.

KEYWORDS:

Cone and seed insects, germination, *Leptoglossus occidentalis*, *Megastigmus* spp., natural stands, seed orchards.

INTRODUCTION

The arrival and establishment of non-native species exponentially increased during the recent decades with the Era of Globalization (Meyerson and Mooney 2007; Hulme 2009). Forests are not spared by this phenomenon, as it is shown by the recent invasion of the Asian emerald ash borer, *Agrilus planipennis,* in North America, or that of the Asian chestnut gall-maker, *Dryocosmus kuriphilus*, in Europe, both species causing significant economic and ecological damage to forested ecosystems and landscape trees (Gandhi and Herms 2010; Bosio et al. 2010). In Europe, almost 1600 alien exotic arthropod species have already established and a number of them are affecting woody plants (Roques 2010). Among them, a true bug, the Western conifer seed bug, *Leptoglossus occidentalis* Heidemann (Heteroptera, Coreidae), may be considered as a serious threat for conifer seeds for both commercial seed crops (Roversi et al. 2011; Bracalini et al. 2013) and natural regeneration of forests (Tamburini et al. 2012).

Leptoglossus occidentalis is native of western North America, its original range being considered to extend from British Colombia to Mexico in latitude and from the Pacific Coast to Colorado in longitude (Koerber 1963; Cibrián-Tovar et al. 1995; Blatt and Borden 1996). Since 1956, the species was introduced accidentally in Iowa (Schaffner 1967), and then spread eastward to reach the East coast in the 1990s (McPherson et al. 1990; Gall 1992; Ridge-O'Connor 2001). After that, it was introduced to Europe, where it was first observed in 1999 in Northern Italy (Taylor et al. 2001; Bernardinelli and Zandigiacomo 2001). It further expanded its range very quickly, and within just a decade, the species has colonized a large part of the continent, from Norway to Sicily and from Portugal to Turkey (Fent and Kment 2011). Recently, the bug was found for the first time in Russia and Ukraine (Gapon 2012) as well as in Eastern Asia (Ahn et al. 2013). It is likely that the present bug distribution in Europe has resulted from a combination of several separate introductions, followed by natural dispersal of the introduced populations, but also probably long-distance translocations through human activities (transport of eggs, nymphs or adults as hitchhikers) (Gall 1992; Malumphy et al. 2008).

Leptoglossus occidentalis exhibits polyphagous habits in its native American range where it has been observed to feed on cones of a large number of conifer species. Although the bug seems to prefer pine species (*Pinus* spp.) and Douglas-fir (*Pseudotsuga menziesii*), it has also been recorded on firs (*Abies* spp.), spruces (*Picea* spp.), larches (*Larix* spp.) and hemlocks (*Tsuga* spp.). In the invaded European regions, scattered observations tended to indicate a large host range, including native pine species such as *Pinus sylvestris*, *P. nigra*, *P.*

halepensis, *P. pinea* but also *Picea abies*, *Larix decidua*, *Abies* spp., and *Juniperus* spp. in addition to introduced exotic conifers (*Cedrus* spp., *Pseudotsuga menziesii*) (Taylor et al. 2001; Fent and Kment 2011; Tamburini et al. 2012). Nevertheless, no trials have yet been carried out to screen the comparative susceptibility of European conifers.

Although capable of feeding occasionally on conifer foliage (Gall 1992; Tamburini et al. 2012), *L. occidentalis* is predominantly related to cones. Both adults and immature nymphs consume individual seeds in developing and mature cones. The insects insert their mouth stylets through the cone tissues directly to the seeds into which they suck the lipid and protein content (Bates et al. 2001). Thus, attacked mature cones do not show any external damage symptoms unlike these of many other cone pests (Strong et al. 2001). In Canada, Bates et al. (2000) therefore developed an X-ray methodology in order to be capable of assigning to bug specific damage patterns observed in Douglas-fir seeds. This technique also allowed to quantify different levels of bug damage on seed content. However, such a characterization of seed damage is still lacking to detect and evaluate bug impact in European conifers. Bug feeding on early stages of cone development before the seeds develop seems also highly detrimental. When affecting 1st year cones, it is susceptible to result in an overall abortion, and thus a total loss of the potential seed yield (Bates et al. 2002b). Early feeding by *L. occidentalis* before the seed coat has hardened was also observed in North America to generate so called "fused" seeds, which are flattened and fused with the supporting cone scale (Bates et al. 2000; Strong et al. 2001). It is assumed that bug feeding alters the physiological process by which the seed coat separates from the ovuliferous scale.

Overall, these impacts result in a significant decrease in seed yield (Bates et al. 2002a). This species is thus regarded as a major economic pest in the seed orchards designed to produce genetically- superior tree seeds in the native American range (Schowalter and Sexton 1990; Bates et al. 2000; Strong et al. 2001). In the invaded European range, this alien species is also considered responsible for a significant decrease of the crop size of stone pine (*Pinus pinea*) seeds that are used for human consumption in Italy (Roversi et al. 2011; Bracalini et al. 2013). However, a precise quantification of the decrease in seed yield following the bug arrival remains to be carried out in most European regions and conifer species, as well as its possible variations with the different steps of the colonization process.

Although the bug is considered as a pest in commercial seed orchards, little is known about its impact on the natural regeneration of conifer forests in the native North American range as well as in the invaded Europe. In Mexico, bug damage was recently reported to be quite low in stands of Engelman pine, *Pinus engelmannii* (Bustamante-Garcia et al. 2012).

The situation in Europe could be totally different because the alien bug and European conifers did not coevolve, and the introduced bug populations apparently escaped from natural enemies (enemy-release hypothesis; (Liu and Stiling 2006)). Tamburini et al. (2012) thus suggested that this invasive species could strongly affect alpine forest ecosystems where it appears to be well established. This impact can also be enhanced through a newly- established association between the alien insect and a native fungal pathogen of conifers, *Diplodia pinea* (Luchi et al. 2012).

Therefore, we intended (i) to test whether the invasive *Leptoglossus occidentalis* is significantly decreasing seed yield and affecting germination potential in a similar way in key conifer species used for afforestation in Western and Central Europe, and, (ii) to look at possible differences in bug damage between commercial seed orchards and natural stands.

MATERIALS & METHODS

All the experiments under controlled conditions were performed in laboratory and in tree nurseries at INRA Orléans (France; 47°49'N; 01°54'E, elevation: 110 m) with insects from a strain reared at our laboratory. The seed bugs were originally collected in the Orléans region in September 2009 and periodically augmented with seed bugs collected in the same area, in order to avoid consanguinity. A fraction of the strain was maintained in a climate chamber under the following conditions: 20 °C with 60% RH and 16:8 h light/dark cycle. This rearing device allowed us to obtain three bug generations per year and to lead to the availability of insects for the whole duration of the bug damage category experiment (see below). Moreover, some insects were installed in an outdoor shelter, mimicking, as much as possible, natural conditions (that is mainly one to two generation per year) and were used in all other feeding bioassays. For that, sexed bugs were let to overwinter outdoors within screened cages (0.5 x 0.5 x 1 m), each containing males or females with extra seeds (pines and Douglas-fir seeds) and Douglas-fir seedlings.

Bug damage categories on seeds of native and introduced exotic conifer species

A total of six conifer species (five European and one north-American) were used in this experiment but, because of limitations in seed availability, we conducted the tests at different times according to the studied species. All the seeds corresponded from the year's crop and were obtained from orchards belonging to the French National Forestry Office (ONF). Seeds were first radiographed using an X-ray apparatus (HP Faxitron - 43855®) and X-ray sensitive films (Kodak ® 'Industrex M'), following the procedures described in Roques and Skrzypczyńska (2003). The empty seeds and these showing chalcid larvae were discarded. Only the seeds showing a fully filled content (megagametophyte and embryo) were kept.

The first series of experiments was conducted with seeds of four conifer species randomly exposed to feeding by adult bugs in spring 2010. In the one hand, we used simultaneously three European species (*Pinus sylvestris*, *Pinus nigra*, and *Abies nordmanniana* subsp. *bornmuelleriana*). In the other hand, we compared seed feeding of the two former European pines with Douglas-fir seeds (*Pseudotsuga menziesii*) which is one of the preferred host in its native area, and also considered as one of the most important exotic species planted in Europe for afforestation. Ten seeds per conifer species were separately fixed at 1 cm- intervals on a polystyrene support and were exposed to a single adult bug in a plastic box (12 cm x 12 cm x 6.5 cm) for a 15-days period. Each treatment was replicated 10 times for each sex. Every two days the seeds, still on their polystyrene support, were submitted to X-raying in order to survey the development of individual damage. Adult preferences for seed species was estimated by counting the total number of predated seeds according to bug sex at the end of both experiments.

An additional experiment was carried out in 2011 to define bug damage category in two additional European conifer species, *Larix decidua* and *Picea abies*. A total of 200 filled seeds were selected per species using X-ray such as above. The seed batch was offered to bug predation in a gauze cage (0.5 x 0.5 x 1 m) including approximately 20 adults. To be capable of comparing the results with these obtained in the previous test, the experiment was also repeated on both pine species and Douglas-fir. After a 15-days exposure, all seeds were placed on an adhesive paper sheet and X-rayed to assess the final damage.

Following the methodology developed by Bates et al (2000), the damaged parts of the megagametophyte and/or of the embryo were visually categorized on radiographs at each 2-days survey, and the size of the damaged area was estimated with regard of the initial seed volume. Taking into account the larger number of tree species, we decided to define a supplementary category corresponding to very light damage compared to bug damage categories developed by Bates et al (2000). In each conifer species, different damage categories were then tentatively defined, gradually increasing in damage extent: (1) no apparent damage (fully filled seed), (2) light damage (less than ⅓ of the whole seed content consumed), (3) moderate damage (consumption between ⅓ - ½), (4) important damage (consumption between ½ - ⅔), (5) severe damage (more than ⅔ of seed consumed). Finally, in order to check whether weighting can also constitute a method to assess seed damage, the seed weight was compared between the damage categories defined by radiography in each species. We also included empty seeds discarded at the beginning of experience in order to compare naturally aborted seeds with those suffering severe damage. Depending on availability, 8 to 85 seeds per damage category and per species were weighted using an analytical balance (Mettler Toledo DeltaRange AG204).

Effect of bug predation on seed germination

The seeds obtained from the experiment conducted in 2011 (see above) and classified according to the five different damage categories were tested for the germination potential. The germination tests were based on the ISTA procedures for each species (International Seed Testing Association 1999). Seeds were placed on Whatman filter paper maintained continuously moistened with sterilized water in Petri dishes (100 x 15), under controlled conditions (20°C, 16:8 L:D, 60% RH). Seeds were considered germinated when the radicle protruded of 2 mm at least. Germination percentage per damage category was calculated after 28 days following the transfer under germinative conditions.

Impact of seed bug feeding on 1st year cone development and seed yield of 2nd year cones

Feeding bioassays were performed on 1st year cones and 2nd year cones of *P. sylvestris* and *P. nigra* planted in nurseries at INRA Orléans, with bugs from the outdoor rearing. Ten trees of each species were randomly selected. 1st year cones and 2nd year cones were enclosed in mesh insect-exclusion bags on 12-13 April 2011 before the emergence of the overwintering adult bugs. It also aimed at preventing any damage by other cone pests. In *P. sylvestris,* each bag contained one 2nd year cone and one to ten 1st year cones whilst it only included one 2nd year cone in *P. nigra* because this species showed quite low flowering in 2010. Each bag was randomly assigned to one of the following five treatments: A- one male bug, B- one virgin female, C- one mated female, D- three 2nd -instar nymphs, and E- control without bugs. The adults were introduced on 31 May and removed on 15 June 2011 whilst the nymphs were let in bags from 29 June to 15 July 2011. Ten replicates using ten different trees were used per treatment and per pine species except for the couples male/*P.sylvestris* and mated female/ *P. nigra* (nine replicates), and control/ *P.sylvestris* (eight replicates).

To obtain mated females, several couples of bugs were formed and placed into individual plastic boxes (12 x 12 x 6.5 cm) with fresh branches and seeds. Once the mating observed, females were transferred into bags two-three days after the observation. In treatment C (mated female), all the eggs laid by the female and the resulting nymphs were removed at the end of the experimentation to prevent any damage not caused by the tested individual.

The bags were inspected after one week. Any dead or missing individuals were replaced, which corresponded to only three adults and four nymphs during the whole experiment. At the end of each 15-days period, all seed bugs were removed and the cages resealed until harvest. First year cone survival was checked in September 2011 when cones were harvested. Cones were air- dried at 35°C for 48 hours. All their scales were finally removed by hand to release the seeds. The seeds were then X-rayed as above in order to assign their content to the previously defined damage categories. Fused seeds (i.e. seeds that could not be removed from the seed scale as described by Bates et al (2000) and Strong et al (2001)) were considered as fully-damaged seeds. To compute the proportion of damaged seeds, we assumed that the bugs only feed on filled seeds but not on empty ones because no information is known. Thus, this proportion was calculated as the ratio of bug-damaged seeds on the number of seeds considered as initially available for insect feeding (i.e. filled seeds plus bug-damaged seeds).

Comparative assessment of seed bug impact in seed orchards and alpine stands

*Seed orchard*s

Bug impact was assessed in several seed orchards located at three different nearby sites in southwestern France (Table 1), where *Leptoglossus occidentalis* was first observed by late autumn 2008 (Roques, unpublished observations). The orchards were selected based on their conifer species content: four different species were surveyed at Lavercantière (*Pseudotsuga menziesii*, *Pinus sylvestris*, *P. nigra* and *Larix decidua*), and two species in the two other localities, *P. menziesii* and *Picea abies* at Calviac, and *P. menziesii* and *Abies nordmanniana* subsp. *bornmuelleriana* at Sousceyrac. All the orchards were surveyed for seed damage from 2010 to 2012, except the Douglas-fir plantation of Lavercantière which was surveyed from 2008 to 2012. Depending on the crop, ten to 50 cones were randomly collected in each plantation. The collections were carried out at seed maturity, the period being different according to the conifer species biology (Douglas-fir: mid-August; fir: September; pines: October; larch: September). Only those showing no damage by other cone and seed insects, such as *Dioryctria* coneworms (Lepidoptera, Pyralidae), *Cydia* tortricids (Lepidoptera, Tortricidae), and cone weevils (*Pissodes validirostris*, Coleoptera, Curculionidae) (Roques et al. 1983), were analyzed. For two species present at Lavercantière, *P. sylvestris* and *P. menziesii* respectively, the total number of cones was annually measured in order to relate insect damage with the yearly fluctuations in cone crop size. Cone crop rating corresponded to: 0 = no cone per tree; 1 = light crop (1 to 49 cones per tree); 2 = medium crop (50 to 199 cones per tree); 3 = important crop (200 to 499 cones per tree); 4 = heavy crop (more than 500 cones per tree).

Cones were dried and individually shaken to extract the seeds. The extractable seeds were placed on an adhesive paper sheet to be X-rayed cone by cone. A total of ca. 49 000 seeds were processed. The number and percentages of empty, filled, insect- damaged seeds was counted per cone. For each seed, bug damage was assigned to one of the damage categories defined above per conifer species. The total percentage of seeds damaged by bugs was calculated as above. In these orchards, insect damage could include seeds infested by larvae of different species of *Megastigmus* seed chalcids in fir, larch, and Douglas-fir. The chalcid infested seeds were identified by X-rays and the percentage of *Megastigmus*-infested seeds was calculated following Auger-Rozenberg and Roques (2012), considering that bugs and chalcids do not attack simultaneously a seed.

Alpine stands

Two mixed pine stands located in southern French Alps (Merdanel and Serre-Ponçon, Table 1) were surveyed for seed bug damage from 2010 to 2012. Both stands were aged of ca. 50 years and consisted of native *Pinus sylvestris* trees growing together with *P. nigra* trees issued from natural regeneration of initially planted seedlings. In each stand, ten trees per species were randomly selected and five 2nd- year cones were collected per tree by September, when the seeds were mature. Seeds were extracted and analyzed for bug damage as in seed orchards. A total of ca. 16 000 seeds were X-rayed. Bug damage was categorized and measured as in seed orchards.

Statistical analysis

All variables were checked for their homoscedasticity (Levene test) and normal distribution (Shapiro–Wilk test). Data not fitting a normal distribution after transformation were analyzed using nonparametric tests.

Weights of the seeds from the different damage categories were √x transformed to ensure normality, and then submitted to one-way ANOVA. Means were separated by Tukey-Kramer honestly significant difference (HSD) multiple comparison test. Kruskal–Wallis test was performed to test for seed bug preferences among conifer species, followed, when significant, by multiple comparisons using Mann–Whitney test with Bonferroni-Holm correction.

To evaluate the effect of the different damage categories on germination, germination rates were compared using pairwise Fisher's exact tests with Bonferroni-Holm correction.

The mean percentages of aborted 1st year cones, and these of filled, empty and bug-damaged seeds obtained from the bagging experiments were analyzed using Kruskal–Wallis tests followed, when significant, by Mann–Whitney test with Bonferroni-Holm correction. The same statistics were applied to compare the percentages of filled, empty and damaged seeds observed in seed orchards and natural stands. Kruskal-Wallis tests were applied in order to test for differences between years, sites and conifer species, except for alpine stands where Mann-Whitney tests were used to test for difference between conifer species. The relationships between the percentage of seed damage by bugs and the yearly variations in cone crop size were tested using correlation analysis in the two plantations where the crop size could be measured. The following variables were tested (i) size of the year's cone crop (N); (ii) size of the cone crop in the previous year ($N - 1$); and, (iii) change in cone crop from one year to the next. In the Douglas-fir plantation of Lavercantière, where *Megastigmus spermotrophus* was susceptible to affect largely the seed yield, the same correlation analysis was applied to the chalcid damage in order to compare bug and chalcid damage, and their annual variations with regard to crop fluctuations.

The significance level, α, was set at 5% in all statistical analyses. Statistical analyses were performed with R software (R Development Core Team 2008).

RESULTS

Bug damage categories on seeds of native and introduced exotic conifer species

We established bug damage categories for the six considered conifers species (Fig. 1). In each species, radiographic interpretations allowed to rank bug damage to initially filled seeds into the five categories previously defined. No completely empty seed, without shrinkled remainings of embryo or megagametophyte, was observed. For light damage, in most of cases, only megagametophyte was affected but not the embryo. Moreover, dissections of seeds sustaining light, moderate, important or severe damage supported the radiographic interpretations, internal tissues showing progressive depletions proportional to the damage level. Seed damage categories followed the same trends and were relatively similar in all conifer species (Fig. 1).

In all species, filled seeds were heavier, on the average, than bug-damaged seeds (Table 2). However, they significantly differentiated from those showing light damage only in *Pinus sylvestris*, *Pseudotsuga mensiezii* and *Picea abies*. Seed mean weight decreased along with the importance of the consumption of the seed content. However, the decrease in mean weight did not fully correspond to the radiographic categories. Some of these categories did not differ significantly in weight in *Pseudotsuga* (severe *vs*. empty), *P. sylvestris* (important *vs*. severe), *P. nigra* (severe *vs*. empty) and *Picea* (moderate *vs*. important). In *Abies* and *Larix*, seed weight appeared to discriminate only seeds without any damage and with light damage from the others.

Figure 2 shows the radiographic monitoring of the development of bug damage over a 15-days period of feeding allowance and the mean numbers of seeds per species damaged by bugs at the end of the experimentation were presented in Fig. 3. When *Pseudotsuga* seeds were offered besides pine seeds, females consumed more seeds of *Pseudotsuga* during the first five days than seeds of *Pinus sylvestris* with an intermediate position for seeds of *P. nigra* (H = 6.578, df = 2, P = 0.037; H = 9.386, df = 2, P = 0.009; respectively for the first and the second record). However, no difference between seed species was observed later ($P >$ 0.05 in all cases). Seeds of *P. menziesii* were less selected by males than these of pines over the 15-days period of feeding ($P < 0.05$ in all cases) except at the 9th day of feeding where no significant difference was observed (H = 5.406, df = 2, P = 0.067). When *Abies* seeds were offered besides pine seeds, no difference in bug damage was observed both for males and females throughout the experimentation.

Effect of bug predation on seed germination

Abies seeds did not germinate at all whatever the damage category, even the seeds without damage. In the other conifers, seeds with more than half of the content consumed (i.e., severe and important damage categories) did not germinate except in *Pinus nigra* where such seeds showed a germination rate of 2.5% (Fig. 4). Germination rates of seeds sustaining moderate damage were very low (comprised between 0.0 to 3.0%) except in *Larix decidua* (23.1%).These rates were significantly lower than these resulting of seeds showing light damage in *P. abies* (Fisher exact test, P = 0.029), *P. menziesii* (Fisher exact test, P = 0.004) and *P. sylvestris* (Fisher exact test, $P < 0.001$) but not in *P. nigra* (Fisher exact test, P = 0.128) and *L. decidua* (Fisher exact test, P = 0.078). With regard to filled seeds, light damage significantly decreased seed germination by 7 times in *P. nigra* (Fisher exact test, $P < 0.001$), by 3 times in *P. sylvestris* (Fisher exact test, $P < 0.001$), and by 3.5 times in *P. abies* (Fisher exact test, $P < 0.001$) but this was not observed in *P. menziesii* (Fisher exact test, $P = 0.421$) and *L. decidua* (Fisher exact test, $P = 0.121$).

Impact of seed bug feeding on 1st year cone development and seed yield of 2nd year cones

First year cones of *Pinus sylvestris* exposed to *L. occidentalis* (adults or nymphs) did not abort significantly more than those in control bags without bugs ($H = 6.686$, df = 4; $P = 0.153$). However, the percentage of abortion in the bags where adults were introduced (ca. 25%) tended (but not significantly) to be higher, compared to those observed in nymph and control treatments (respectively 3.0 and 8.3%).

The percentage of filled seeds was significantly lower in 2nd year cones exposed to bugs than in control ones for both *P. sylvestris* ($H = 30.403$, df = 4, $P < 0.001$) and *P. nigra* ($H = 26.938$, df = 4, $P < 0.001$) (Fig. 5). In *P. sylvestris*, cones exposed to feeding by mated females presented the lowest percentage of filled seeds (only 0.3%) although not statistically different from these submitted to males and virgin females. In *P. nigra*, there was no significant difference between the bug treatments, the percentage of filled seeds ranging from 1.3 to 8.2% whereas it reached ca. 60% in controls. The percentages of empty seeds were not significantly different between treatments both for *P. sylvestris* ($H = 12.379$, df = 4, $P = 0.055$) and for *P. nigra* ($H = 4.529$, df = 4, $P = 0.339$) (Fig. 5). The percentages of damaged seeds differed significantly among mature cones offered to bugs and control ones in *P. sylvestris* ($H = 28.378$, df = 4, $P < 0.001$) as well as in *P. nigra* ($H = 27.272$, df = 4, $P < 0.001$). No damaged seeds were observed in control cones in *P. sylvestris* but fused seeds were observed in these of *P. nigra* (6.2%). The percentage of damaged seeds reached ca. 99% in cones of *P. sylvestris* offered to mated females. This value was significantly higher than in cones offered to nymphs, whereas virgin females and males resulted in intermediate values (Fig. 5). No significant difference was observed in *P. nigra*. However the bug treatments induced different types of damage to the seeds (Fig. 5). In both species, cones exposed to adult feeding contained a majority of fused seeds whereas the percentage of fused seeds was low in the cones exposed to nymphs. Consequently, for both pine species and regarding only the extractable seeds (i.e. not taking into account fused seeds), the proportions of damaged seeds were quite low (1 to 31%) except for nymphs where the mean values were approximately 50% (data not shown).

Comparative assessment of seed bug impact in seed orchards & alpine stands

Seed orchards

Seed bug damage was observed in all of the surveyed seed orchards, and affected all conifer species except *Larix decidua* in 2011 where most flowers were quite not pollinated (less than 2% of the seeds available for bug consumption) (Table 1). However, bug damage was rather limited in the seeds orchards of Calviac and Sousceyrac, with less than 5% of the available seeds consumed whatever the conifer species. By contrast, at Lavercantiere, bug damage decreased by up to 25.7 % the potential seed yield in the Douglas-fir plantation in 2010, and by more than 15% these of *Pinus nigra* and *P. sylvestris* in 2011 (Table 1). At this site, *L. decidua* was less damaged (H = 50.060, df = 3, P < 0.001) than the 3 other conifers. However, larch offered only a few filled seeds to bug feeding, considering that empty seeds represented 82.7 to 93.2% of the total seeds. No bug preference could be drawn between the 3 other species (H = 4.013, df = 2, P = 0.134). When the four conifer species were pooled together, the highest percentages of bug damage were observed in 2010 (H = 33.280, df = 2, P < 0.001) while the percentages observed in 2011 and 2012 were not statistically different. Bug damage appeared either to be stable from 2010 to 2012 at Calviac and Sousceyrac or to decrease during the same period at Lavercantière in *Pinus nigra* (Table 1). In the Douglas-fir plantation of Lavercantière, bug damage largely fluctuated over the four years following the insect arrival by late 2008. After a sharp increase during the first two years of presence, it largely decreased in 2011 and 2012 (Fig. 6).

Concerning added seed chalcids damage, the combined attack of bugs and both exotic and native fir chalcids, *Megastigmus rafni* and *M. suspectus* respectively, affected more than 25% of the available seeds, resulting in a very low seed yield in 2011 (Table 1). In Douglas-fir, the combined attack of the alien *M. spermotrophus* and bugs resulted in a total percentage of damaged seeds fewer than 10% at Calviac and Sousceyrac (Table 1). However, their combined damage reached 40% in 2010 and 37% in 2012 at Lavercantière (Fig. 5).

Yearly variations in the percentage of bug- damaged seeds were not correlated with any of the crop variables recorded in the plantation of *P. sylvestris* (Table 3). In the Douglas-fir plantation of Lavercantière, the percentage of bug-damaged seeds was positively correlated with the change in cone crop size from one year to the next (Pearson r^2 = 0.894, P = 0.041) (Table 3). In this plantation, no correlation was observed between the percentages of *M. spermotrophus* damaged seeds and any of the crop variables. By contrast, seed chalcid damage was negatively correlated to bug damage observed the year before (Pearson r^2 = -0.963, P = 0.037) (Table 4).

Alpine stands

Several bugs were observed every year feeding on mature cones of *P. nigra* and *P. sylvestris* at both sites. In both tree species, the percentages of filled and empty seeds largely varied from year to year and the highest percentages of bug- damaged seeds were observed at Serre-Ponçon (U = 63697, P < 0.001) (Table 1). In this stand, *P. nigra* seeds were more damaged (77.36 % in 2011) than those of *P. sylvestris* (U = 11473.5, P = 0.005) whereas the last species was preferred at Merdanel (U = 8587.5, P < 0.001).

The additive effects of bug damage and empty seeds led to a dramatic decrease in potential seed yield of both pine species at Serre-Ponçon, where filled seed to be released finally represented only 10 to 30% of the initial seeds.

DISCUSSION

Laboratory and bagging experiences confirmed the polyphagous status of *L. occidentalis*. Seeds of native European species (i.e. pine, larch, spruce and fir) could be exploited by the bugs as well as these of the exotic Douglas-fir. Using radiography, a five-level damage category has been experimentally defined on seeds of six of the most important conifer species used for afforestation in Western and Central Europe. Field observations confirmed that the damage patterns detected at the laboratory are similar to these noticed in seeds fed upon within mature cones. However, characterizing the status of empty seeds remained a problem because radiographic interpretations do not allow a clear differentiation between seeds sustaining severe bug damage and empty seeds which have naturally aborted because of a lack of pollination, fertilization problems or any other reasons not linked to insects (Schowalter and Sexton 1990). This could probably led to underestimate bug damage. Empty seeds represented ca. 40% in *P. sylvestris* and 30% in *P. nigra* of the seeds at the end of the 15-days long experiment but it cannot be excluded that their proportion may increase with a longer exposure of cones to bugs as it is the case over the field season until seeds are released. Nevertheless, this five-level damage category constitutes a tool to survey the seed quality in commercial crops, the more as densitometry does not provide so reliable results, especially in case of light damage.

Tamburini et al. (2012) suggested that this invasive bug can affect natural regeneration in alpine forest ecosystems. Our survey of the development of *Leptoglossus* damage over three years in two pine stands of the French Alps tended to confirm this assumption. The bug was first observed at the southern location, Serre-Ponçon, by October 2007 (Dusoulier et al. 2007), i.e. three years before the survey. At this place, bug damage dramatically decreased by more than 70% the potential seed yield for natural regeneration in both *Pinus nigra* and *P. sylvestris* in 2011, and damage fluctuated between 24.2 and 44.2% the other years. The other site was less impacted but seed damage in *P. sylvestris* nevertheless reached approximately 53.5% in 2011. These results must be related to these regarding the germination capability of damaged seeds. In Canada, Bates et al. (2001) noticed that only 14% of partially- filled seeds following bug damage were capable to germinate in Douglas-fir. Even a light damage (less than ⅓ of the whole content consumed) was here proved to reduce significantly germination by at least 70% in both pine species as well as in native spruce. Moreover, when more than ⅓ of the internal content was consumed, the major part of the seeds (>95%) were not capable of germinating. Furthermore, bug damage has been probably underestimated during this survey. Indeed, because of the sampling volume to be treated, cones were only dried and shaken to extract seeds but not completely dissected to check fused seeds possibly induced by adult feeding early in the season, and no survey of possible 1st year cone abortion was realized. Therefore, bug damage must be considered as a major threat for natural regeneration in these pine stands at least.

In North America, bug damage ranged from <5 to 40% in different surveys (Pasek and Dix 1988; Schowalter 1994; Cibrián-Tovar et al. 1995; Blatt and Borden 1996; Kegley et al. 2001). In our seed orchard survey, bug damage never exceeded 25 % while in natural stands the percentages of bug damaged seeds could be very high. Nonetheless, damage appeared to be variable from one site to another. Site-related factors such as local climatic conditions could influence bug population (Schowalter et al. 1985; Tamburini et al. 2012). However, the two-years survey of three sites of *P. menziesii* showed a "site effect" in 2010 but not in 2011 and suggested more a "year effect". Although the relative abundance of bugs at each site was not available, the intensity of bug damage seemed to be more dependent on the variation in population density.

The low mortality observed in the bagging experiment as well as the large seed consumption within cones showed that both *P. nigra* and *P. sylvestris* constitute an adequate food source for adults and nymphs. Moreover, natural damage by *Leptoglossus* was observed in all of the six conifer species exploited in the seed orchards of Southwestern France. These results confirmed these of Tamburini et al. (2012), who noticed *Leptoglossus* feeding on *P. sylvestris*, *P. nigra*, *P. mugo* and *L. decidua* in Northern Italy. However, no clear host preference was found during our study. Although seeds of *P. menziesii* were less selected by males than these of pines, further experiences on seeds within cones and not on extracted seeds are needed to confirm this result. Furthermore, European larch appeared much less attacked in the seed orchards but this species was only present in Lavercantière orchard, and at this location, female seed cones suffered every year from a very low rate of natural pollination, and as a result produced only a few filled seeds except when manually pollinated (G. Philippe, pers. comm.). Therefore, this drastic limitation in resources susceptible to be exploited by bugs does not allow to conclude about a lower susceptibility of larch, the more as this species was well fed upon in the seed tests.

The type and extent of damage of *L. occidentalis* appears to differ with the period of the year, and subsequently the development stage of the female cone, and the bug life stage. Conversely to Bates et al. (2002b) in Western white pine (*Pinus monticola* Dougl. ex D. Don), bug feeding on 1st-year cones in *P. sylvestris* did not result in a more important abortion rate than natural processes acting in the insect-exclusion bags. Moreover, feeding on developing 2nd-year significantly reduced the number of filled seeds per cone, with each bug reducing yield by approximately 0.8 seeds per day in *P. sylvestris* and 0.7 in *P. nigra*. These values were lower than these found by Strong (2006) on lodgepole pine (*Pinus contorta* var. *latifolia*) in the bug native range, where each female bug reduced the number of filled seeds by ca. 1.7 seeds per day from 6 May to 29 June in British Columbia. However, Strong (2006) showed that average female damage then decreased to 1.0-1.3 seeds per day from 29 June to 10 August, and dropped to 0.3 per day after 10 August, whereas the reduction caused by 3rd-5th instar nymphs averaged 0.6 to 1.2 seeds per day from late June to early August. Our experiments were carried out during the first two weeks of June for adults (which overwintered) and the first two weeks of July for nymphs (recently hatched). Therefore, it was impossible to confirm whether bug damage in the invaded range is more important early in the season and decreases during summer such as in the native area.

In the native British Columbia, Bates et al. (2002a; 2002b) and Strong (2006) noticed that female bugs exhibited a greater rate of seed consumption, independently of their mating status. In contrast, our bagging experiments did not reveal any differences in total seed damage between male and female and a group of three 2nd instar nymphs, nor between virgin and mated female. The experiment using individual seeds confirmed, but only partly, these findings. No significant difference was observed between sexes in the total number of predated seeds but damage caused by males mostly affected less than ⅓ of the seed content ('light' category) whilst females predominantly consumed more than ⅔ of it ('severe' damage). In the bagging experiment, the only significant difference was observed between mated females and nymphs in *P. sylvestris*. Nevertheless, patterns of adult and nymph damage largely differed in native European pines. The 2nd-year cones attacked by adults mostly produced fused seeds whereas the percentage of such seeds was significantly lower in those offered to nymphs. However, cones were presented to nymphs about one month after the exposure to adults (first two weeks of July *vs*. first two weeks of June), and such cones were obviously physiologically more advanced in maturation. The presence of fused seeds as a result of bug damage is well known in the native area, e.g. in *Pinus monticola* (Connelly and Schowalter 1991), *P. contorta* (Strong et al. 2001; Bates et al. 2002a) and *Pseudotsuga menziesii* (Bates et al. 2000). Strong (2006) found that this damage in lodgepole pine was most prevalent in early May, less prevalent in late May and nonexistent after the beginning of June. Bug food uptake early in the season, before fertilization and seed coat hardening, was suggested to alter the physiological process by which the seed coat separates from the ovuliferous scale. Bates et al. (2000) showed that nymph feeding in June but not in July and August increased the number of fused seeds in Douglas-fir. In our experience, the control cones of *P. nigra* also showed a small proportion of fused seeds. They may have resulted from bug feeding on 1st-year cones – one year before the experimentation, when the insect exclusion bags were not present. Bates et al. (2002b) showed that the extractable seed set of the surviving 1st year cones of western white pine exposed to nymph feeding was reduced by 47% compared to unexposed 1st year cones.

Since the arrival of *L. occidentalis* in Europe, new competitive interactions for seed resource could have emerged with the entomofauna already associated with cones and seeds. In Douglas-fir, the combined attack of the exotic *Megastigmus spermotrophus* and bugs resulted in a total percentage of damaged seeds fewer than 10% in two of the surveyed plantations but reached 40.7% in 2010 and 37.5% in 2012 at the Lavercantière plantation. Looking at the interactions between *L. occidentalis* and *M. spermotrophus* in Douglas-fir in the native range, Blatt and Borden (1998) showed that the impacts of the two species are segregrated and additive. In the Douglas-fir plantations surrounding the Lavercantière seed orchard where both insect species are invading, Auger-Rozenberg and Roques (2012) showed that bug arrival by late 2008 resulted in an increase in total seed damage whereas the specific impact of *M. spermotrophus* appeared to have significantly decreased. Here, we found that the infestation of *M. spermotrophus* is negatively correlated to the extent of bug damage the year before. However, this correlation may actually correspond to confounding factors acting on the two species. Moreover, change in crop size from one year to the next in the Douglas fir plantation appeared to affect both bug and chalcid damage. Auger-Rozenberg and Roques (2012) have already observed a negative correlation between seed damage by *M. spermotrophus* and the annual variation in cone crop size in the surrounding plantations over a 25-years survey. The limited time of this study (five years) may have prevented such an observation. However, we found that bug damage was related to the annual change in crop size and appeared to be more important when the cone crop decreased from one year to the next. In *Abies,* the combined attack of *Megastimus rafni* and *M. suspectus* and bugs affected up to 25% of the available seeds in 2011 but the bug damage was stable over years at low level unlike the large yearly fluctuations in chalcid infestation.

Without an estimation of the actual size of the bug populations, it is difficult to draw any definite conclusions about the population dynamics in the invaded area. However, yearly damage by bugs appeared to largely fluctuate since the insect arrival. In the Douglas-fir plantation of Lavercantière, where it arrived by late 2008, damage showed a sharp increase during the first two years of presence but largely decreased in the following years. In the other seed orchards of the same region, damage either stabilized or decreased to a very low level in 2012. Direct visual observations, although without quantitative estimation, revealed that the presence of adults seemed more scarce in these areas in 2012 compared to the previous years (C. Blazy, pers. comm.). A quite similar situation was observed in the pine stands of the French Alps where the species arrived two years before at least but also in the area of first record in Europe, Northeastern Italy, where it was difficult to find any bugs during autumn 2011. No mechanism has been proved to explain those collapses. It may proceed from massive migrations at rather long-distance owing to strong flight capability of adults. However, population fluctuations may be related to specific pattern in the life cycle observed in Europe. Except in Mexico (Cibrián-Tovar et al. 1995), the species was considered to be monovoltine in North America (Koerber 1963; Hedlin et al. 1980) with the possibility of a partial second generation (Bates et al. 2002b) whereas one to three generations were noticed in Europe (Bernardinelli et al. 2006; Tamburini et al. 2012). Tamburini et al. (2012) suggested that climatic conditions in late summer and autumn may led a large part of the nymphs of the last generation to be unable to reach the adult stage before winter, and therefore cannot survive overwintering.

This study confirmed that *L. occidentalis* can represent a threat in the continent and we highlighted the fact that the impact of invasive seed bug must be taken into account in future forest strategies. Moreover, our data suggests a possible competition between bugs and seed chalcids. Further investigations are necessary to clarify the relationships of the invasive bug with the other insects exploiting the same resource.

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FIGURE LEGEND

Fig. 1 *Leptoglossus occidentalis* damage categories on seeds established for the six following conifer species: (a) *Pinus sylvestris,* (b) *Pinus nigra,* (c) *Larix decidua*, (d) *Pseudotsuga menziesii*, (e) *Picea abies* and (f) *Abies bornmulleriana*

Fig. 2 Radiographic monitoring of the damage development in seeds offered to individual *Leptoglossus occidentalis* according to sex over a 15 days period of feeding allowance. (a) female and (b) male.

Fig. 3 Mean number (+SE) of seeds per host species damaged by *Leptoglossus occidentalis* for each sex: (a) female and (b) male. Seeds were offered to individual bugs over a 15-days period according to two different groups of host species (experience 1 and experience 2). Different letters within an experience indicate significant differences between the proportions of damaged seeds per host species, not considering the levels of damage after Kruskal–Wallis tests followed by Mann–Whitney test with Bonferroni-Holm correction.

Fig. 4 Percentage of germinated seeds according to radiographic damage categories for key conifer species used for afforestation in Western and Central Europe. Different letters within a species indicate significant differences ($P < 0.05$, Fisher's exact test).

Fig. 5 Comparison of the impact of *Leptoglossus occidentalis* on the final seed content of 2nd year cones of two pine species: *Pinus sylvestris* (left), *P. nigra* (right). Vertical axes represent mean percentages (+SE) of (a) filled seeds, (b) empty seeds and (c) bug-damaged seeds respectively. For each histogram, five treatments are compared: control, nymphs and adults with different reproductive status (fed individually on cones for 15-days period in late spring). Different letters (latin and greek) indicate significant differences between treatments within each species after Kruskal–Wallis tests followed by Mann–Whitney test with Bonferroni-Holm correction. Specifically, for (a) filled seeds histograms: Latin letters indicate significant differences after bug damage whereas Greek letter indicates non-significant difference before bug damage. For (c) bug damage seeds, statistical tests were made on the proportions of damaged seeds per species, not considering the levels of damage.

Fig. 6 Comparative annual fluctuation in seed damage by bugs and chalcids with regard to the cone crop size in a seed plantation of *Pseudotsuga menziesii* located at Lavercantière (Southwestern France) from 2008 to 2012 (for cone crop rating see Materials and methods).

TABLES

Table 1. Variation in the sanitary status of seed crops on selected French seed orchards and alpine stands from 2010 to 2012.

Table 2. Mean weight of mature seeds exposed to feeding by Leptoglossus occidentalis sorted by radiographic damage categories. Means followed by different letters within lines indicate a significant difference ($P < 0.05$,Tukey-Kramer HSD test).

Table 3. Pearson's correlations between Leptoglossus damage and the yearly variations in cone crop size in two plantations located at Lavercantière (Southwestern France).

Table 4. Pearson's correlations between *Megastigmus* infestation and the yearly variations in cone crop size and *Leptoglossus* damage in a plantation of *Pseudotsuga menziesii* located at Lavercantière (Southwestern France).

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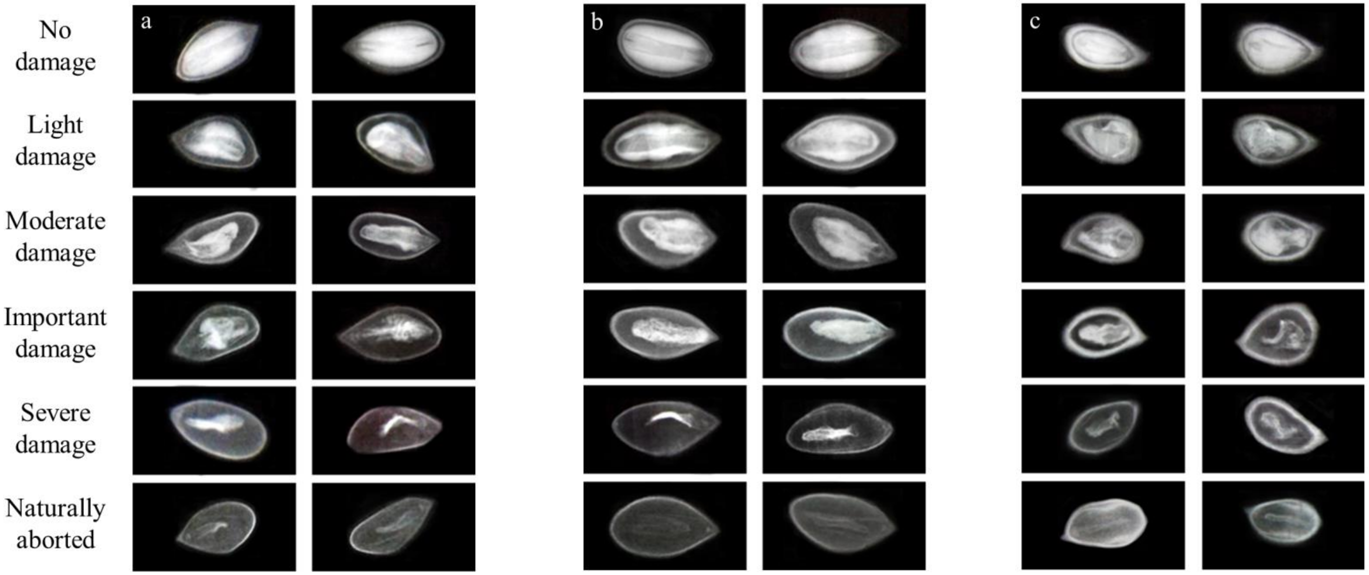

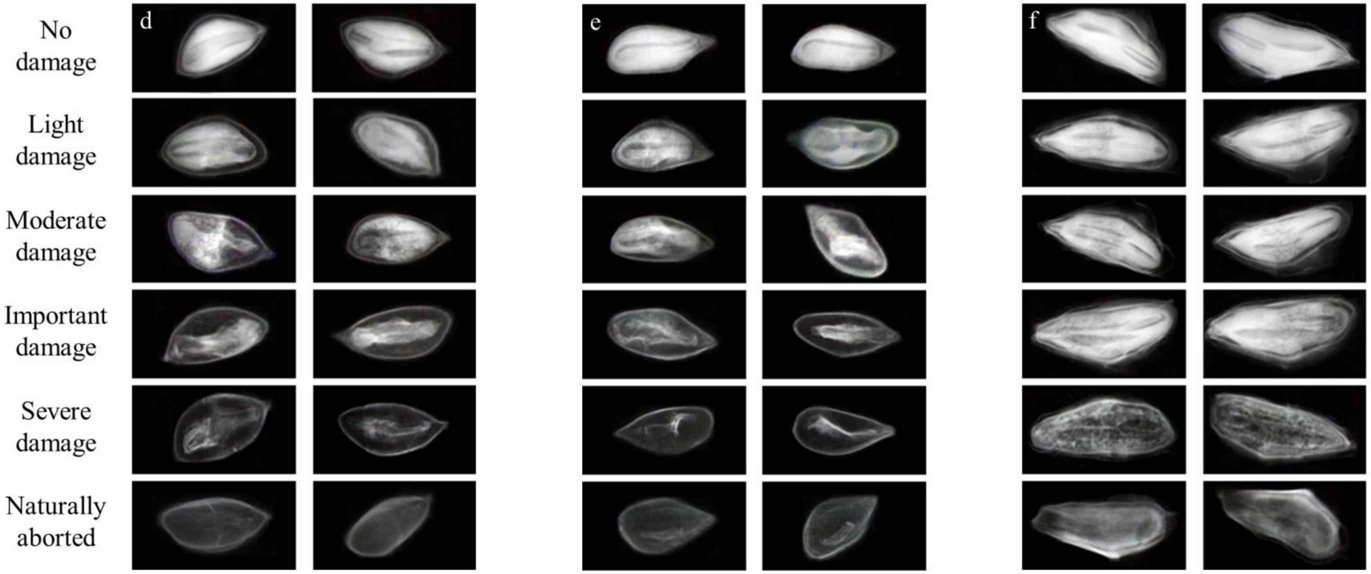

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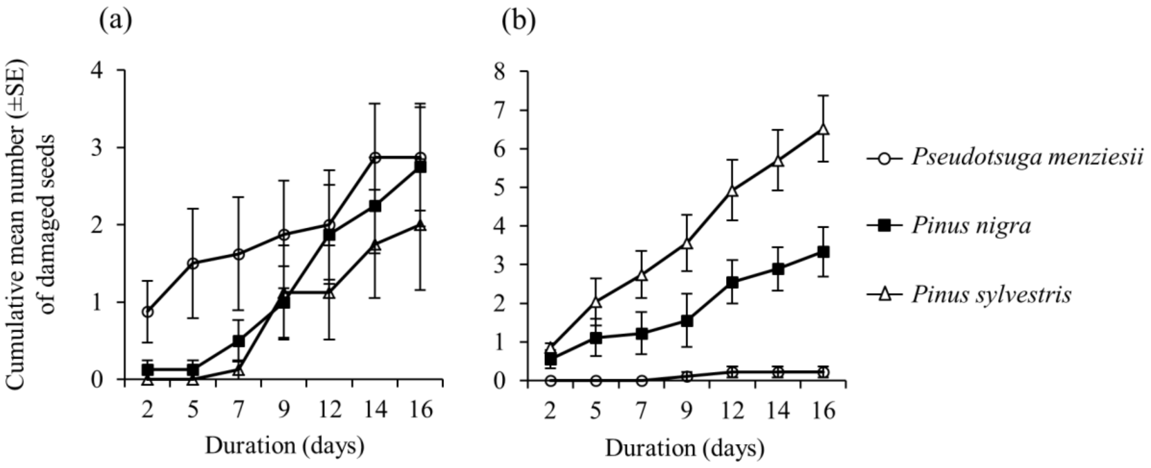
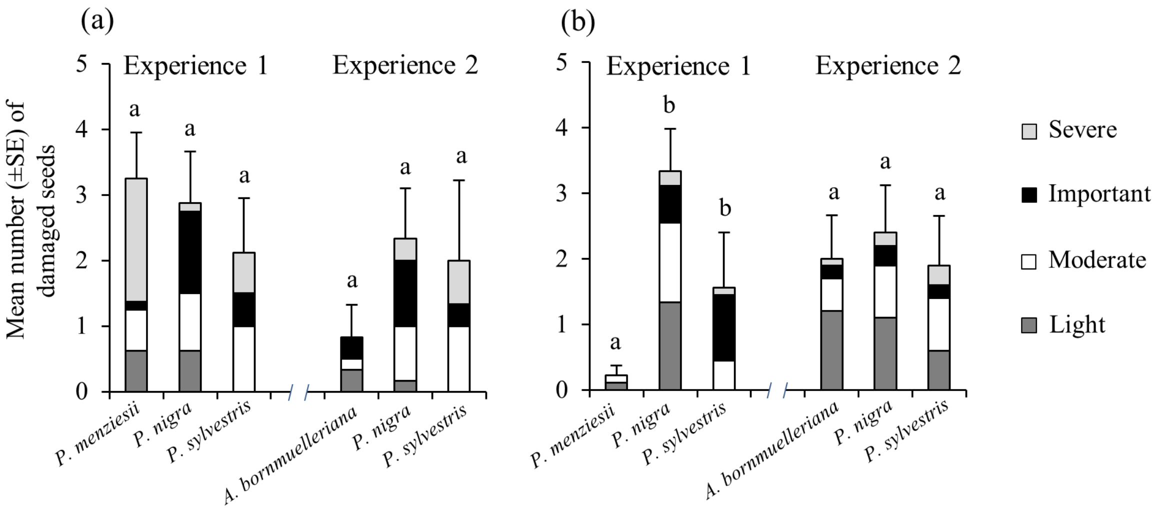
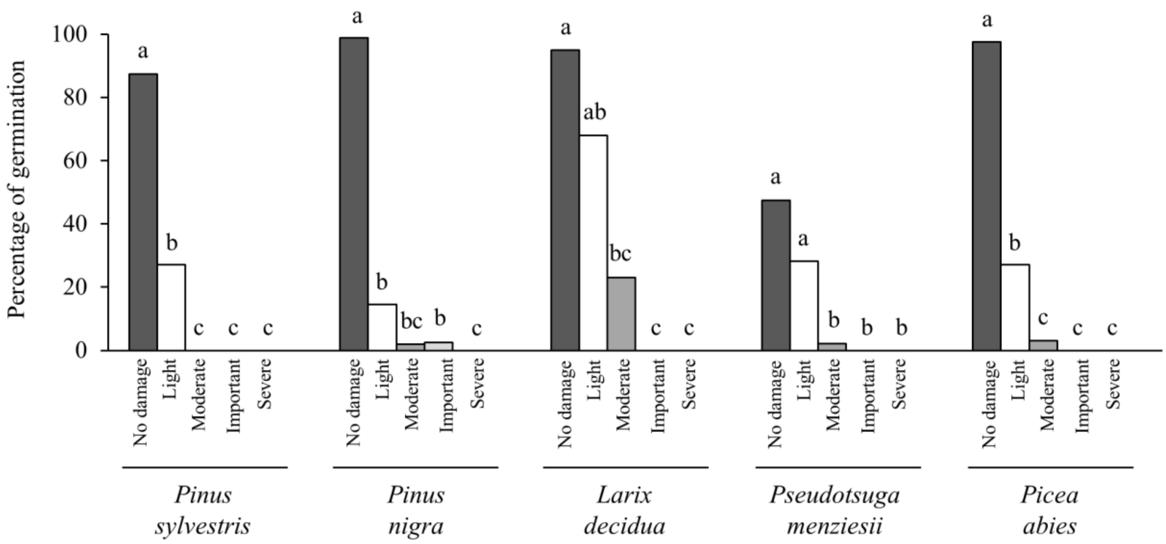
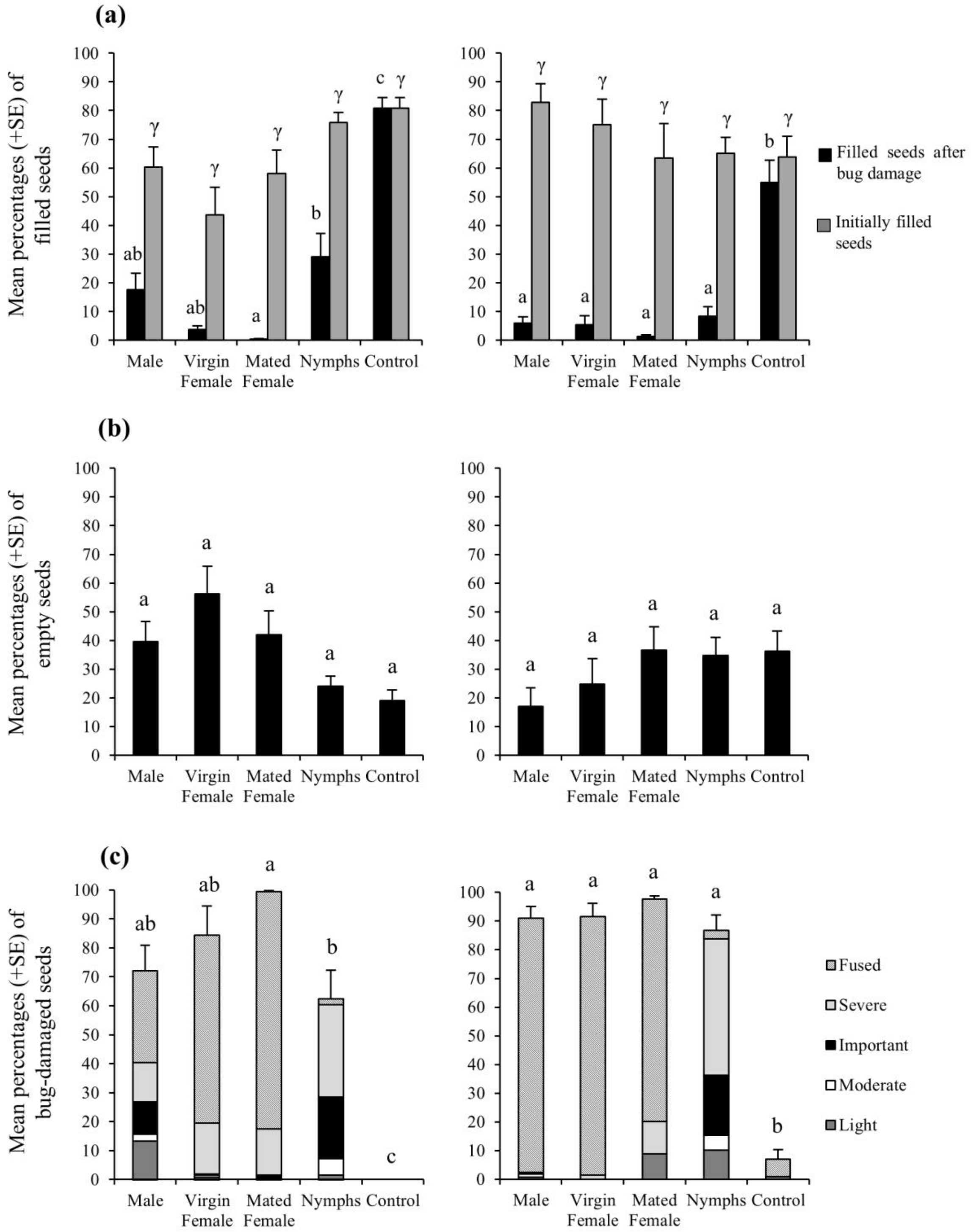
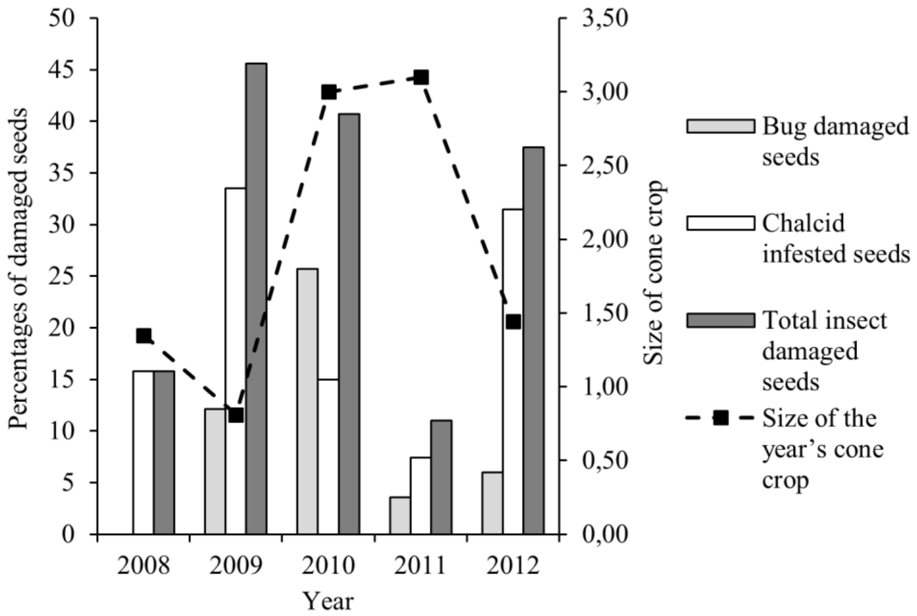

(a)
(b)
Cumulative mean number (±SE) of damaged seeds
Duration (days)
2 5 7 9 12 14 16
Pseudotsuga menziesii
Pinus nigra
Pinus sylvestris


(a)
(b)
Experience 1
Experience 2
Mean number (±SE) of damaged seeds
Severe
Important
Moderate
Light
P. menziesii
P. nigra
P. sylvestris
A. bornmuelleriana
P. nigra
P. sylvestris


Percentage of germination
No damage
Light
Moderate
Important
Severe
Pinus sylvestris
Pinus nigra
Larix decidua
Pseudotsuga menziesii
Picea abies


(a)
Mean percentages (+SE) of filled seeds
Male
Virgin Female
Mated Female
Nymphs
Control
ab
ab
a
b
c
γ
a
b
Filled seeds after bug damage
Initially filled seeds
(b)
Mean percentages (+SE) of empty seeds
a
(c)
Mean percentages (+SE) of bug-damaged seeds
ab
ab
a
b
c
a
b
Fused
Severe
Important
Moderate
Light



50
45
40
35
30
25
20
15
10
5
0
Percentages of damaged seeds
3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,00
Size of cone crop
2008
2009
2010
2011
2012
Year
Bug damaged seeds
Chalcid infested seeds
Total insect damaged seeds
Size of the year's cone crop

6

Type of sampled site	Location	Species	Total number of seeds examined			Mean number of seeds per cone			% Empty		
			2010	2011	2012	2010	2011	2012	2010	2011	2012
Seed	Calviac	*Picea abies*	8730	-	1461	178,16	-	48,7	29,99	-	59,81
Orchards	44°54' N; 2° 3' E	*Pseudotsuga menziesii*	2614	1023	-	52,28	34,10	-	39,29	41,75	-
	Sousceyrac	*Abies bornmülleriana*	7618	6459	2493	253,93	229,47	249,30	40,08	86,01	71,64
	44°51' N; 2° 6' E	*Pseudotsuga menziesii*	2329	912	-	46,58	30,40	-	36,69	32,57	-
	Lavercantière	*Larix decidua*	2631	768	1960	52,62	38,40	39,20	88,39	93,15	82,74
	44°37' N; 1°20' E	*Pinus nigra*	1260	779	1085	25,20	21,69	36,17	52,27	20,32	18,90
		Pinus sylestris	989	306	824	19,78	9,87	27,47	45,45	24,70	44,86
		Pseudotsuga menziesii	1626	1647	1144	31,62	32,94	22,88	65,99	43,22	80,90
Alpine	Merdanel	*Pinus nigra*	1838	2273	2130	36,76	45,31	43,36	50,19	33,04	37,21
stands	44°41' N; 6°37' E	*Pinus sylestris*	1193	825	934	23,86	16,50	21,55	28,78	45,83	51,68
	Serre Ponçon	*Pinus nigra*	606	1783	1385	24,56	35,66	27,70	53,87	24,06	55,30
	44°31' N; 6°20' E	*Pinus sylestris*	931	885	1073	18,62	17,35	21,46	39,82	55,00	71,24

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						Insect Damage					
% Fully available †			% Filled			% Bug ‡			% Chalcid *		
2010	2011	2012	2010	2011	2012	2010	2011	2012	2010	2011	2012
70,01	-	40,19	69,21	-	19,17	1,10	-	1,08	0,00	-	0,00
60,71	58,25	-	57,13	54,13	-	2,93	4,52	-	3,37	3,94	-
59,92	13,99	28,36	58,15	9,06	26,25	1,17	2,68	1,20	2,90	22,71	2,15
63,31	67,43	-	60,28	64,51	-	3,73	2,41	-	0,77	1,04	-
11,61	6,85	17,26	11,12	1,85	16,99	6,30	0,00	1,39	0,00	0,00	0,00
47,73	79,68	81,10	40,48	67,51	78,62	14,10	15,75	3,18	NA	NA	NA
54,55	75,30	55,14	50,94	63,72	51,69	9,38	15,66	6,15	NA	NA	NA
34,01	56,78	19,10	21,27	51,76	8,91	25,73	3,59	6,02	14,98	7,39	31,48
49,81	66,96	62,79	48,90	57,70	57,45	2,04	14,27	8,92	NA	NA	NA
50,19	33,04	37,21	67,94	23,80	40,05	4,80	53,55	19,05	NA	NA	NA
46,13	75,94	44,7	23,86	17,71	23,65	43,68	77,36	44,18	NA	NA	NA
53,87	45,00	55,3	46,59	10,87	12,48	24,23	70,11	33,55	NA	NA	NA

† Mean % of fully available seeds per cone was calculated as the number of seeds considered as available for insect development (i.e. filled-seeds plus insect damaged seeds) on the total number of seeds

‡ Mean % of bug damage was calculated as the ratio of bug damaged seeds on the number of seeds considered as available for insect development (i.e. filled-seed plus insect-damaged seeds)

* Mean % of chalcid infestation was calculated as the ratio of chalcid-infested seeds on the number of seeds considered as available for insect development (i.e. filled-seed plus insect-damaged seeds)

NA: No Megastigmus species is known to infest seeds of *P. nigra* and *P. sylvestris*

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Species	No damage	Light	Moderate	Important	Severe	Empty
Abies bornmülleriana	99,46 a	99,65 a	80,70 ab	69,19 b	69,49 b	64,11 b
Pinus sylestris	8,22 a	6,02 b	4,50 c	3,55 d	3,01 d	2,41 e
Pinus nigra	16,16 a	13,87 a	9,16 b	8,66 bc	7,25 cd	6,26 d
Larix decidua	9,67 a	8,08 ab	7,20 b	6,84 b	6,70 b	6,57 b
Pseudotsuga menziesii	12,25 a	10,17 b	8,28 c	5,94 d	4,56 e	5,11 e
Picea abies	9,09 a	6,79 b	5,05 c	4,49 c	3,67 d	2,67 e

Means followed by different letters within lines indicate a significant difference ($P < 0.05$,Tukey-Kramer HSD test)

	Pinus sylvestris		*Pseudotsuga menziesii*	
Variables	r^2	*P*	r^2	*P*
Size of the year's cone crop	-0.494	0.671	0.228	0.771
Size of the previous cone crop	-0.982	0.122	-0.909	0.091
Change in cone crop	-0.032	0.979	0.894	0.041 *

Significant relationship is asterisked.

Variables	r^2	*P*
Size of the year's cone crop	-0.814	0.093
Size of the previous cone crop	-0.210	0.735
Change in cone crop	-0.367	0.543
Leptoglossus damage of the year	0.061	0.922
Leptoglossus damage of the previous year	-0.963	0.037*

Significant relationship is asterisked.