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Plant neighbour identity and invasive pathogen infection affect associational resistance to an invasive gall wasp

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Abstract Theory predicts that mixed forests are more resistant to native pests than pure forests (i.e. associational resistance) because of reduced host accessibility and increased top-down control by natural enemies. Yet, whether the same mechanisms also apply to invasive pests remains to be verified. We tested the hypothesis of associational resistance against the invasive Asian chestnut gall wasp (ACGW, *Dryocosmus kuriphilus*) by comparing ACGW infestation rates on chestnuts (*Castanea sativa*) in stands varying in species composition (chestnut alone or associated with oaks, pines or ashes). We investigated the effects of reduced chestnut density and frequency in mixed stands, as well as the effect of biotic interactions between ACGW, its parasitoids and the chestnut blight disease (caused by *Cryphonectria parasitica*). ACGW infestation rates were significantly lower in chestnut–oak and chestnut–ash mixtures than in pure chestnut stands and chestnut–pine mixtures. Infestation rate decreased with decreasing chestnut relative proportion. The composition of native parasitoid communities emerged from galls significantly differed between pure and mixed chestnut stands, but not the species richness or abundance of parasitoids. The abundance of the introduced parasitoid *Torymus sinensis* was not correlated with ACGW infestation rates and was independent of stand composition. Blight symptoms modified ACGW infestation rates with taller trees being preferred when they were asymptomatic but avoided when they presented blight disease damage. Our results suggest that conservation biological control based on tree species mixtures could contribute to reducing the damage of invasive forest pests.

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Introduction

Tree diversity provides support to multiple functions and services in forest ecosystems (Balvanera et al. 2006; Gamfeldt et al. 2013). Yet, biodiversity is increasingly threatened by invasive species, which have already caused alarming decline and local extinctions of native species (Clavero and Garcia-berthou 2005; Galil 2007). A common view in invasion ecology is that communities with higher species richness are more resistant to the establishment and spread of invasive species (the diversity–invasibility relationship, Elton 1958). This idea received support (Case 1990; Erneberg 1999; Kennedy et al. 2002) mainly from plant ecologists (Levine et al. 2004), while much less is known about ecosystem resistance to invasive pest insects (Wilsey and Polley 2002). A few recent studies suggest that tree diversity in native communities may reduce the establishment and spread of invasive pests (Rigot et al. 2014; Guyot et al. 2015), but identifying the underlying mechanisms remains a major challenge (Nunez-Mir et al. 2017).

Associational resistance (*AR*) is a likely mechanism explaining the greater resistance of species-rich plant communities to invasion by herbivorous insects. *AR* describes the lower risk of a given plant being infested when surrounded by heterospecific neighbours (Jactel and Brockerhoff 2007; Barbosa et al. 2009). Although the opposite pattern—associational susceptibility (*AS*)—can also be observed (White and Whitham 2000; Schuldt et al. 2015), AR seems a more common phenomenon in forest ecosystems (Jactel and Brockerhoff 2007; Castagneyrol et al. 2014a; but see Kambach et al. 2016). *AR* has been described mainly for native pest species but it can be hypothesized that similar mechanisms are involved for invasive pests (Jactel et al. 2006; Guyot et al. 2015).

AR primarily occurs when the presence of heterospecific neighbours reduces the probability of a plant being colonized by herbivores, because these neighbours can reduce host plant concentration (i.e. species-specific density), frequency (i.e. relative

proportion) and apparency (i.e. relative size). These mechanisms, however, are more likely to explain AR to herbivore specialists, with heterospecific neighbours being unsuitable hosts (Castagneyrol et al. 2014a, b). The "resource concentration hypothesis" (Tahvanainen and Root 1972; Hambäck et al. 2014) posits that herbivores are more able to locate and remain on hosts that are growing in high density or in nearly pure stands than in more diverse stands where their host plants are more diluted. How easily a host plant is found by herbivores, i.e. its apparency (Strauss et al. 2015), has been described as a key mechanism driving AR (Castagneyrol et al. 2013). Both visual (Dulaurent et al. 2012; Damien et al. 2016) and chemical (Zhang and Schlyter 2004; Jactel et al. 2011) cues used by insect herbivores to locate and colonize a host tree can be disrupted by the presence of heterospecific neighbours.

Host apparency may further be altered by biotic and abiotic factors interacting with plant diversity. For instance, fungal infections may modify plant quality (e.g., Hatcher et al. 1994; Cardoza et al. 2003), defensive traits (e.g., Cardoza et al. 2003), or even the emission of plant volatile compounds (e.g., Cardoza et al. 2002), that can be used by herbivores' natural enemies (e.g., Tack et al. 2012), resulting in the potential reduction of herbivorous insect performance and preference for a determined host plant (Tack and Dicke 2013). Infection of a host tree by fungal pathogens is thus an important but often neglected factor that can drive associational resistance. For instance, Schuldt et al. (2017) reported a positive relationship between damage of foliar herbivores and pathogens, which resulted in additive negative effects on tree growth. However tree species richness lead to *AS* to insects but *AR* to pathogens, with an overall reduced impact of both damaging agents combined on tree growth.

Associational effects may also result from top-down biotic interactions involving herbivores' enemies. The "enemies hypothesis" (Elton 1958; Root 1973) posits that plant communities with higher species richness provide more resources and habitats and thus can shelter more diverse predator or parasitoid communities (Wilby and Thomas 2002; Schuldt et al. 2011; Castagneyrol and Jactel 2012), which could in turn provide a better control of herbivore populations (Riihimäki et al. 2005; Leles et al. 2017). A spill-over of natural enemies from

associated to target trees is expected if associated and target trees share common or alternative prey or hosts (Cappuccino et al. 1998). However, a greater abundance or diversity of herbivores' enemies does not necessarily result in higher predation rates as there is a wide range of enemy–enemy interactions that can be positive, negative or neutral (reviewed by Letourneau et al. 2009). Moreover, one of the proposed reasons for the success of alien herbivores (Shea and Chesson 2002) relates to the "enemy release hypothesis" (Keane and Crawley 2002) which states that their co-evolved natural enemies are virtually absent in newly colonized areas (Meijer et al. 2016). By contrast, the "exotic prey naïveté hypothesis" (Sih et al. 2010) posits that exotic, naïve species may suffer from higher predation pressures than native prey species because of its naïveté towards native enemies (Li et al. 2011). However, a few studies have taken into account pressure by native natural enemies on exotic species in invaded areas (but see Cox and Lima 2006; Li et al. 2011; Cabrera-Guzmán et al. 2012; Wilcox and Fletcher 2016).

Since 2002, European chestnut (*Castanea sativa*, Mill.) forests and orchards have been severely affected by the Asian chestnut gall wasp (ACGW), *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae). ACGW is considered the most important pest of *Castanea* species worldwide. It can cause chestnut productivity losses up to 80% (Battisti et al. 2014). Severe and repeated infestations on young trees can even lead to tree death (Moriya et al. 2003). Chestnut tree mortality may also occur when ACGW infestations are combined with strong chestnut blight infections (*Cryphonectria parasitica,* Murrill). ACGW is a micro-Hymenoptera (2–3 mm) native to China, which was introduced to Italy in early the 2000's from where it colonized almost all the European chestnut geographical range (Aebi et al. 2007; Quacchia et al. 2008). All members of the Cynipidae family are obligatory parasites of plants, either by inducing gall formation or by developing as inquilines within galls induced by other gall wasps (Stone et al. 2002).

Guyot et al. (2015) showed that crown defoliation caused by ACGW was significantly reduced in mixed chestnut stands compared to pure stands. However, little is known about underlying mechanisms of this *AR* and particularly the role of other interacting organisms. For example, the chestnut blight, is now widely spread in all chestnut forests in Europe

(Rigling and Prospero 2017). This invasive fungus causes extensive necrosis in the cortical tissue (cankers) which can girdle trunks and branches and result in the death of the distal part. Recently, Meyer et al. (2015) showed that attacks by ACGW could increase the incidence of chestnut blight, because the latter can colonize abandoned galls and then establish in shoots usually not affected by the fungus. A reciprocal effect of chestnut blight on ACGW searching behavior may also occur but has not been documented yet. Interactions between two invaders in a new area may result in an 'invasional meltdown' process, i.e. invader–invader mutualism (Simberloff and Von Holle 1999). In particular a non-native pathogen (e.g., chestnut blight) may facilitate the establishment of a subsequent invader (e.g., ACGW), leading to exacerbated impact on native ecosystems (Simberloff and Von Holle 1999; Simberloff 2006). However the associational effects on co-occurring invasive pests remain largely unknown.

Here, we further investigate the pattern of reduced chestnut crown defoliation by ACGW in mixed stands found by Guyot et al. (2015) by examining whether ACGW infestation rates are altered by the identity of co-occurring tree species, tree apparency, or by biotic interactions among ACGW, its parasitoids, and chestnut blight disease. In particular, we predicted that: (1) heterospecific neighbours trigger *AR* to ACGW; (2) *AR* results from the dilution, i.e. reduced density and relative abundance, of chestnuts in mixed stands, (3) *AR* is stronger in the presence of tree species also infected by Cynipidae galls that are likely to share parasitoids with ACGW, in particular *Quercus* species, and (4) infection by chestnut blight may modify ACGW infestation through changes in chestnut apparency. To test these hypotheses, we assessed ACGW infestation in chestnut trees naturally growing in pure versus two-species mixtures of different compositions. In the mixed stands, chestnut trees were associated with one of the three following native tree species: the turkey oak (*Quercus cerris* L., Fagaceae), a broadleaved species colonized by a high diversity of oak cynipid gall wasps, with associated parasitoids that have been already observed in ACGW galls (Aebi et al. 2007) and for which ACGW could present a "naïve prey"; the flowering ash (*Fraxinus ornus* L., Oleaceae), another broadleaved tree which is not known to be attacked by cynipid gall wasps, and the maritime pine (*Pinus pinaster* Aiton, Pinaceae), a

conifer for which no cynipid gall maker is known and which is phylogenetically much more distant from chestnut than ash is. In each forest plot, we measured the density and relative abundance of chestnut trees. On each sampled chestnut tree we estimated ACGW infestation, chestnut blight severity, tree height and collected galls of ACGW to characterize parasitoids community.

Materials and methods

Study species

The current distribution of European chestnut ranges from Southern Europe (Iberian Peninsula, Italy, Balkans, Mediterranean islands) and North Africa (Morocco), to North-Western Europe (England, Belgium, Netherlands) and eastward to Western Asia (North East Turkey, Armenia, Georgia, Azerbaijan, Syria) (Conedera et al. 2016).

ACGW is a thelytokous and univoltine species (i.e. its populations are composed only by females which reproduce asexually once a year). At the time of chestnut bud-burst (around mid-April), ACGW larvae induce the formation of galls on different vegetative organs (Maltoni et al. 2012) within which they feed, develop and pupate. Between mid-June and mid-August adults hatch from the galls and lay eggs in new developing chestnut buds. Adult longevity can be up to 10 days. Following eggs hatching in summer, first instar larvae overwinter within dormant buds until the following spring (Bernardo et al. 2013).

ACGW populations are mainly regulated by hymenopteran parasitoids (Cooper and Rieske 2007). In Europe, ACGW galls are parasitized by native parasitoids of oak gall wasps (Panzavolta et al. 2013; Palmeri et al. 2014; Francati et al. 2015), and by a parasitoid wasp from its natural range, *Torymus sinensis* (Hymenoptera, Torymidae), which has been introduced in Europe as a biocontrol agent (see also Aebi et al. 2007; Borowiec et al. 2014; Matošević et al. 2014). Native to China, this parasitoid is univoltine (with about 3% of its population having a 12 months diapause period, Quacchia et al. 2014a, b; Ferracini et al. 2015), with adults emerging in early spring and females laying eggs in newly-formed ACGW galls. The ectoparasitoid larva feeds on the host larva and adults emerge in the following spring (Quacchia et al. 2014a).

Cryphonectria parasitica is an invasive plant pathogen native to Eastern Asia (Rigling and Prospero 2017). Its native range partly overlaps with that of ACGW in China (Zhang et al. 2009). This necrotrophic pathogenic fungus infects in the cortical tissues, causing extensive necrosis and cankers which can girdle trunks and branches. This results in the blight and then the death of the distal part (Rigling and Prospero 2017). Blight was first reported in American chestnut (*Castanea dentata*) in the United States in 1904 and in European chestnuts in Italy in 1938 (Rigling and Prospero 2017). It is a necrotrophic pathogen, which colonizes bark and cambium of chestnut trees through wounds, thereby destroying the function of these tissues in susceptible chestnut species such as *Castanea sativa* (Rigling and Prospero 2017).

Study area

The study was performed in natural forest stands in Southern Tuscany (Italy). These forests are mainly composed of chestnut (*Castanea sativa*), European hornbeam (*Ostrya carpinifolia*), oaks (*Quercus ilex, Q. petraea, Q. suber, Q. pubescens* and *Q. cerris*), ash (*Fraxinus ornus*) and pines (*Pinus pinaster*). In these stands, the chestnut was part of the original mixed broadleaved forests (native), then transformed into mostly pure stands for the production of wood and fruits. ACGW was officially reported in the study area for the first time in 2009 and *T. sinensis* was first released in 2010 (Maltoni, *personal communication*).

In February 2015, we selected 32 forest plots (30 × 30 m) representing 10 chestnut monocultures and 22 two-species mixtures (Fig. S1). Mixtures associated chestnut with *Q. cerris* (n = 10), *F. ornus* (n = 6) or *P. pinaster* (n = 6). Mean plot elevation was 438 m above sea level. All plots were in similar conditions of soil and type of parent rock, e.g., siliceous crystalline quartzites and anagenites of "Verrucano" formation that are widespread in the study area (Lazzarotto 1993; Carmignani and Lazzarotto 2004). Soils deriving from this formation belong to the broad category of Cambisols (V.V.A.A. 2008), and are generally nutrient-poor and subject to acidification and moderate summer drought. Comparable site conditions among plots was also recently

verified for the purpose of the FundivEurope project which involved the study of 36 forest plots close to those used in the present study (Baeten et al. 2013). Plots are located in the same vicinity as the study by Guyot et al. (2015), although only one plot from their study was used here.

In each plot, we sampled three focal chestnut trees, with either conspecific neighbours only (in monocultures) or both conspecific and heterospecific (in mixtures) neighbours in their immediate vicinity (i.e. tree canopies overlap; total: n = 96 sampled chestnut trees). Spatial coordinates of each focal chestnut tree and corners of plots were recorded using a Trimble® Geo 7X. We measured the diameter at breast height of all trees present in the plots. We also measured the total height of each focal chestnut tree, which was comparable for each type of plot composition (Table 1; see Table S1 for more details).

Assessment of blight symptoms

Severity of chestnut blight was assessed by recording cankers on stems and main branches. *Cryphonectria parasitica* was isolated in all accessible cankers, confirming they were caused by this pathogenic fungus.

Assessment of ACGW infestation

In May 2015, we collected 15 branches per focal chestnut tree, randomly selected around the tree crown. For each collected branch, we recorded the number of galls per shoot in all new shoots. New shoots, i.e. those produced during spring 2015, corresponded to buds of the previous year, i.e. those susceptible to be attacked by ACGW the previous year. As such, gall infestation observed in 2015 corresponded to eggs laid in summer 2014. Infestation rate by ACGW was estimated as the number of infested shoots (i.e. presenting at least one gall).

Parasitoid collection

In winter 2015, we randomly collected 25 galls on eight branches per focal chestnut tree (total: n = 200 galls per tree). In laboratory "winter galls" were stored in cardboard boxes (one box per tree) with extractable tubes exposed to light. Boxes were maintained in a climatic chamber at 25 °C, with 12 h daylight. Boxes were surveyed every 2–3 days and emerged parasitoids were collected, stored at − 20 °C, and then transferred in 96% ethanol for further identification. In May 2015, we also collected 100 green galls per tree. These "spring galls" were surveyed for parasitoid emergence as described above for the winter galls.

Parasitoids emerging from winter and spring galls were identified at least to the genus level under a stereomicroscope (Leica M205C). Winter galls were expected to host overwintering parasitoids, and in particular *T. sinensis*. Spring galls were sampled to collect native parasitoids of oak gall wasps that were expected to parasitize ACGW larvae. They belong to six main families of chalcid wasps (Eulophidae, Eupelmidae, Eurytomidae, Ormyridae, Pteromalidae, Torymidae), and have been identified on oak cynipids (Askew et al. 2013) or on ACGW (Aebi et al. 2006, 2007; Panzavolta et al. 2013; Al Khatib et al.

Table 1 Mean (± SD) plot characteristics for each type of composition

Plot composition	Chestnut basal area (cm²/m² plot)	Total trees basal area (cm²/m² plot)	Chestnut relative proportion (in basal area)	Focal chestnut height (m)	*C. parasitica* symptomatic trees
CS (n = 10)	44.59 ± 44.58	45.66 ± 45.18	0.97 ± 0.05	11.92 ± 1.83	60% (n = 30)
CS–FO (n = 6)	13.50 ± 11.52	24.29 ± 28.32	0.62 ± 0.23	10.36 ± 3.85	56% (n = 18)
CS–PP (n = 6)	43.80 ± 32.86	70.92 ± 43.17	0.60 ± 0.20	10.89 ± 4.33	72% (n = 18)
CS–QC (n = 10)	23.35 ± 30.13	46.24 ± 29.31	0.38 ± 0.26	10.13 ± 3.03	50% (n = 30)

CS, Castanea sativa (monocultures); *CS–PP, C. sativa + P. pinaster*; *CS–FO, C. sativa + Fraxinus ornus*; *CS–QC, C. sativa + Quercus cerris*

2014, 2015, 2016; Palmeri et al. 2014). It is important to note that for some wasps, molecular characterization (Cytochrome Oxydase I) revealed the presence of probable cryptic species (Borowiec, unpubl. data), especially for *Ormyrus nitidulus* and *O. pomaceus* (Kaartinen et al. 2010; Lotfalizadeh et al. 2012; Goméz et al. 2017). For this reason, specimens of *Ormyrus* were not identified at species level.

Statistical analysis

General approach. Data analysis was performed with generalized linear mixed models (GLMMs) following the procedure recommended by Zuur et al. (2009). Plot identity was included as a random factor (1|Plot.ID in *R* syntax) to account for the correlated data structure arising from the three replicate focal trees sampled per plot (Schielzeth and Nakagawa 2013). Significance of parameters was assessed using χ^2 tests by comparing models with and without the term to be tested. We applied model simplification by starting with the highest order interaction and sequentially removing non-significant predictors. After the most parsimonious model had been reached, parameter estimates corresponding to fixed effects were estimated by restricted maximum likelihood (REML). Contrast analyses were used to compare levels of significant factors. To estimate model fit, R^2 were calculated following Nakagawa and Schielzeth (2013). For each model, we calculated the marginal R^2 (R_m^2, corresponding to the proportion of variance explained by fixed effects) and the conditional R^2 (R_c^2, corresponding to variance explained by fixed *plus* random effects). All analyses were conducted in 3.2.3 version of R (R Core Team 2015), using the g*lmer* function from the *lme4* package (Bates et al. 2015).

First, we tested the effect of chestnut forest composition as a categorical fixed-effect factor with four levels (pure chestnut stand, CS; mixture of chestnut and ash, CS–FO; mixture chestnut and pine, CS–PP; mixture chestnut and oak, CS–QC) on ACGW infestation. We analyzed ACGW infestation rate as the proportion of shoots with at least one gall by using a bivariate response variable consisting in number of shoots with at least one gall versus number of shoots with no gall. We used a GLMM with binomial error distribution and logit function such that fitted values were bounded between 0 and 1.

Second, to assess mechanisms that might explain the effect of chestnut forest composition, we ran another model with the general structure described above but with different predictors. This model included three types of predictors referring to (1) tree species composition, (2) bottom-up and (3) top-down associational resistance effects. The composition effect (1) was described by two continuous variables: chestnut density (i.e. chestnut basal area) and chestnut frequency, as its relative proportion to other species in the plot (measured as the ratio between chestnut basal area and basal area of all tree species present in the plot). Bottom-up processes (2) were accounted for, at the tree level, by including as fixed effects the presence/absence of blight symptoms, chestnut height and their interaction. Top-down processes (3) were accounted for, at the tree level, by including the number of parasitoids that emerged from winter and spring galls as separate fixed effects. This approach resulted in one model that was simplified as explained above. Despite the weak correlation between chestnut density and proportion (Pearson's $r = 0.22$), both were introduced in the same model (Dormann et al. 2013). A marginal test of predictor significance was used by testing whether some variance remained explained by each predictor once other sources of variation in the response variable were accounted for. *Torymus sinensis* was the most common parasitoid emerging from winter galls (94% of individuals). So, in order to avoid correlation between *T. sinensis* and total winter parasitoid abundance, the effect of *T. sinensis* as biocontrol agent on ACGW was specifically tested in a separated model with ACGW infestation rates as a binomial response variable, as explained above.

Third, we analyzed the effect of plot composition on parasitoid abundance (number of parasitoids from winter galls per tree in one model, number of *T. sinensis* specifically in a second one, and number of parasitoids from spring galls in a third one) with a *Poisson* error distribution.

Finally, a non-metric multidimensional scaling (NMDS) was performed on parasitoid data in order to test whether and how the composition of spring gall parasitoid communities varied according to the tree species composition of plots. This was not investigated with the communities of parasitoids emerging from winter galls because they were dominated by *T. sinensis*. The significance of tree composition in the

plot on parasitoid community composition was tested with a PERMANOVA (Anderson 2005) using the 'adonis' function from package 'vegan' (Oksanen et al. 2017). We performed 1000 permutations using Bray–Curtis' dissimilarity measure. Parasitoid diversity was estimated by calculating Shannon's index with the 'diversity' function. Significance of composition effect on Shannon indices was tested by using GLMMs as described above, using *Gaussian* error distribution, after linearization of the index to obtain the 'effective species number' (Jost 2006).

Results

All the 96 sampled chestnut trees were infested by ACGW. The proportion of shoots infested by ACGW (i.e. infestation rate) was on average 78% and varied between 30 and 100% per tree. Infestation rates significantly differed among plots of different tree species composition ($\chi^2 = 15.66$, $df = 3$, $P = 0.001$, $R_m^2 = 0.15$, $R_c^2 = 0.34$) being significantly lower in ash– and oak–chestnut mixtures as compared to chestnut monocultures (Fig. 1a). In pine–chestnut mixtures, infection rates were intermediate between chestnut monocultures and chestnut–ash mixtures (Fig. 1a).

The infestation rate by ACGW significantly increased with the relative proportion of chestnut trees in plots (Table 2, Fig. 1b), but was independent of chestnut density (Table 2).

The infestation rate by ACGW varied with chestnut blight symptoms in interaction with tree height (Table 2). In asymptomatic trees, ACGW infestation rate increased with chestnut tree height (Fig. 2; Table 2) while ACGW infestation rates decreased with chestnut height in symptomatic trees (Fig. 2; Table 2).

A total of 1619 parasitoids were collected from winter galls, with a mean number of 16 (varying from 0 to 300) parasitoids emerging from 200 winter galls per tree. Nine trees did not provide any parasitoid. In winter galls, *T. sinensis* represented 94% of total parasitoid abundance.

From spring galls, a total of 814 parasitoids emerged, with a mean number of 8 parasitoids per 100 galls (varying from 0 to 35). Ten trees had no parasitoids in their spring galls. The most abundant

species were *Torymus auratus* and *Torymus flavipes* that represented respectively 34 and 26% of the total number of parasitoids collected (see also Table S2 for additional information about parasitoids species found). The effect of tree species composition of plots was significant but explained only 16% of variance in the composition of parasitoid communities emerging from spring galls (PERMANOVA *pseudo-F* = 1.76, $P = 0.021$, Fig. 3).

The infestation rate by ACGW was independent of total parasitoid abundance in winter or spring galls (Table 2). ACGW infestation rate was also independent of *T. sinensis* abundance ($\chi^2 = 1.78$, $df = 1$, $P = 0.18$). Total parasitoid abundance was independent of plot composition for winter ($\chi^2 = 4.22$, $df = 3$, $P = 0.238$, $R_m^2 = 0.08$, $R_c^2 = 0.58$) and spring galls ($\chi^2 = 2.77$, $df = 3$, $P = 0.428$, $R_m^2 = 0.03$, $R_c^2 = 0.27$). The species richness and diversity of parasitoids were also independent of plot composition ($\chi^2 = 0.877$, $df = 3$, $P = 0.831$ and $\chi^2 = 1.08$, $df = 3$, $P = 0.781$ respectively; Table 3). The abundance of *T. sinensis* found in winter galls was also independent of plot composition ($\chi^2 = 6.31$, $df = 3$, $P = 0.098$).

Discussion

ACGW infestation rates depend on tree neighbour identity

We not only confirmed previous observations reporting *AR* to the ACGW (Guyot et al. 2015), we provide evidence that this effect is dependent on the identity of the tree species associated with chestnut trees. *AR* occurred only in the two-species mixtures where chestnut trees were associated with another broadleaved species, with infestation rates on average 13.9% lower than in monocultures (Fig. 1a). Such reduction in ACGW infestation may be important for chestnut survival, even if mean infestation rates in mixtures with oak and ashes were still high (71.5 and 74.1% respectively; Fig. 1a). Infestation rates in chestnut–pine mixtures, however, were comparable to that in monocultures (Fig. 1a). This last finding conflicts with the prediction that *AR* should be stronger in mixtures of more distantly related species such as mixtures of conifers and broadleaved species
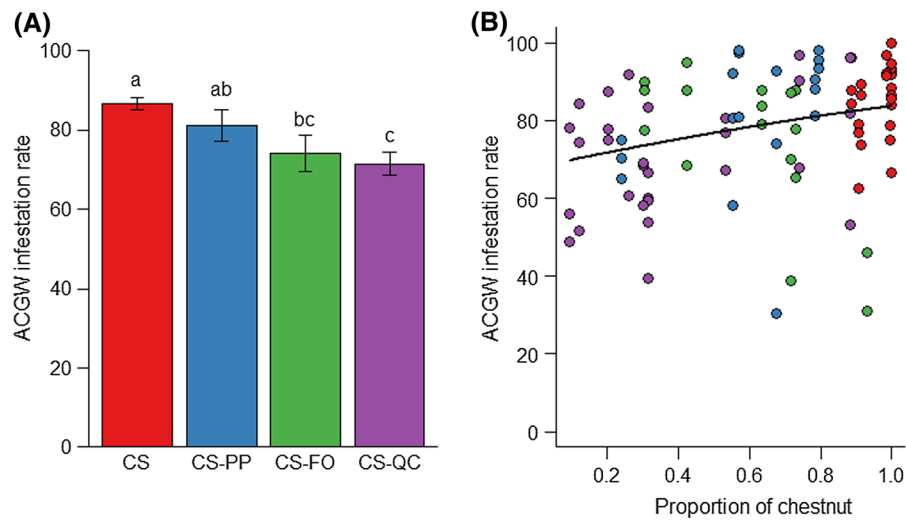
Fig. 1 Mean ACGW infestation rate per chestnut tree as a function of, **a** tree species composition and **b** chestnut frequency (i.e. relative proportion of chestnut basal area to basal area of all tree species in the plot). In **a**, different letters indicate significant differences between treatments (at $P < 0.05$). In **b** the line represents model predictions after back-transformation of the logit link applied to binomial GLMMs. *CS Castanea sativa* (monocultures); *CS–PP C. sativa + Pinus pinaster*; *CS–FO C. sativa + Fraxinus ornus*; *CS–QC C. sativa + Quercus cerris*. Colors in B correspond to tree species compositions in **a**

Table 2 Summary of generalized linear mixed effect model evaluating the effects of stand structure and composition, natural enemies pressure and two-way interactions, on ACGW infestation rate

Fixed effects	χ^2	df	P	Estimate	SE
Intercept				1.31	0.14
Chestnut density	2.24	1	0.137	0.20	0.13
Chestnut relative proportion	4.05	1	**0.046**	0.27	0.13
Chestnut blight symptoms (symptomatic)	5.82	1	**0.015**	− 0.21	**0.13**
Height	0.03	1	0.918	− 0.20	**0.09**
Parasitoids abundance in spring galls	0.09	1	0.838	0.01	**0.06**
Parasitoids abundance in winter galls	0.14	1	0.651	0.03	**0.07**
Chestnut density × relative proportion	0.65	1	0.419	− 0.16	**0.20**
Chestnut blight symptoms × height	16.49	1	**< 0.001**	− 0.47	**0.12**

Model parameter estimates and standard errors for the intercept correspond to the reference level for chestnut blight: asymptomatic trees. Marginal R^2_m represents the variance explained by fixed factors, while conditional R^2_c is interpreted as variance explained by both fixed and random factors. For the final model (retained after model selection), they equaled 0.14 and 0.36, respectively

Explanatory variables in bold characters had a significant effect for χ^2 values (at $P < 0.05$)

(Castagneyrol et al. 2014a). However, ACGW is a highly specialized pest with only one host species in the study area (Bernardo et al. 2013). In this context, Castagneyrol et al. (2014a) showed that host frequency should be the main driver of *AR*.

Host dilution in mixed stands resulted in lower ACGW infestation rates

Chestnut density per se had no significant effect on ACGW infestation rates (Table 2), which goes against the resource concentration hypothesis (Root 1973;

Fig. 2 AGCW infestation rate per chestnut tree as a function of chestnut height, in symptomatic versus asymptomatic chestnut blight trees. Lines represent model predictions after back-transformation of the logit link applied to binomial GLMMs. Different point colors represent different plot composition: chestnut monocultures (red) and pine (blue), ash (green) and oak (purple) two species chestnut mixtures
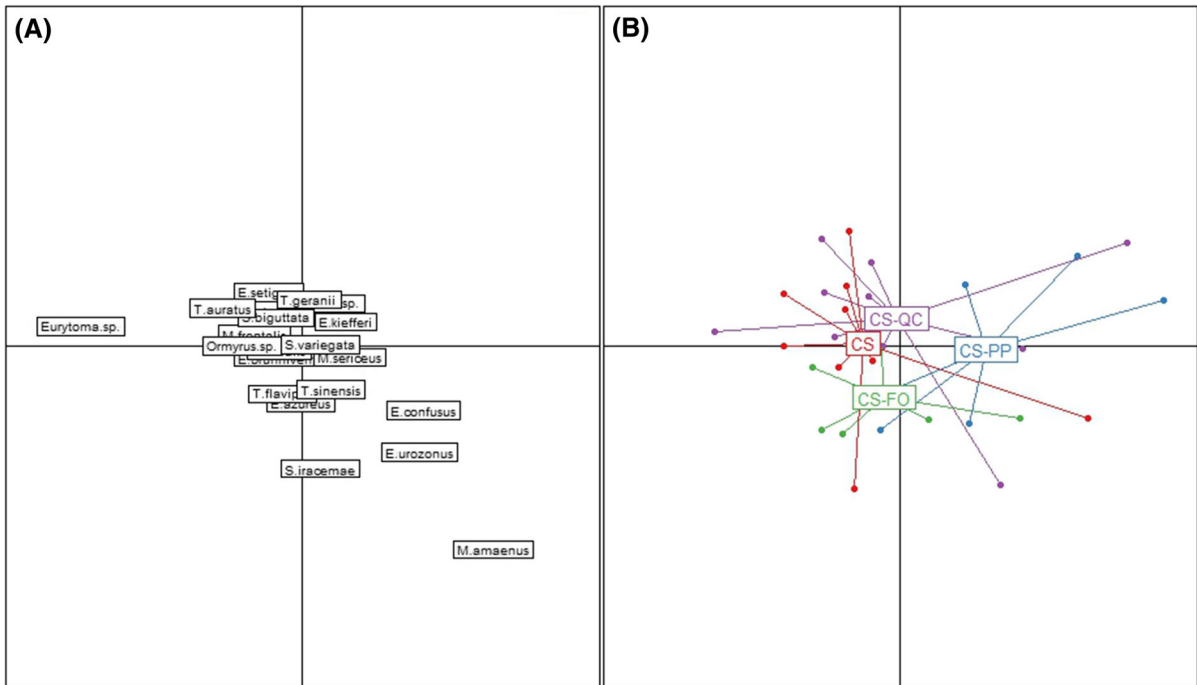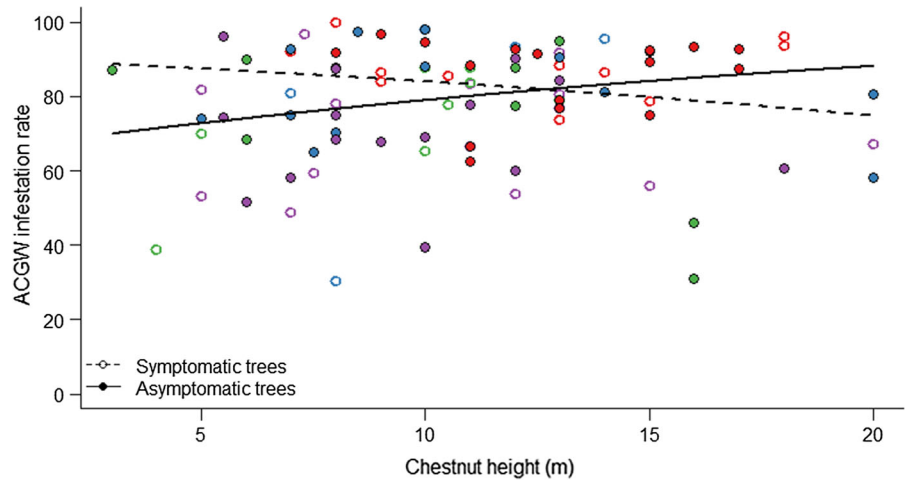

Fig. 3 NMDS of parasitoid communities from spring ACGW galls. In **a** species identity distribution and in **b** sites relative distribution. *CS*, *Castanea sativa* (monocultures); *CS–PP*, *C.* *sativa + Pinus pinaster*; *CS–FO*, *C. sativa + Fraxinus ornus*; *CS–QC*, *C. sativa + Quercus cerris*. The stress value associated with this representation was 0.167

Hambäck et al. 2014). However, we demonstrated that chestnut infestation by ACGW was reduced when chestnut was less frequent, i.e. more diluted among non-host neighbours (Fig. 1b; Table 2). An increasing dilution between non-host trees is an important driver of *AR* (Hambäck and Beckerman 2003; Jactel et al. 2006; Damien et al. 2016). This dilution effect on ACGW infestation rates could be explained by heterospecific neighbours acting as physical (Castagneyrol et al. 2014b; Damien et al. 2016) or chemical barriers (Tahvanainen and Root 1972; Randlkofer et al. 2010; Jactel et al. 2011) to chestnut localisation by ACGW *(see also* Germinara et al. 2011).

Table 3 Mean parasitoid abundance (± SE), species richness and Shannon diversity per plot for each plot composition: chestnuts monocultures (CS) and ash (CS–FO), pine (CS–PP) and oak (CS–QC) mixtures

	CS (n = 10)	CS–PP (n = 6)	CS–FO (n = 6)	CS–QC (n = 10)
Abundance	27.10 ± 1.30	15.00 ± 4.99	23.50 ± 4.65	27.10 ± 0.68
Species richness	5.70 ± 3.16	5.33 ± 0.99	5.00 ± 0.97	6.00 ± 0.68
Shannon diversity	1.23 ± 0.04	1.44 ± 0.20	1.16 ± 0.18	1.29 ± 0.13

Blight symptoms in interaction with chestnut apparency modifies ACGW infestations

In our study, we show that ACGW infestation rates were higher in taller trees than on smaller ones for trees without blight symptoms (Fig. 2). This is consistent with previous observations by Maltoni et al. (2012), who found that vigorous chestnut trees are more attacked than dominated or weak chestnuts. These results suggest that taller, more apparent and more vigorous trees are preferred by female ACGW for laying eggs. However, because we counted galls and not oviposition events, we cannot exclude that differences in observed infestation rates were driven by differential mortality after oviposition. It remains possible that ACGW females did not choose among stands and trees, but larvae developed more successfully in more vigorous trees.

However, the host apparency effect was not observed on trees severely infected by chestnut blight. In these trees, ACGW infestation rates were higher in smaller trees than in taller ones (Fig. 2). The presence of *C. parasitica* in cankers of American chestnut (*C. dentata*) results in stomatal closure, possibly as a direct result of xylem dysfunction (McManus and Ewers 1990), thus, in trees with blight symptoms water transport might be more impaired than in asymptomatic trees. This might be more severe in large than in small trees. As a consequence, foliar tissues might be of lower quality as breeding substrate for ACGW in large and blight symptomatic trees. Fungal infection of a plant can also modify host visual and chemical cues used for the insect to locate its host (Cardoza et al. 2002, 2003; Rizvi et al. 2015), thus interfering with tree apparency. Knowing how chestnut blight modifies chestnut traits used by ACGW to locate and exploit host trees (e.g., Germinara et al. 2011) would provide useful information about their combined impact.

The co-occurrence of blight and ACGW on smaller chestnut trees could be a case of invasional meltdown, with a more severe, combined adverse effect on survival and growth of young chestnut trees, thus jeopardizing natural regeneration. However, our data does not allow us to properly test such invasional meltdown as we could not reported independent impact of each exotic aggressors (all trees were infected by ACGW and likely by *C. parasitica*).

Abundances of ACGW parasitoids were independent of forest composition

Invasion success of exotic pests are usually attributed to the absence of co-evolved natural enemies in the new colonized area ('enemy release hypothesis', e.g., Keane and Crawley 2002). At the time of its establishment in Italy, ACGW found an 'enemy free space', allowing the pest to rapidly build up its populations. However few years later a spill-over of native parasitoids was already observed from oak gall onto ACGW galls (Aebi et al. 2006, 2007; Panzavolta et al. 2013), suggesting that ACGW became a 'naïve prey' (Sih et al. 2010) for local, native enemies.

Due to this spill-over process, we expected a better top-down control of ACGW by natural enemies in chestnut-oak mixed stands ('enemies hypothesis', Elton 1958; Root 1973). However, we did not detect any correlation between parasitoid abundance and ACGW infestation rate (Table 2), and the parasitoid abundance was independent of stand composition. We found native parasitoids in all stands (Fig. 3), even though no cynipid gall wasps are known on *Pinus* or *Fraxinus* species (Csoka et al. 2005). The more likely explanation is the presence of oak species (*Q. petraea*, *Q. pubescens*) in the vicinity of our plots, which was not controlled in our study, and may have represented nearby sources of native cynipid parasitoids.

Moreover, the parasitism rate by native species was on average of ca. 8 individuals per 100 galls, which was relatively low compared with the mean parasitism rate of oak cynipids as reported in the literature (e.g., Fernandes and Price 1992). This suggests that oak trees may have also acted as a sink for native parasitoids, i.e. oak galls being more suitable hosts. Some parasitoid species could have indeed avoided ACGW galls and concentrated on native hosts as infecting a new introduced host may incur a fitness cost for native parasitoids (Jones et al. 2015; Knoll et al. 2017).

We did not detect either an effect of *T. sinensis* abundance on ACGW infestation rate. However, the introduction of this exotic parasitoid, originating from the native area of ACGW in Asia, in Tuscany was relatively recent (2010), which is consistent with the low abundance of *T. sinensis* observed on ACGW galls (on average 15 parasitoids for 200 galls). Yet, it took 8 years following the first releases of *T. sinensis* to achieve an effective reduction of 75% of ACGW infestations in the Piedmont Region, Italy (Quacchia et al. 2014b). We may expect that the combined effects of native and exotic parasitoids will contribute to better reduce ACGW populations in the future. However, whether native parasitoids only (those produced by oak galls) would have been effective enough to control ACGW outbreaks in mixed forests remains an open question.

Last, it must be also acknowledged that parasitoids represent a fraction of ACGW' enemies and that variability in ACGW infestation rates could be explained by the effects of endophytic fungi (ex. *Gnomoniopsis castanea*, Vannini et al. 2017) or of competing generalist herbivores (Cooper and Rieske 2010; Tosi et al. 2015).

Conclusion

Our study provides new empirical data supporting the hypothesis that mixed forests are more resistant to biological invasions by exotic pests than tree monocultures. Our results suggest that the strength of associational resistance to invasive insects depends on both the quality (i.e. the identity of associated tree species) and the quantity of non-host trees in mixed stands. In addition, our observations suggest that complex biotic interactions might mediate these

mechanisms. For example, plant infection by a pathogenic fungi may modify *AR* to pests.

Classical biological control, i.e. the introduction of natural enemies native to the area of origin of the pest for its permanent establishment in a new area (Eilenberg et al. 2001), is the most common strategy used to regulate ACGW populations worldwide. However, although *T. sinensis* was present in winter galls from almost all trees that we sampled, the parasitism rates are still low and we thus observed a high level of ACGW damage, ranging from 30 to 100% infested shoots. Our findings suggest that forest management practices, based on tree species mixtures, would provide a complementary protection against ACGW, along the lines of the conservation biological control strategy (Barbosa 1998). As additional advantage of mixed-species forests represent a preventive method which might help to reduce the risk posed by other natural disturbances, including further biological invasions (Jactel et al. 2017).

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