Seed removal decrease by invasive Argentine ants in a High Nature Value farmland

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Abstract

Seed dispersal by ants is an important ecological process that maintains the structure and diversity of natural communities, however, it is vulnerable to biological invasions. Argentine ants are one of the worst invasive ant species and cause severe changes in ecosystem processes and native ant biodiversity declines in invaded sites. Here, we studied seed removal by ants combining observations and a cafeteria experiment with seeds of four myrmecochorous plant species (*Centaurea sphaerocephala, Rosmarinus officinalis*, *Silybum marianum*, and *Ulex australis*) in two sites (invaded and uninvaded) located in the Mediterranean *Montado* ecosystem and classified as High Nature Value farmland (HNV). Significant differences in daily seed removal rates were found between the two study sites. In uninvaded sites, several native ant species were attracted to the seeds, resulting in all seeds being removed rapidly. The majority of seed removal events were carried out by two key seed disperses *Pheidole pallidula* (71%) and *Aphaenogaster iberica* (26%) with a clear preference for diaspored with larger and heavier elaiosome (i.e., *C. sphaerocephala*, *S. marianum*). By contrast, while the Argentine ant showed some interest (68% of seeds were interacted with), no seed removal events were observed. The extirpation of the local ant fauna by the Argentine ant and its inability to ensure seed dispersal services may lead to the interference and eventually to the collapse of seed dispersal of the four studied myrmecochorous plants in the invaded site in the future. We argue that

these discrete but severe consequences of an invasive species on a key ecological process may strongly affect the functioning of the *Montado* ecosystem.

Key words: seed dispersal, Argentine ant, myrmecochory, *Montado* ecosystem, seed removal collapse, mutualism disruption

Introduction

Over 11,000 plant species worldwide rely on ants for their seed dispersal (Lengyel *et al.*, 2010), a mutualistic process named myrmechocory. The presence of a nutrient rich appendage (elaiosome) on their seeds facilitates this process as it attracts ants as potential seed dispersers. Once the seed is carried to the ant nest, the ants eat the elaiosome and often leave the seed inside the nutrient rich nest or disperse it in its surroundings (Giladi, 2006). In this way, plants benefit from this interaction since ants may contribute to decreased competition between the seeds and the parent plants, transport seeds to more fertile soils (ant nests) that favor seed germination and/or decrease seed predation (Giladi, 2006; Noriega *et al.,* 2018).

However, seed dispersal by ants may be impacted by the presence of invasive ant species (Rodriguez-Cabal *et al.*, 2009). Invasive species colonization may lead to native biodiversity decline, especially when they occur at the same trophic level as the native species (Bradley *et al.*, 2019), and may cause the loss of important ecosystem functions and processes (e.g. Rodriguez-Cabal *et al.*, 2009). For example, Ness et al. (2004) found that invaded sites by the invasive red fire ant, *Solenopsis invicta*, are characterized by a decrease in seed dispersal distance and ant mean body size. This may be linked to the physical constrains, e.g. smaller body sizes and mandible gap (Oliveras *et al.,* 2005), characterizing many invasive ant species, which may lead to a disruption of native ant-seed dispersal mutualisms (Ness *et al.,* 2004).

The Argentine ant (*Linepithema humile* (Mayr)), originally from South America but today with a cosmopolitan distribution, is listed as one of the most invasive ant species worldwide (Lowe *et al.*, 2000). Its occurrence and spread have been associated with anthropogenic activities and once established, it may severely impact native biodiversity (Holway *et al.*, 2002). Due to invasive ant characteristics (e.g. high abundance, ability to monopolize resources and higher aggressiveness) native species are often outcompeted (Holway *et al.*, 2002). In Mediterranean ecosystems, seed dispersal and invertebrate communities are severely affected by the presence of invasive Argentine ants (Devenish *et al.*, 2019 and references therein), although their net effects on seed dispersal remain unclear, since alongside negative effects (Rodriguez-Cabal *et al.*, 2009; Devenish *et al.*, 2019), neutral and positive outcomes have been reported too (Blight *et al.*, 2018).

This invasive species arrived in Portugal more than 100 years ago and established mostly along the coast of the country, particularly in Mediterranean ecosystems that present some anthropogenic disturbance (Silva Dias, 1955), such as the man-made silvo-pastoral *Montado* ecosystem (Cammell *et al.*, 1996).

In this seminatural ecosystem, we studied the effects of Argentine ants on seed dispersal of four myrmecohorous native plants by assessing seed removal rates in an invaded and an uninvaded site. In the invaded site, we expected to have lower seed removal rates due to the dominance of the invasive species while in the uninvaded site, several native ant species may contribute to higher rates of seed removal.

Materials and Methods

Study site and ant communities

The study was conducted in the *Montado* ecosystem at Companhia das Lezírias (38°50′ N, 8°49′ W), a Long-Term Socio-Ecological Research (LTSER) station near Lisbon, Portugal. The *Montado* is an agroforestry system characterized by high farmland biodiversity maintained through sustainable anthropogenic activities (Pinto-Correia *et al.*, 2011), in fact at the European level it is recognized as a High Nature Value (HNV) farmland (Keenleyside *et al.*, 2014). The study area is classified as an HNV farmland since several low-intensity management practices, such as livestock grazing and cork harvest, coexist there contributing to high habitat heterogeneity, high levels of farmland biodiversity and the occurrence of several threatened species (http://www.ltsermontado.pt).

We selected two study sites: one dominated exclusively by the Argentine ant (invaded) and the other without the presence of the invasive species uninvaded). The two sites are 4.7 km apart and present similar habitat characteristics regarding the density of cork oak trees and understory cover (http://www.ltsermontado.pt), but the invaded site is subject to low-intensity livestock grazing (Fig. 1). The two sites have been targeted by several biodiversity and ecological studies in recent years (e.g., Listopad *et al.*, 2018; Frasconi Wendt *et al.,* 2021; Köbel *et al.,* 2021). Furthermore, with regards to ant community composition, a recent study conducted in the two same study sites, solely reported the occurrence of the Argentine ant in the invaded site, while several native species were found in the uninvaded site (Frasconi Wendt *et al.,* 2021). The Argentine ant is extremely abundant in the invaded site and seemed to have outcompeted the native species, thus leading to the taxonomic and functional homogenization of the ant community (Frasconi Wendt *et al.,* 2021). Most of the native ant species sampled in the uninvaded site belong to the *Aphaenogaster*, *Camponotus*, *Pheidole* and *Temnothorax* genera (Frasconi Wendt *et al.,* 2021).

Seed removal experiments

In the two study sites, we performed seed removal observations and set up cafeteria experiments during September 2018 to investigate seed removal rates by ants using four myrmecochorous plant species: *Centaurea sphaerocephala* L. (Asteraceae), *Rosmarinus officinalis* L. (Lamiaceae), *Silybum marianum* (L.) Gaertn. (Asteraceae), and *Ulex australis* Clemente (Fabaceae). We selected these four species because they are native and occur spontaneously in the area where we performed the experiment, they have an elaiosome (myrmecochorous plant species) and we also aimed to ensure both taxonomic and growth-form diversity (herbaceous: *S. marianum* and *C. sphaerocephala*; shrubs: *R. officinalis* and *U. australis*). The diaspores and elaiosomes of the four study plant species also differed in length, width and weight (Table S1). Seeds were collected in June and July and used in September of the same year.

Daily seed removal rates (over a 24h period) were assessed by establishing three linear transects in each site, distanced at least by 50 m from each other. Along each transect, we placed 15 seeds per species on the soil with each seed distanced by 10 cm from the next ones to prevent ant recruitment. To avoid potential bias in the seed removal rates, seeds of the different plant species were set in a random order along the transect. Overall, 180 seeds were set in each site (45 seeds per plant species) and after 24h, the ones remaining were counted and re-collected.

To evaluate the role of the different ant species as seed dispersal agents of the four study plants, we performed direct observations of seed removal (cafeteria experiments) by setting three observational grids (70 cm x 40 cm each; 50 m apart from each other) in each sampling site (Fig. 1). For each observational grid, we used 40 seeds (10 seeds per plant species) regularly spaced from each other by a distance of 10 cm to avoid ant recruitment and with the seed species randomly distributed. Grids were continuously surveyed to record ant-seed interactions during the morning (900 - 1300 h) and the afternoon (1400 -1700 h), encompassing the peak of ant foraging, for a total of 28h of observation per site. The 28 hours of observation per site were hap hazardously distributed during a period of several days. We took care to sample equal numbers of morning and afternoon periods per site to account for differences in activity between ant species. For each observation, we recorded the number and type of interactions and the seed and ant species involved. Whenever a seed was removed by ants, we placed a new seed of the same plant species in the grid. Ant-seed interaction types were classified following Takahashi & Itino (2012): (Interaction I) Ignore: ant touches the seed but then ignores it, (Interaction II) Interest: ant picks the seed but drops it immediately after, (Interaction III) Removal: ant picks the seed and carries it away (> 5 cm).

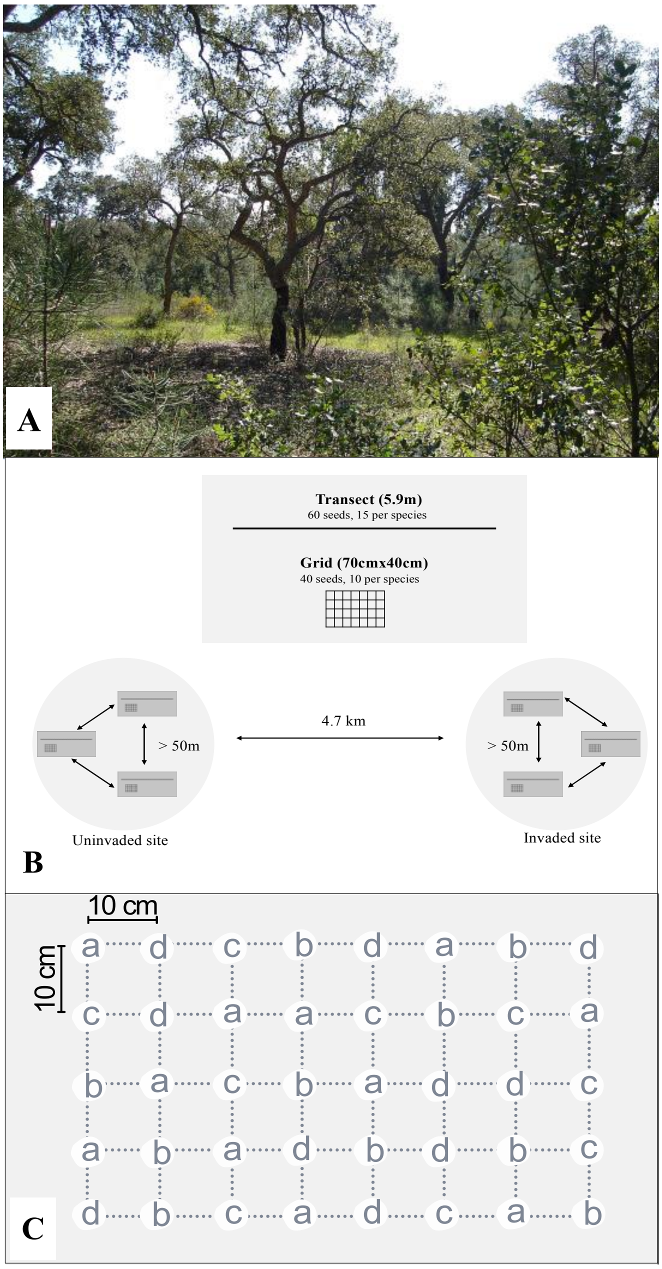

Fig. 1. Sampling site and schemes of the transect and the grids for the 24h and cafeteria experiment, respectively. A) Sampling site; B) scheme of the transect and grids and C) example of the disposition of the seeds (white circles) of the four different plant species (indicated with different letters) along the grids.

Data analysis

We assessed differences in seed removal rates over the 24h period and differences in the total number of ant-seed interactions (with no distinction between interaction type) between the two sampling sites using Wilcox-tests (alpha = 0.05).

To visualize the distribution of the seed removal at the uninvaded site, we built bipartite networks using the "bipartite" package and measured two indices at the species level, namely "species specificity", which refers to the association of ant species towards seed species and "d", which stands for the "specialization of each species based on its discrimination from a random selection of partners" (Dormann *et al.*, 2008). All analyses were conducted in R environment (R Core Team, 2017).

Results

Daily seed removal rates were significantly different between the invaded and the uninvaded site (Wilcox-test, p-value = 0.02). In the invaded site only some seeds were removed (44% for *R. officinalis,* 24% for *C. sphaerocephala,* 18% for *S. marianum* and 14% for *U. australis*; Fig. 2a), while in the uninvaded site, all seeds were removed (Fig. 2b).

The cafeteria experiment showed significant differences in ant-seed interactions between the two sampling sites (Wilcox-test, p-value = 0.02), regardless the type of interaction (Table 1).

Table 1. Total number of ant-seed interactions from cafeteria experiments (regardless interaction type) in the invaded and uninvaded site. Data is presented as mean ± standard deviation.

Plant species	Invaded site	Uninvaded site	Total number of seeds in invaded site	Total number of seeds in uninvaded site
S. marianum	10.6 ± 5.1	59.2 ± 100.9	30	296
C. sphaerocephala	5.3 ± 5.7	155 ± 144.6	30	620
U. australis	6.3 ± 3.2	67 ± 89.6	30	267
R. officinalis	6.0 ± 7.8	18.6 ± 22.8	30	56

When we accounted for the type of interactions in the invaded site, only Interaction I (Ignore, 32%) and Interaction II (Interest, 68%) were recorded (Fig. 2c), and no observation of seed removal by the Argentine ant was made. On the other hand, in the uninvaded site we recorded Interaction III

(Removal) only, meaning that whenever ants discovered a seed, they picked it. In the uninvaded site interaction III was, performed by seven native species, namely *Aphaenogaster gibbosa*, *A. iberica*, *Crematogaster auberti*, *Formica subrufa*, *Pheidole pallidula*, *Temnothorax recedens* and *Tetramorium semilaeve*. In the uninvaded site, two ant species, namely *P. pallidula* and *A. iberica* were involved in most of the interactions and accounted for 71% and 26% respectively of the total interactions recorded (Fig. 2d).

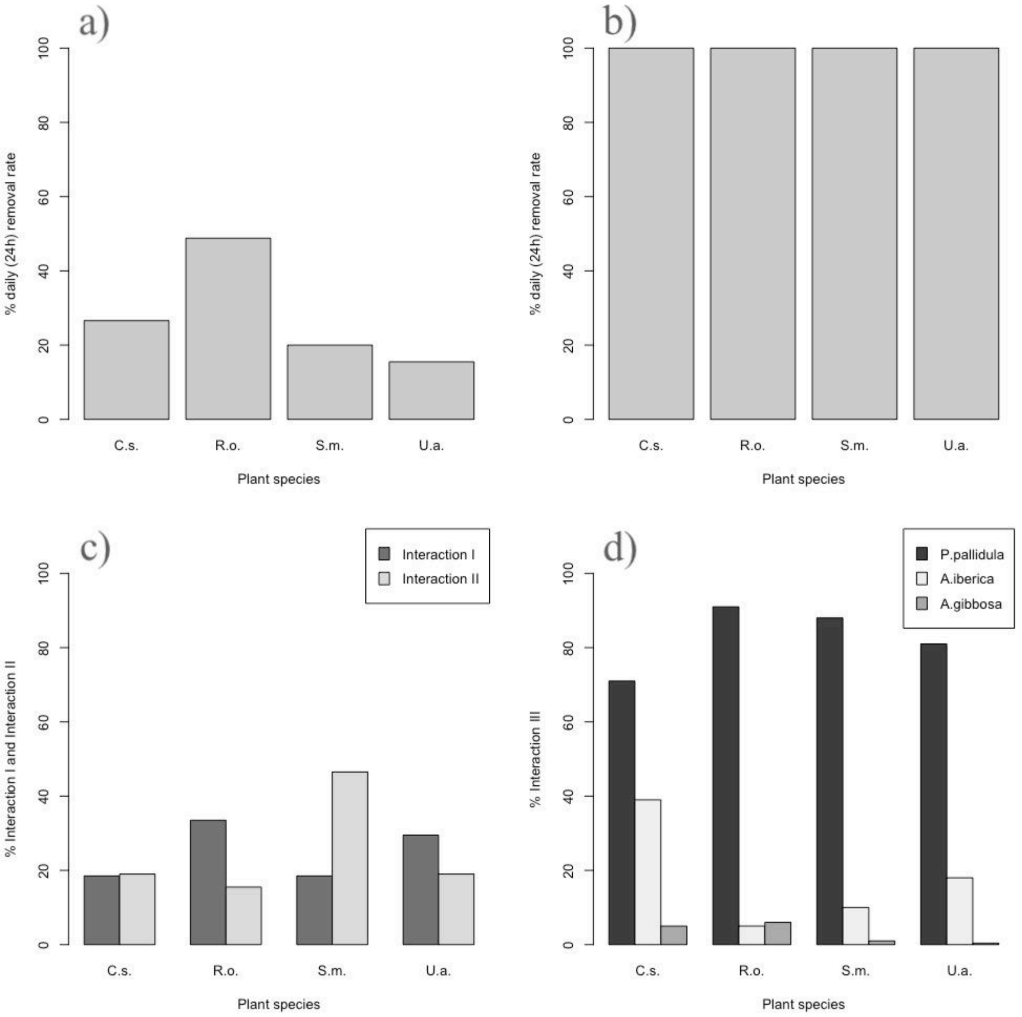

Fig. 2. Percentages of daily seed removal rates and of ant-seed interactions in the invaded and uninvaded sites. Daily seed removal rates (over 24h) for the four myrmecochorous plant species in the invaded site (a) and in the uninvaded site (b). Percentages of Interaction I (ignore) and Interaction II (interest) for the four myrmecochorous plant species in the invaded site (c), and percentages of Interaction III (removal) by different ant species for the four myrmecochorous plant species in the

uninvaded site (d) - ant species with < 3 interactions were not included here. Abbreviations: C.s.= *Centaurea sphaerocephala*, R.o.= *Rosmarinus officinalis*, S. m.= *Silybum marianum* and U.a.= *Ulex australis*. Note: Interaction III did not occur in the invaded site.

High levels of species specificity were found for *A. iberica* and *A. gibbosa*: These two ant species were mainly associated to seeds of *C. sphaerocephala.* In contrast, *P. pallidula* workers did not show preferences for a particular seed species (Fig. 3 and Table 2). The percentage of seeds removed by *A. gibbosa* was low (3%), thus, *P. pallidula* and *A. iberica* stand out as key seed dispersers both considering the higher number of transport events and the wider spectrum of plant species dispersed.

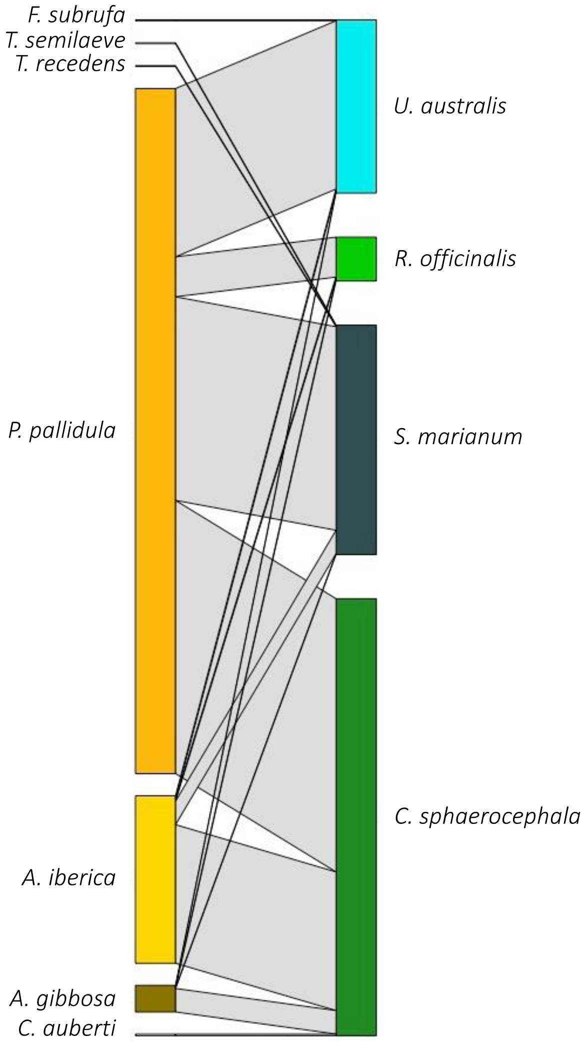

Fig. 3. Bipartite graph showing seed removal (Interaction III) in the uninvaded site. Ant species are on the left and the four different seed species on the right of the network. The width of each rectangle

is proportional to the sum of interactions for each species. Connections in grey indicate seed removal events and their width is proportional to the number of seed removal mediated by each ant species.

Table 2. Species specificity and specialization "d" indices for the interaction network between seed and ant species (more than three observations) in the uninvaded site.

Ant species	Species Specificity Index	d (specialization)
P. pallidula	0.2841	0.0629
A. iberica	0.6725	0.1053
A. gibbosa	0.7507	0.0738

Discussion

This study sheds light on the interactions of two ant communities with seeds of four different plant species in an Argentine ant invaded and in an uninvaded site in a High Nature Value farmland. Our findings support previous studies that showed significant changes in seed removal rates in ant-invaded sites when compared to uninvaded (e.g. Bond & Slingsby, 1984; Christian, 2001; Gómez & Oliveras, 2003).

In our study, we witnessed no seed dispersal by ants in the invaded site as a consequence of the extirpation of the local ant fauna by the Argentine ant (Christian 2001; Rowles & O'Dowd, 2009; Frasconi Wendt *et al.,* 2021) and the incapability of the invasive species to perform effectively this ecological process. The Argentine ant ignored the seeds or showed some interest on them but provided no seed removal. Previous studies conducted in Mediterranean-type ecosystems also reported a significant decrease in myrmecochory in Argentine ant invaded sites (e.g. study in NE Spain conducted by Gómez & Oliveras, 2003). For example, Rowles and O'Down (2009) reported the decrease but not the collapse of native seed dispersal following the invasion by the Argentine ant in southeastern Australia. In contrast, Blight *et al.* (2018) reported high seed removal rates of *Anchusa crispa* by the Argentine ant on the island of Corsica (France), suggesting that this invasive ant functionally replaced the native seed dispersers. In this case, seeds are provided with a "handle" that may have facilitated the transport by the invasive ant species. Thus, diaspore morphology and weight seem to play a role in seed removal success.

Ant body size is also an important driver of seed dispersal by ants: many invasive species present a smaller body size relative to native species, thus performing poorly as seed dispersers, particularly for large seeded plants (Christian, 2001; Rodriguez-Cabal *et al.*, 2009). For example, Rowles and O'Dowd (2009) found that in southeastern Australia the Argentine ant was uncapable to remove the large seeds of the native *Acacia sophorae* at distances over 5cm, feeding on the elaiosomes and leaving the seeds *in situ*. Interesting, Ness *et al.* (2004), based on a global analysis of ant species, showed that the reduction in mean ant body size in invaded sites (compared to uninvaded ones) leads to a decrease in seed dispersal distances with potential negative consequences for seed fate.

In our study, we argue that the small body size and low mandible gap of the Argentine ant (Ness *et al.,* 2004; Oliveras *et al.,* 2005) limit its capacity to transport the seeds of the four study species. The physical constraint of the invasive species (jointly with the extirpation of the local ant fauna) may be the major cause for the disruption of seed removal by ants in the invaded site (e.g. Christian, 2001; Ness *et al.,* 2004).

In the uninvaded site, seed removal was carried out by different native species, but two of them, *P. pallidula* and *A. iberica*, were responsible for most of the transport and removal of all seed species. *Pheidole pallidula* and *Aphaenogaster* ants disperse seeds of many myrmecochorous plant species in temperate forests and Mediterranean ecosystems and are considered keystone dispersers in these ecosystems (Espadaler & Gómez, 1996; Ness *et al.*, 2009). These key seed disperser species literally vacuum the soil surface in search for food being extremely efficient in detecting and removing seeds (Espadaler & Gómez, 1997). These native species removed the larger and heavier diaspores of *C. sphaerocephala* and *S. marianum* at much higher rates compared to the smaller and lighter co-occurring diaspores of *R. officinalis* and the larger seeds may be more attractive to ants since they bear a well-developed elaiosome. We are aware that some ants involved in seed removal are granivorous, but they may still contribute to seed dispersal by dropping seeds during transport to the nest and by discarding viable seeds in the refuse piles (Arnan *et al.,* 2010; Bulot *et al.,* 2016). In our study, we did not record the destination of the seeds once they were picked up and carried away by the ants, although observations on seed fate are important to assess long-term changes at community level (e.g. Gorb & Gorb, 2003; Giladi, 2006; Tanaka *et al.,* 2015). In the Argentine ant invaded site, we observed no seed removal during the cafeteria experiments, but we found that some seeds were removed during the 24h observation period. We are aware that other groups of animals besides ants (e.g. beetles, birds, rodents) may remove elaiosome-bearing seeds. However, previous studies using exclusion experiments have shown that the seed removal rates by vertebrates are extremely low in neighboring areas (Boieiro, 2012) while ants play a major role as dispersers of myrmecochorous plants in these Mediterranean habitats (e.g. Espadaler & Gómez, 1996, 1997). Nevertheless, we cannot rule out the probability that some seeds were removed by nocturnal granivorous ground beetles (e.g. *Amara*, *Harpalus*, *Pseudophonus* spp.), which are known to be abundant in the close vicinity of study sites (Martins da Silva *et al.,* 2008, 2009).

Finally, we acknowledge that this study has some limitations since our observations were carried out over a short period (approximately 2 weeks), thus not accounting for temporal variation in ant diversity and activity, and we sampled in only three replicates per sampling site. Ant activity shows a high sensitivity towards environmental temperature (e.g. Cerdá *et al.,* 1998) and we decided to perform the observations after the summer temperature peak, which may attain over 42ºC and severely constrain ant foraging activity during the day. During summer months, the Argentine ants mostly rely on sugar-rich liquid food (e.g. Abril *et al.*, 2007) probably not being that interested in collecting the seeds. Ant diet preferences shift across the season to match colony demands and this can also determine differences in seed removal rates between plants that release their seeds in spring compared to those (like our study species) that shed them in summer.

In terms of further research, repeating this study in time may help to better understand what the consequences for the myrmecochorous plant structure are. For example, repeating the study in time, may show low seed dispersal and high seed mortality levels of myrmechocorous plants with potential implications on plant recruitment for the plant species considered. Community-level consequences of the invasion by the Argentine ant resulting in a shift in plant species compositions have already been reported (Christian, 2001) and authors fear that invasive species may affects myrmecochorous plant abundance, distribution and population dynamics and even lead to extinction rare narrow endemic species (Bond & Slingsby, 1984; Quilichini & Debussche, 2000). Furthermore, a future experiment in the same study area may include sites where seed-harvester ants occur, given that in our uninvaded site no ants with a seed-based diet were recorded. In this way another factor besides invasive ant species, that may affect the dispersal and distribution of myrmecochorous plant species, would be present. Different species of ants may show a selective diet, which may in turn determine their role in seed dispersal. For example, harvester ants or species of the genus *Aphaenogaster*, are mainly seed eater, however they contribute to seed dispersal by dropping seeds towards the way back to the nest (Arnan et al., 2010) or by increasing seed density and richness in their refuse piles (Bulot et al., 2016).

In conclusion, in High Nature Value farmlands such as our study area, efforts are being made to maintain crop diversity through farming practices and heterogeneous landscapes with the ecological goal of supporting high levels of biodiversity and halting biotic homogenization. Nevertheless, the Argentine ant may benefit from human-assisted dispersal to establish and spread in these disturbed habitats ultimately outcompeting native arthropod fauna and disrupting ecological processes, thereby discreetly triggering a cascade of events throughout the community. For this reason, it is crucial to establish indicators and monitoring programs for the early detection and rapid response to species invasions in HNV farmlands.

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