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Analysis of introduced and autochthonous populations of the red wood ant *Formica paralugubris*: ecological interactions and conservation issues

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INDEX

Summary

English version

Red wood ants (RWA) are ecologically important keystone species that affect a multitude of taxa at different trophic levels. In the past century, some RWA species were used as biological control agents and exported outside their native range. One of these species is *Formica paralugubris*, which was transplanted from the Italian Alps to the Apennines (Central Italy) but also to Quebec (Canada). Recently, it has been demonstrated that some of the introduced populations have acquired some invasivity features.

This PhD thesis investigated the ecology of *Formica paralugubris*, aiming to assess its impact at multiple levels, from single taxa to the forest ecosystem as a whole. To do this, I combined different techniques, from direct observations to gas analysis and stable isotopes analysis. I started with assessing the effect of the presence of this species on epiphytic lichen communities, and I analyzed the myrmecophilous fauna inhabiting the nest mounds of introduced and autochthonous populations. I then compared the trophic position of native and introduced populations of this species, using Stable Isotopes Analysis (SIA) techniques. Finally, I characterized the microbial communities hosted within the nest mounds using molecular techniques and I measured the gas emissions from the mounds. To conclude, I have done an overview of the protection status of RWA throughout Europe.

Overall, the results of this thesis confirm the ecological importance of *F. paralugubris*. This species was shown to affect the composition of lichen communities, both from a taxonomic and a functional point of view. Its nest mounds host a rich myrmecophilous fauna and diverse microbial communities. The species was also found to occupy key positions in the trophic web and to play an important role in the carbon dynamics at the forest scale. These results were also discussed in light of the nature of *F. paralugubris* as a peculiar invasive species. Finally, the complex status of RWA protection was highlighted, also pointing out paradoxical situations in which the introduced populations are protected whereas the native and sometimes declining ones are not.

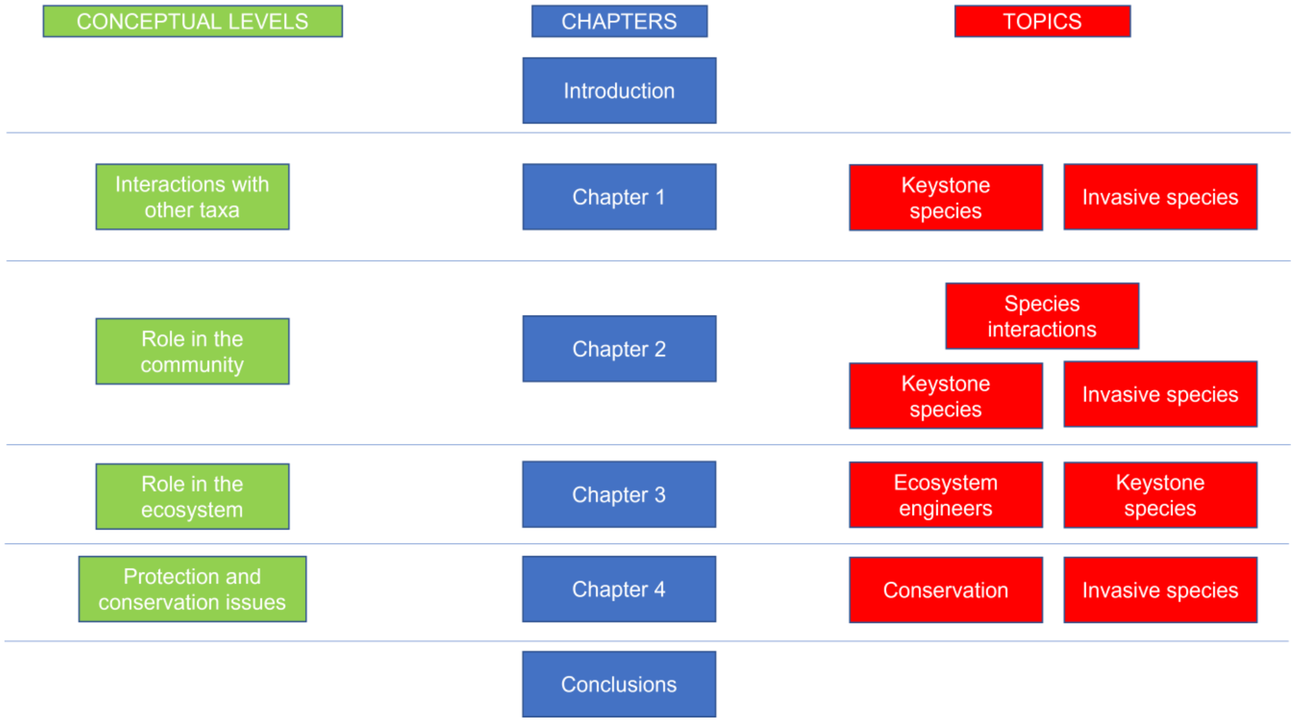
Italian version

Le formiche del gruppo *Formica rufa* (red wood ants, RWA) sono specie chiave di volta ecologicamente importanti che influenzano una moltitudine di taxa a diversi livelli trofici. Durante il secolo scorso alcune specie di RWA sono state impiegate come agenti di controllo biologico ed esportate al di fuori del loro areale nativo. Una di queste specie è *Formica paralugubris*, che è stata traslocata dalle Alpi italiane agli Appennini (Italia centrale) ma anche in Quebec (Canada). Recentemente è stato dimostrato che alcune delle popolazioni introdotte hanno acquisito alcuni tratti di invasività.

Questa tesi di dottorato ha investigato l'ecologia di *Formica paralugubris*, collo scopo di valutare il suo impatto a molteplici livelli, da singoli taxa all'ecosistema forestale nel complesso. Per questo scopo ho combinato tecniche differenti, dall'osservazione diretta all'analisi dei gas e all'analisi degli isotopi stabili. Ho iniziato valutando gli effetti della presenza di questa specie sulle comunità di licheni epifitici ed ho analizzato la fauna mirmecofila abitante gli acervi dei nidi delle popolazioni introdotte ed autoctone. In seguito, ho comparato la posizione trofica di popolazioni native ed introdotte di questa specie, usando tecniche di analisi degli isotopi stabili (stable isotope analysis, SIA). Infine, ho caratterizzato le comunità microbiche presenti all'interno degli acervi usando tecniche molecolari ed ho misurato le emissioni gassose degli acervi. Per concludere, ho fatto una panoramica sullo stato di protezione delle RWA in Europa.

In generale, i risultati di questa tesi confermano l'importanza ecologica di *F. paralugubris*. È stato dimostrato che questa specie influenza la composizione delle comunità licheniche, sia da punto di vista tassonomico che funzionale. I suoi acervi ospitano una ricca fauna mirmecofila e comunità microbiche diverse. È stato anche dimostrato che la specie occupa posizioni chiave nella rete trofica ed ha un ruolo importante nella dinamica del carbonio a scala forestale. Questi risultati sono stati discussi anche alla luce della natura di *F. paralugubris* come specie invasiva peculiare. Infine, è stato sottolineato il complesso stato di protezione delle RWA, mettendo in evidenza situazioni paradossali in cui le popolazioni introdotte sono protette mentre quelle native, e talvolta in declino, non lo sono.

Graphical scheme of the thesis



CONCEPTUAL LEVELS	CHAPTERS	TOPICS
	Introduction	
Interactions with other taxa	Chapter 1	Keystone species Invasive species
Role in the community	Chapter 2	Species interactions Keystone species Invasive species
Role in the ecosystem	Chapter 3	Ecosystem engineers Keystone species
Protection and conservation issues	Chapter 4	Conservation Invasive species
	Conclusions	

Introduction

General introduction

In the field of animal ecology, the interactions among species play an extremely important role. Different species can interact with each other through predation (*sensu lato*, i.e. including herbivory), parasitism, mutualism, or competition (Lang & Benbow 2013). Considering a simplified situation with pairwise interactions, predation and parasitism are the relationships in which one species increase its fitness by negatively affecting the one of another species (i.e. by eating all or a part of the prey and by directly exploiting the host to acquire resources, respectively; Chesson 2013; Raffel et al. 2008). Instead, mutualistic relationships are those interactions that result in an increase of both species' fitness and can be either obligate or facultative (Holland & Bronstein 2008). On the other hand, in competitive relationships both species have their fitness reduced. This occur when the shared resources (e.g. food, shelters, reproductive sites) are limiting (Chase et al. 2002). Two mechanisms of competition are distinguished: interference competition and exploitative competition. In the former, species directly interfere with each other for the access to the resource, while in the latter two species indirectly compete by reducing the available resources for the other species (Jensen 1987). Additionally, there can be commensalism, amensalism, and neutralism relationships, that are less studied and not completely understood. In these interactions, one species positively (commensalism) or negatively (amensalism) affects the other one, while the first species (or both in case of neutralism) is almost unaffected (Mathis & Bronstein 2020).

All these mentioned interactions, in turn, affect many aspects of the ecology of each species, and together with other processes (e.g. biogeography, environmental filtering, intraspecific interactions), contribute to the structuring of communities (Weiher et al. 2011). Indeed, communities are set of species co-occurring in the same habitat, thus tightly interacting among each other (Underwood 2008). The interspecific interactions occurring within a community structure the trophic webs, where species occupy a trophic level and a trophic niche (Leibold et al. 1997; Pringle & Hutchinson 2020). The trophic level occupied defines both the competition for food and predatory relationships among species. Indeed, each species generally compete with other species of the same trophic level (with which they constitute a 'guild') and predates on individuals belonging to the trophic level below its one. In this way, species belonging to each trophic level are regulated by both the lower trophic level (that provides them energy and nutrients), and the upper trophic level (that exerts on them a predatory pressure), that is what is called bottom-up control and top-down control, respectively (Preisser 2008). However, this simplistic assumption is not always supported, since omnivorous species predate on individuals belonging to different trophic levels. Consequently, omnivorous species occupy an intermediate trophic level between the one of herbivores and the one of predators (Thompson et al. 2007). As the trophic levels are discrete by definition, the concept of a continuous measure has been introduced, namely the trophic position (Levine 1980). Similarly, competition among members of the same trophic level is not straightforward, as there could be small differences in the trophic position of each species, or in their trophic niche (e.g. Arribas et al. 2015). Finally, other exceptions are represented by intraguild and mutual predation, that are more common than previously thought (e.g. Kinzler et al. 2009; Parimuchová et al. 2021).

Within communities, some species play a critical role. For example, keystone species are defined as those species affecting the community of which they are part more strongly than expected from their biomass. Typically, keystone species are those that exert a strong predatory pressure on the trophic level below them, in other words exerting a strong top-down control. Therefore, the removal or exclusion of keystone species from the community results in a trophic cascade, potentially leading to the collapse of some populations and the disruption of the functioning of the community (Libralato 2008). Another important concept is that of ecosystem engineers, that are species that change the physical structure or other properties (e.g. chemical) of the habitat where they live or create new habitats (Jones et al. 1994).

This complex structure of interspecific interactions can be disrupted by many anthropogenic factors. Among these, the intentional or accidental introduction of new (allochthonous) species play a major role (e.g. David et al. 2017). Indeed, some introduced species establish viable populations in the new area, starting to interact with the native species present in the recipient community. Some of these allochthonous species become invasive, meaning that they exert an ecological, economic, or health impact where they are introduced (Pyšek et al. 2020). The multifaceted impacts of invasive species are increasingly recognized (e.g. Mazza & Tricarico 2018; Walsh et al. 2016; Ahmed et al. 2021). In particular, invasive species can have detrimental ecological impacts on communities, for example by modifying their composition (e.g. Sanders et al. 2003), competing with (Balzani et al. 2016) or predating on (Duenas et al. 2021) native species.

Ants represent an ideal taxon to investigate all these topics. Indeed, they strongly affect other species with a plethora of interactions, ranging from predation (Floren et al. 2002; Clark et al. 2016), competition with other ants (Pontin 1963; Davidson 1985) or taxa (Brown & Davidson 1977; Supriya et al. 2020), and mutualism (Way 1963; Mueller et al. 2001). Also, they are important keystone species (Underwood & Fisher 2006) and ecosystem engineers (Cammeraat & Risch 2008; Del Toro et al. 2012; Meyer et al. 2013; Swanson et al. 2019). Finally, but not less important, some ants are relevant invasive species, causing large ecological impacts on the native species, both invertebrates (McNatty et al. 2009; Plentovich et al. 2021) and vertebrates (Davis et al. 2008; Darracq et al. 2017; Lach et al. 2022). They also are causes of important economic damages

(Gutrich et al. 2007; Angulo et al. 2021) and affect human health, too (Kemp et al. 2000; Vanderwoude et al. 2021).

Ecology of red wood ants (RWA)

Red wood ants (RWA) are a group of morphologically and ecologically similar species belonging to the subgenus *Formica sensu stricto* or *F. rufa* group, with at least 13 species described in the Palearctic and a minimum of 19 species reported in the Nearctic (Stockan et al. 2016; Trager 2016; Seifert 2021). They are typical dwellers of Eurasian woods, where they inhabit coniferous or mixed forests (Baroni Urbani 1971; Risch et al. 2016; Sondej et al. 2021). Although there are some species-specific differences in their habitat requirements or preferences (Pavan et al. 1971; Glanzmann et al. 2019; Perron et al. 2019; Fitzpatrick et al. 2021) and altitudinal range where they can be found (Freitag & Cherix 2009; Freitag et al. 2016a; Vandegehuchte et al. 2017; Antonova & Marinov 2021), RWA nests location is mainly driven by their ecological constraints. Indeed, all species generally require some degree of exposition to sun, thus preferring forest edges and clearings to completely closed canopies (Risch et al. 2016).

Above the subterranean part of the nest, RWA build a characteristic structure called *mound* (Figure 1), using vegetal material (mainly coniferous needles and twigs) mixed with soil (Risch et al. 2016). The mound has a key role in the colony thermoregulation, as it must be exposed to sun so that the internal mound temperature is kept higher than that of the external air throughout the year, without dropping below 0 °C during the winter (Frouz 2000; Frouz & Finér 2007; Frouz et al. 2016). Furthermore, though their abandonment and relocation are common phenomena (Punttila 1996; Sorvari & Hakkarainen 2007a; Punttila & Kilpeläinen 2009; Burns et al. 2020), RWA mounds can be long-lasting structures. Indeed, in stable and favorable conditions, a mound can persist decades, thus becoming part of the forest landscape (Breen 1979). Consequently, they can also be used to easily estimate RWA abundance and monitor their populations (Freitag et al. 2016b; Frizzi et al. 2018; Klimetzek et al. 2021). However, RWA mound density can strongly vary depending on the local environmental and habitat conditions, reaching in Eurasia up to 20, but rarely exceeding 5, mounds per hectare (Risch et al. 2016).

Figure 1. Nest mound of *Formica paralugubris* in the Foreste Casentinesi, Monte Falterona e Campigna National Park, Italy (photo provided by Alberto Masoni).

The diet of RWA is based on two types of food resources: animal preys (even scavenged) as protein source for the queen and larvae, and honeydew from homopteran aggregations as the primary carbohydrate source for workers (Domisch et al. 2016). This latter source is part of a mutualistic relationship, called trophobiosis, in which homopterans offer ants food in exchange for their protection from predators and parasites (Domisch et al. 2016). The exploitation of this persistent, renewable, and highly energetic food source (sugars), allows RWA to build up very large colonies, extend their control over large areas, and maintain elevate activity levels (Davidson 1997; Gibb & Johansson 2010). Therefore, RWA play a double role in food webs, behaving both as generalist predators, and as "herbivores" that consume plant-derived carbohydrates (i.e. aphid honeydew; Domisch et al. 2016).

Ecological role of RWA in the community

Most European RWA species are ecologically dominant (Johansson & Gibb 2016) and have a key role in the structure and functioning of invertebrate communities in temperate and boreal forests (Savolainen & Vepsäläinen 1988; Punttila et al. 2004). They are aggressive and territorial species (Le Moli et al. 1984; Mabelis 1984), defending their nests, territories, and the resources within them from other colonies of the same (Skinner 1980; Sorvari & Hakkarainen 2004) or different species (Mabelis 1983; Savolainen & Vepsäläinen 1988, 1989), excluding them from their area (Savolainen et al. 1989; Czechowski et al. 2013; Maák et al. 2021).

Also other taxa are negatively affected by RWA presence, being excluded, predated, or outcompeted (Robinson et al. 2016). While herbivorous and xylophagous insects are directly impacted by predation (Skinner & Whittaker 1981; Fowler & McGarvin 1985; Punttila et al. 2004; Trigos-Peral et al. 2021), predatory arthropods like harvestmen, spiders, and beetles suffer RWA competition or aggression (Gridina 1990; Brüning 1991; Halaj et al. 1997; Laakso 1999; Hawes et al. 2002; Reznikova & Dorosheva 2004). Carabid beetles, for example, are less abundant (Niemelä et al. 1992) and show reduced individual fitness and reproductive success in proximity of RWA mounds (Hawes et al. 2013). It has been demonstrated that also parasites (i.e. ticks) are negatively correlated with RWA presence, because of direct predation, repellency of the sprayed formic acid, or reduction of the vertebrate tick hosts (Zingg et al. 2018).

Even vertebrates are affected by RWA presence. Small mammals have been found in lower number and with a reduced activity within RWA territories (Panteleeva et al. 2016), although establishing whether this pattern is due to direct or indirect effects is not straightforward (Boryakova & Melnik 2017). On the other hand, insectivorous birds were found to avoid the areas occupied by RWA both as feeding sites, as a result of prey depletion by ants, i.e. through exploitative competition (Haemig 1992, 1994, 1996; Aho et al. 1997), and as nesting sites, as a result of direct attacks by ants (Haemig 1999). It was also demonstrated that birds nesting within RWA territories had a diminished reproductive success (Aho et al. 1999; Jäntti et al. 2007).

Moreover, given the above-described relationships with other organisms, RWA also affect plants. However, the net balance of their presence on trees' growth is variable and depends on local factors, in particular on the food source availability for ants. Indeed, while the predation on herbivorous arthropods represents a benefit for the plant (Ito & Higashi 1990, 1991), the trophobiotic relationship with aphids constitutes an increased cost (Kilpeläinen et al. 2009; Domisch et al. 2016), as aphid population generally increase when protected by RWA (McNeil et al. 1977; Mahdi & Whittaker 1993). Yet, predation on aphids by RWA can also occur in some circumstances, for example when there is a lack of other available preys (Billick et al. 2007; Robinson et al. 2016).

Ecological role of RWA in the ecosystem

RWA can be defined as keystone species, given their role in seed dispersion (Gorb & Gorb 1995, 1999, 2000; Heinken & Winkler 2009; Englický & Šera 2018), their effect on tree growth and ant and invertebrate assemblages from multiple trophic levels (Johansson & Gibb 2016; Robinson et al. 2016; Thunes et al. 2018). The keystone role of RWA was demonstrated in a long-term study where the exclusion of *Formica aquilonia* in a boreal forest in Sweden led to an increase of the herbaceous plants coverage, due to the lack of seeds removal by ants, and of the microbial activity, leading to increased decomposition rates (Wardle et al. 2011).

Yet another aspect of their biology that makes RWA keystone species is the fact that their nest mounds represent unique biodiversity hotspots for many invertebrate species (Parmentier et al. 2014; Robinson et al. 2016). This is a consequence of the relatively stable microhabitat (Rosengren et al. 1987; Jones & Oldroyd 2006; Kadochová & Frouz 2014; Jílková et al. 2015a), with temperature and humidity kept almost constant even under harsh external environmental conditions (Frouz & Finér 2007; Jílková et al. 2015a), and the preys or food resources that RWA mounds provide (Parmentier et al. 2015). These ant-associated organisms can be obligate or facultative (if they occur in the nest mound as well as in the soil, for example) hosts of these microcosms (Fiedler 2006; Robinson & Robinson 2013; Parker 2016; Härkönen & Sorvari 2018; Parmentier & Claus 2019), so they can be defined as myrmecophiles *sensu lato*. Myrmecophiles can be involved in a plethora of trophic interactions with the host ants or the other myrmecophiles, ranging from predation to parasitic relationships, detritivory, or scavenging (Parmentier et al. 2016, 2018).

The role of RWA extends beyond the biotic part, also affecting the abiotic compartment of the ecosystem they are part of. In fact, RWA can affect the soil properties and the nutrient cycling by accumulating high concentrations of nutrients in their mound and in the close proximities (Frouz et al. 2005; Domisch et al. 2008; Frouz & Jílková 2008; Jurgensen et al. 2008; Finér et al. 2013; Frouz et al. 2016). The accumulation of nutrients is the result of the huge quantity of food (aphid honeydew and animal preys) that the ants carry to the nest (Domisch et al. 2009, 2016; Gibb & Johansson 2010; Jílková et al. 2012; Frouz et al. 2016) and the organic material (coniferous needles, twigs, and resin) that is used by ants to build and maintain the mound itself (Jílková et al. 2011, 2012; Finér et al. 2013; Frouz et al. 2016). This leads to an increase in the availability of nutrients like phosphorus and nitrogen and in the pH in RWA mounds compared to the surrounding soil, and to a decrease in these parameters with the distance from the mound (Frouz et al. 1997; Lenoir et al. 2001; Risch et al. 2005; Kilpeläinen et al. 2007; Jílková et al. 2011; Tsikas et al. 2021) – partly due to the microbial activity within the mound (see below). Plants have been shown to exploit this high nutrient concentration by extending their roots in the proximity of and even within the RWA mounds (Ohashi et al. 2007). Moreover, these accumulated nutrients are leached and released in the soil once the mound is abandoned (Kristiansen et al. 2001; Jílková et al. 2015b), thus becoming available again for the surrounding plants.

The very favorable conditions and the microhabitat provided, together with the high amount of nutrients and organic matter transported by ant workers, make RWA mounds perfect candidates for hosting rich microbial and fungal communities (Jílková et al. 2015c; Siedlecki et al. 2021). Indeed, such communities have been found to be much more abundant and active in mounds compared with the near soil (Laakso & Setälä 1997; Frouz et al. 1997; Frouz 2000; Jílková et al. 2015a; Sorvari & Hartikainen 2021) and are supposed to actively contribute to the heat production and resource metabolization (Jílková & Frouz 2014). These communities have profound effects on the mound dynamics and biogeochemistry, for example they accelerate the decomposition rate (Frouz et al. 1997; Jílková & Frouz 2014) and strongly contribute to high nutrient content of the mounds, through the action of methanotrophic and nitrogen-fixing bacteria (Frouz et al. 1997; Jílková et al. 2016).

Finally, due to the high number of workers that a single colony can host (over a million; Rosengren et al. 1987) and to all the organisms cohabiting in these microcosm, RWA mounds are also hotspots of CO_2 emissions, producing more than the surrounding soil (Ohashi et al. 2005; Risch et al. 2005; Domisch et al. 2006; Frouz et al. 2016). As nutrient and carbon dioxide hotspots, RWA mounds increase the spatial heterogeneity of forest ecosystems (Jouquet et al. 2006; Kilpeläinen et al. 2007), therefore, taking all the above-discussed aspects together, we can define RWA as ecosystem engineers (Lenoir 2001).

RWA conservation and introductions

All RWA are threated mainly by habitat loss and alteration (Sorvari 2016). Indeed, they need wide foraging areas to sustain their large colonies (Sorvari 2016), but also, as discussed above, not completely closed forest habitats to let solar radiation reach their mounds (Punttila 1996; Risch et al. 2016). Forest management activities such as clearcutting have been shown to negatively impact on RWA (Sorvari 2013; Sorvari & Hakkarainen 2005, 2007b, 2009), because of both reduced food sources availability and quality (Sorvari & Hakkarainen 2009; Johansson & Gibb 2012) and the lack of suitable nesting sites (Juhász et al. 2020), causing high frequency of nest abandonment (Sorvari & Hakkarainen 2007a; Juhász et al. 2020). The effect of clear cutting extends also to the myrmecophile fauna associated with RWA mounds, that is impoverished in clear fells compared to mature forests (Elo & Sorvari 2019).

On the other hand, RWA have been – and are still – used as biological control agents against forestry pests (Nielsen et al. 2018; Trigos-Peral et al. 2021). Starting in the 50s of the past century, large experimental introductions of different RWA species have been carried out in some European countries, such as Germany and Italy, to study the suitability of RWA species as useful predators in the fight against forest pests (Gösswald 1951; Pavan 1959). One of the mostly employed species in these introductions was *F. paralugubris* (Seifert 1996; Figure 2), that was repeatedly transplanted from its original areas in the Alps to other sites of the Italian peninsula, where they were formerly absent (Pavan 1959; Ronchetti & Groppali 1995). Most introductions occurred in the Apennines mountains, where climatic conditions provided habitats suitable to this cold-climate species, thus allowing its successful establishment (Ronchetti et al. 1986; Groppali & Crudele 2005; Masoni et al. 2019). The success of these introductions and the effectiveness of *F. paralugubris* as a predator against defoliator insects, together with the echo of the national and international propaganda, led to further, overseas, introductions. Indeed, in 1971, *F. paralugubris* was imported from the Italian Alps to Quebec, Canada, and successfully established (Finnegan 1975; Seifert 2016).

Figure 2. Worker of *Formica paralugubris* (photo provided by Christian Bernasconi).

These introduced and established populations have considerably grown. For example, the Canadian population, estimated to one million workers in the year of introduction (Finnegan 1975), became composed by 95 nests with an estimated worker number of eight million in 2005 (Storer et al. 2008), though this was considered an underestimate, with a more accurate estimate (from the same data) that is around 19 million workers (Seifert 2016). Likewise, some populations in the Apennines, within the Foreste Casentinesi, Monte Falterona e Campigna National Park (Italy), that were censused across years, have quickly grown from a total of 70 mounds in 1968, to 135 in 1983, to 275 in 1999, to 423 in 2015 (Frizzi et al. 2018). The increase in the total number of mounds was also accompanied by a spatial expansion and a strong increase in the total volume of mounds, that was 44 m^3 in 1968, then 100 in 1983, 239 in 1999, and lastly 749 m^3 in 2015 (Frizzi et al. 2018; Figure 3).

This increasing trend followed an initial lag-phase, in which the introduced populations remained stable or even decreased for several years, after which the expansion phase started. This dynamic is common in invasive species (Ward 2007; Hui and Richardson 2017), with the time lag that could be the result of both external (i.e. environmental) as well as internal (i.e. adaptation) processes (Groppali & Crudele 2005; Hui and Richardson 2017).

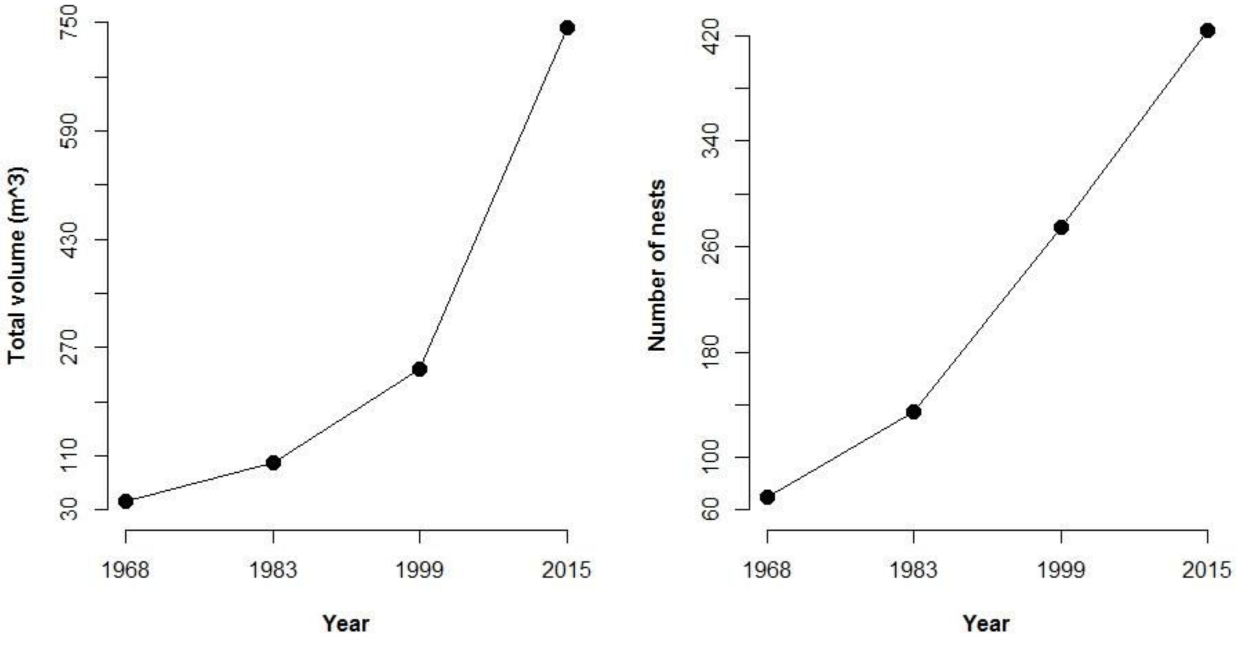


Figure 3. Temporal increase in the total volume and number of nests of *Formica paralugubris* in one site within the Foreste Casentinesi, Monte Falterona e Campigna National Park, Italy (modified from Frizzi et al. 2018).

The transplants of *F. paralugubris* colonies, performed by moving entire nests together with the mound material (Pavan 1959), could have resulted in the accidental co-introduction of mound-dweller species, as discussed for the beetle *Monotoma conicicollis*, whose first (and only) record South of the Alps has been found in the mound material of an introduced *F. paralugubris* population (Cianferoni et al. 2019).

Moreover, these introductions affected the recipient communities. In the Foreste Casentinesi, Monte Falterona e Campigna National Park, many taxa (such as Diptera, carabid beetles, spiders, and harvestmen) were significantly less abundant in the areas where *F. paralugubris* occurred compared to the control areas. Likewise, all other ant species except for one, were excluded from the invaded areas. Furthermore, due to *F. paralugubris* activity, the predation rate was experimentally demonstrated to be significantly higher in the invaded areas compared to the control ones (Frizzi et al. 2018).

Aims

Understanding how introduced ant species affect the recipient community and environment at various levels is key for improving both the basic knowledge on their ecology as well as the determinants of their success (Krushelnycky et al. 2010), subsequently allowing to assess their impacts (Lach & Hooper-Bui 2010) and develop efficient management policies (Hoffmann et al. 2010). Moreover, through metanalyses or the screening of their ecological traits, such results could provide a basis for the prediction of new potential ant invaders (Fournier et al. 2019) and their associated impacts (Lach & Thomas 2008; Helms 2013; Lach et al. 2020).

In general, I aimed to disentangle the effect of the introduced RWA *F. paralugubris* on the native community by evaluating the interactions with multiple taxa (Chapter 1), and to quantify the ecological role of introduced *F. paralugubris* within the recipient trophic web (Chapter 2) and forest ecosystem (Chapter 3). Finally, I aimed to perform an overall revision of the current protection status of RWA throughout Europe (Chapter 4).

In Chapter 1, I investigated the effect of *F. paralugubris* on other organisms: the lichen assemblage growing on forest trees (Chapter 1.1) and the myrmecophilous (*sensu lato*) fauna living within the mounds (Chapter 1.2):

a) In Chapter 1.1, I evaluated the effect of the presence of introduced populations of *F. paralugubris* on the structure of epiphytic lichen communities in two white fir forests. For this purpose, I compared the lichen species diversity and the taxonomic and functional composition in occupied and unoccupied areas within the two sites in the Apennine mountains.

b) In Chapter 1.2, I studied the invertebrate fauna associated with *F. paralugubris* nest mounds by collecting soil and mound material from three populations of this RWA species, one native (from the Alps) and two invasive (from the Apennines). Invertebrates were extracted with Berlese funnels and identified, and comparisons were made between the mound-living and soil-living organisms as well as across sites. Since springtails and oribatid mites are commonly used as ecological indicators of soils and RWA mounds (Lenoir et al. 2003; Elo et al. 2016), a focus on these two taxa has been posed. I aimed to i) confirm the biodiversity hotspot role played by *F. paralugubris* mounds also of introduced populations; and ii) compare native and introduced populations to assess if myrmecophilous species relocation could have occurred together with the transplantation of entire RWA nests.

In Chapter 2, I assessed the trophic positioning and the trophic niche of two native (from the Alps) and three invasive (two from the Apennines and one from Canada) populations of *F. paralugubris*. The aim was to assess whether the pattern found in the diet of other invasive ant species, namely an increased consumption of aphid honeydew in the invasive compared to native populations (Tillberg et al. 2007; Wilder et al. 2011), was followed by *F. paralugubris*, too. For this, for each site I performed carbon and nitrogen stable isotope analysis (SIA) on ant workers as well as arthropods belonging to clearly identified trophic levels (herbivores and predators), and compared their trophic positioning as determined by their nitrogen stable isotope signatures. Moreover, I compared *F. paralugubris* isotopic niche width across sites, to test if invasive populations relied on a more specialized diet respect to the native ones, as previously demonstrated on other invasive ants (Wilder et al. 2011). Additionally, for each site the isotopic niche overlap between *F. paralugubris* and other co-occurring ant species was assessed, to test for potential competition or niche partitioning for food resources.

In Chapter 3, I assessed the role of nest mounds of an invasive population of *F. paralugubris* within a white fir forest ecosystem. To study the dynamics of mound metabolism, I quantified the CO_2 efflux and internal concentration and characterized its carbon isotopic signature. I also checked for temporal (diurnal and seasonal) trends in these measures, related them to the mound topology, and estimated the contribution of *F. paralugubris* mounds to the total CO_2 production at the forest scale. Moreover, to characterize the broad functional role of the microbiota associated with the mound material, the carbon isotopic signature of the produced CO_2 was experimentally determined and compared between summer and winter seasons. Additionally, I experimentally quantified the relative proportion to the overall nest mound gas emissions of its two most important contributors, namely ants and microbiota (Jílková & Frouz 2014). Finally, I characterized the microbiota associated with *F. paralugubris* mounds, and tested for seasonal differences in taxonomic composition and diversity.

Lastly, in Chapter 4, I reviewed the threat status and the protection level of RWA across European countries, highlighting exemplary cases. The aim was to resume the complex and scattered situation in which RWA are not protected in every country, while in some cases the invasive populations attracted more attention and preservation efforts compared to the native ones. Further, I aimed to highlight the lack of a coordinated framework for the protection of this species group.

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Chapter 1

Interactions between *Formica paralugubris* and other organisms

Red wood ants shape epiphytic lichen assemblages in montane white fir forests

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Abstract

The *Formica rufa* group comprises several ant species which are collectively referred to as ‘red wood ants’ (hereafter RWA). These species have key roles in forest ecosystems, where they are ecologically dominant and greatly influence the dynamics of the habitat they colonise. Various studies have shown how their trophic activity may affect other organisms, which include both other invertebrates and plants. We can therefore hypothesize that their presence could affect the taxonomic and functional composition of epiphytes, despite clear information on such an effect is lacking. This study aimed to fill this research gap by evaluating whether the presence of red wood ants could affect the structure and composition of lichen communities. We selected two sites on the Apennine Mountains in Italy, where the red wood ant *F. paralugubris* was introduced from the Alps more than 50 years ago. In each site, lichen assemblages on *Abies alba* trees located within the colonised areas were compared to those from nearby, non-occupied areas. The results allowed for the identification of significant effects of *F. paralugubris* on the structure of lichen communities. Although there was no detectable impact on lichen species richness, a significant difference in their community composition between colonised and control sites was detected. Furthermore, ant presence seemed to be associated with specific lichen functional traits such as asexual reproduction. We argue that RWA could affect the lichen community either directly, e.g. by actively dispersing the species capable of asexual reproduction through their movements on trees (ant-mediated dispersion), or indirectly through herbivore exclusion. Finally, we also observed differences in β-diversity among the colonised and non-colonised sites.

Keywords: *F. paralugubris*, red wood ants, lichen diversity, impact, introduced species, functional diversity

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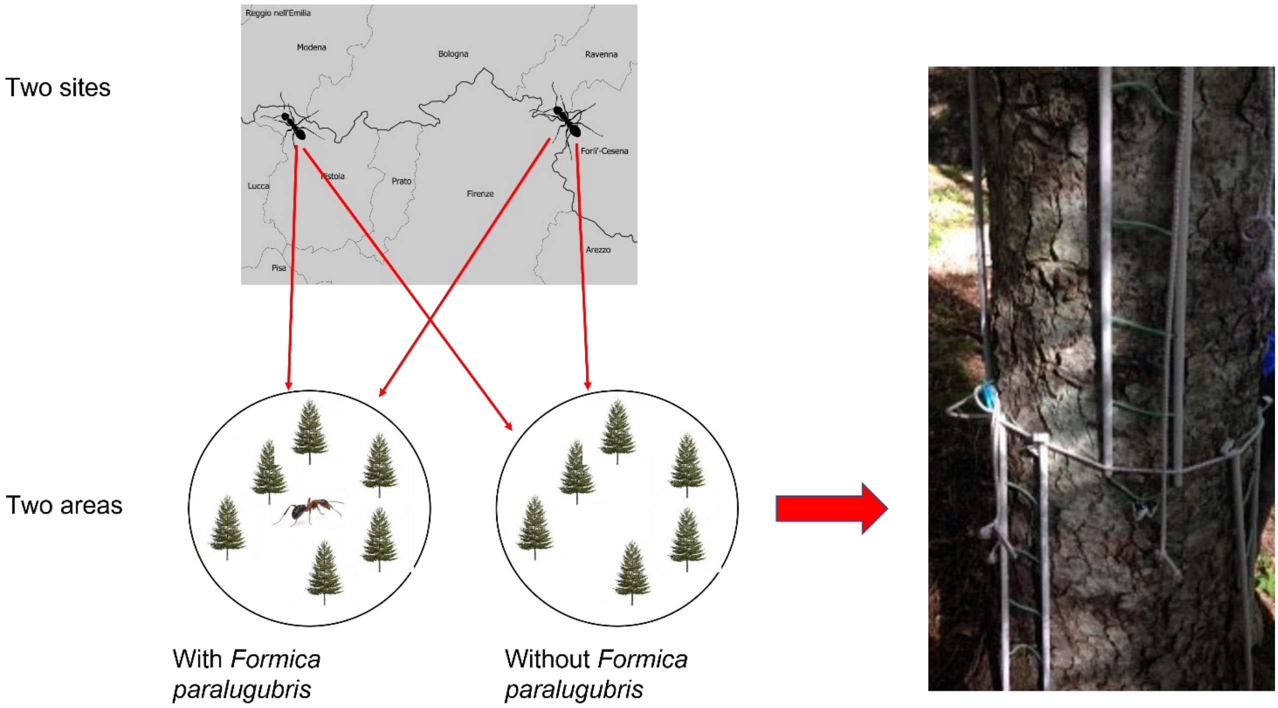

Introduction

Animal–plant interactions are widespread and extend beyond the known consumer–resource association in a wide array of relationships. As such, these interactions have long ago attracted the interest of scientists and today constitute an important field of research (Herrera & Pellmyr 2009, CaraDonna et al. 2021). Among the many documented examples, those involving arthropods, particularly ants species, are the most intriguing (Rico-Gray & Oliveira 2007). Ants are one of the most diverse, abundant, and ecologically dominant animal groups worldwide, and their impact on ecosystem function is correspondingly significant (Lach et al. 2010). Most of the available literature is biased towards angiosperms, and comparatively little is known about other organisms such as lichens.

Lichens are symbiotic poikilohydric organisms formed by a fungus, the mycobiont, and one or more algae, the photobiont (Nash 2008). Together with bryophytes, these cryptogamic organisms represent an important component of forest ecosystems and biodiversity. They are key organisms in ecosystem functioning, since they are primary producers involved, for example, in water and nutrient cycles (Pike 1978, Porada et al. 2018). Several interactions are known to occur between lichens and animals (Asplund & Wardle 2017). Lichens can be a feeding substrate for both some vertebrates and several invertebrates. For example, gastropods are known to feed on lichens (Benesperi & Tretiach 2004) and some species are specialised lichen-feeders (Kerney 1999). On the other hand, lichens provide a microhabitat where an organism could find particular temperature or moisture conditions. Invertebrates could find protection and food in the interface between the thallus and substrate, especially in the case of foliose lichens (André 1985). Moreover, although lichens rely mainly on wind for dispersion, several forms of zoochory by both invertebrates and vertebrates have been documented. In particular, ants can disperse both lichen soredia (Lorentsson & Mattsson 1999) and thallus fragments (Heinken et al. 2007). Soredia can attach to ants' bodies during their passage on the surface of lichen thalli, or in some cases, wind-borne spores are carried in contact with ant body (Bailey 1970). In the case of thallus fragments, some ants use them to build nests, collecting fragments in the surrounding areas and thus providing maintenance of epigeous lichen diversity (Heinken et al. 2007). This harvesting behaviour has also been investigated to explain the negative correlation between epiphytic macrolichen richness and ants (Thunes et al. 2018). Nevertheless, to the best of our knowledge, the effect of ants on the overall lichen epiphytic community has never been investigated.

Red wood ants (hereafter RWA) are ecologically dominant species belonging to the *Formica rufa* group, with seven species described in Europe and at least 19 species reported in

North America (Stockan & Robinson 2016). RWA are cold-adapted species inhabiting coniferous woodlands (Risch et al. 2016), and in the southern part of their range they are restricted to mountain areas, rarely occurring below 900 metres a.s.l.. In Italy, these species are widespread along the Alpine chain and only the more thermophilic *Formica pratensis* (Retzius,1783) naturally occurs south of the Alps in the Apennine mountains. Moreover, starting in the mid-1900s, colonies of *F. lugubris* (Zetterstedt, 1838), *F. polyctena* (Foerster, 1850) and *F. aquilonia* (Yarrow, 1955) were repeatedly transplanted as a biocontrol agent for forest insect pests from their original areas in the Alps to other sites in the Apennines, where they were formerly absent. The current status of most of the introduced populations is unknown. In a few cases, local extinctions have been documented, especially in less suitable and warmer areas, whereas some of the introduced populations have grown considerably (Frizzi et al. 2018).

Most RWA have a strong influence on forest ecosystems (Wardle et al. 2011), affecting both nutrient cycling and ecosystem function (Frouz et al. 2016). They also affect a wide range of cooccurring animal taxa, which includes other ant species (Stockan & Robinson 2016), herbivorous insects (Punttila et al. 2004), predatory arthropods (Robinson et al. 2016) and birds (Aho et al. 1999). Moreover, RWA can affect plant communities both indirectly, mediated by their action on aphids or other plant parasites (Domisch et al. 2016), and directly through seed dispersion or soil enrichment (Ohashi 2007). In particular, RWA can be effective seed dispersers for plants with elaiosome-bearing seeds or fruits that represent a valuable trophic resource (Gorb & Gorb 2003). More recently, Thunes et al. (2018) suggested that *F. aquilonia* colonies can impact lichen species richness, removing them from the tree bark along their foraging trails to aphids in the canopy, and sometimes collecting them as nest material. Because of these effects, RWA are interesting candidates to explore ant–lichen interactions.

In this study, we investigated the effects of *F. paralugubris* populations on lichen assemblages at two sites in the Central Apennine Mountains, in Italy. In particular, we compared the lichen species richness, lichen assemblage composition, community functional traits, and β-diversity between areas within and outside of the range occupied by *F. paralugubris*. The estimation of β-diversity has proven to a be an effective tool to better understand mechanisms determining differences between communities (Nascimbene et al. 2015).

Materials and Methods

Study areas and sampling

The study was carried out in Abetone forest (AB, 44°08′50″ N, 10°40′24″ E) and in the Campigna Biogenetic Nature Reserve (hereafter referred to as Campigna, CA, 43°52′20″ N, 11°44′ 41″ E), where RWA populations have been transplanted since the late '50 (Groppali & Crudele 2005, Fig. 1). According to IV level Corine Land Cover for the Tuscany region, both sites can be classified as coniferous woodlands (CLC code 3123) dominated by white fir (*Abies alba*). Both sites have similar elevational range (1200-1350 m asl) and the mean annual precipitation is 2325 mm and 1682.1 mm for Abetone (Bartolini et al. 2018) and Campigna (Gonnelli & Bottacci 2009), respectively.

Figure 1. Location of the study areas.

In Campigna, the RWA population is subdivided into several independent sub-populations displaced in the area, one of which is located in Avorniolo Alto, occupies an area of ~8 hectares, and was chosen for this study (see Frizzi et al. 2020). In the area of Abetone, a single population exists and covers an area of ~10 ha. Despite previous identifications described RWA populations in both areas as *F. lugubris* based on morphological characteristics (Groppali & Crudele 2005), here the target populations were identified as *F. paralugubris* following the methods described in Masoni et al. (2019). The nest density per hectare was similar between sites (AB ~13 nest/ha, CA ~12 nests/ha). For each area, we randomly selected three *F. paralugubris* nests spaced at least 150 metres from each other. Six *Abies alba* (Mill., 1759) trees located within 20 m from each nest were chosen to sample lichen communities. The fir trees were as evenly spaced in all directions around the nests as possible. Additionally, we randomly selected as control sites three points from nearby non-occupied areas, where *F. paralugubris* was known to be never introduced or present (Groppali & Crudele 2005). As a result of their reproductive strategy based on colony budding, populations of this ant have sharp, easily recognisable boundaries (Chapuisat & Keller 1999). Moreover, as these populations are under study since 2015, their boundaries are well mapped (Frizzi et al. 2018). The control areas shared similar environmental features of the occupied areas (i.e., altitude, type of forest stand, undergrowth, orientation, and slope). For each site, the lichen communities present on the randomly selected *Abies* trees were assessed.

Epiphytic lichens were sampled using four standard frames of 10×50 cm subdivided into five 10×10 cm quadrants as sampling grids, which were vertically attached to the tree trunk at three different heights above the soil (with the lower edge at 0, 50, and 100 cm). At 0 and 100 cm, the frames were attached at the cardinal points, while at 50 cm, they were rotated by 45° with respect to the cardinal points. All lichen species occurring within the frames were listed, and their frequency was recorded as the number of quadrats in which the species occurred. Most species were identified in the field, while critical specimens were collected and identified in the laboratory using standard stereo- and light microscopy techniques and chemical reagents. Following Nimis & Martellos (2020), we described lichen diversity considering three functional traits evaluated for each species: growth form (GF), reproductive strategy (RS), and presence of lichenic acids (LA) (Table S1), which have been previously used to compare lichen communities (Giordani et al. 2016, Hurtado et al. 2020).

Data analysis

Differences in observed lichen species density (defined as the number of species observed in each sampled tree), carried out by comparing trees with and without RWA at both sites, were assessed using Generalised Linear Mixed Models (GLMM) with Poisson error distribution, including the nest as a random variable (Zuur et al. 2009).

Compositional differences within sites were investigated using multivariate techniques. The multivariate distances among samples were computed using the Bray-Curtis dissimilarity index after fourth root transformation to reduce the influence of extreme values. We then performed non-Metric Multidimensional Scaling (nMDS) ordination analysis on the resulting distance matrix, according to Clarke and Warwick (2001). As described in Anderson (2001), differences in species composition were tested within each site using a permutation-based non-parametric Multivariate Analysis of Variance (npMANOVA), using the presence of the ants as the main fixed factor and the tree replicate as a nested random factor.

For the analysis of functional traits, we computed the Community Weighted Mean (CWM), which represents the weighted mean trait in a community and accounts for the abundance of the species that carries the considered trait (Pla et al. 2012). The CWM of categorical traits was measured as the relative abundance of the category or group in the community, while the CWM of continuous traits was calculated as the trait average value (Lavorel et al. 2008). The computed CWM values were fitted onto the ordination axes using the function "envfit" of the Vegan/R package.

β-diversity was computed to evaluate the lichen species diversity and turnover among the habitats tested. We applied the SDR simplex approach (Podani & Schmera 2011), comparing for both areas the plots with and without *F. paralugubris*. We computed the three additive components of β-diversity: the relativized species replacement (R), the relativized richness difference (D), and similarity (S). This method proceeded by comparing all pairs of plots and computing these three components of β-diversity to analyse lichen species presence.

All analyses were carried out in R software v.3.6 using the packages vegan, lme4, FD, and BiodiversityR.

Results

On the 72 *A. alba* sampled trees we found 65 lichen species (Tab. S1), belonging to 40 genera, whose density in both sites did not vary according to RWA presence ($z = -0.572$, $P = 0.567$).

Focusing on lichen species composition within each site, nMDS ordination plots (Fig. 2) for both AB (stress = 0.18) and CA (stress = 0.13) showed a clear separation of ant-visited trees compared to those from control sites. In both AB and CA sites, npMANOVA revealed a significant effect of ant presence ($F_{1,2}$ = 27.77, P = 0.001; $F_{1,2}$ = 11.15, P = 0.001; AB and CA, respectively), but also significant variability among plots within each site ($F_{1,4}$ = 5.50, P = 0.001; $F_{1,4}$ = 6.68, P = 0.001; AB and CA, respectively).

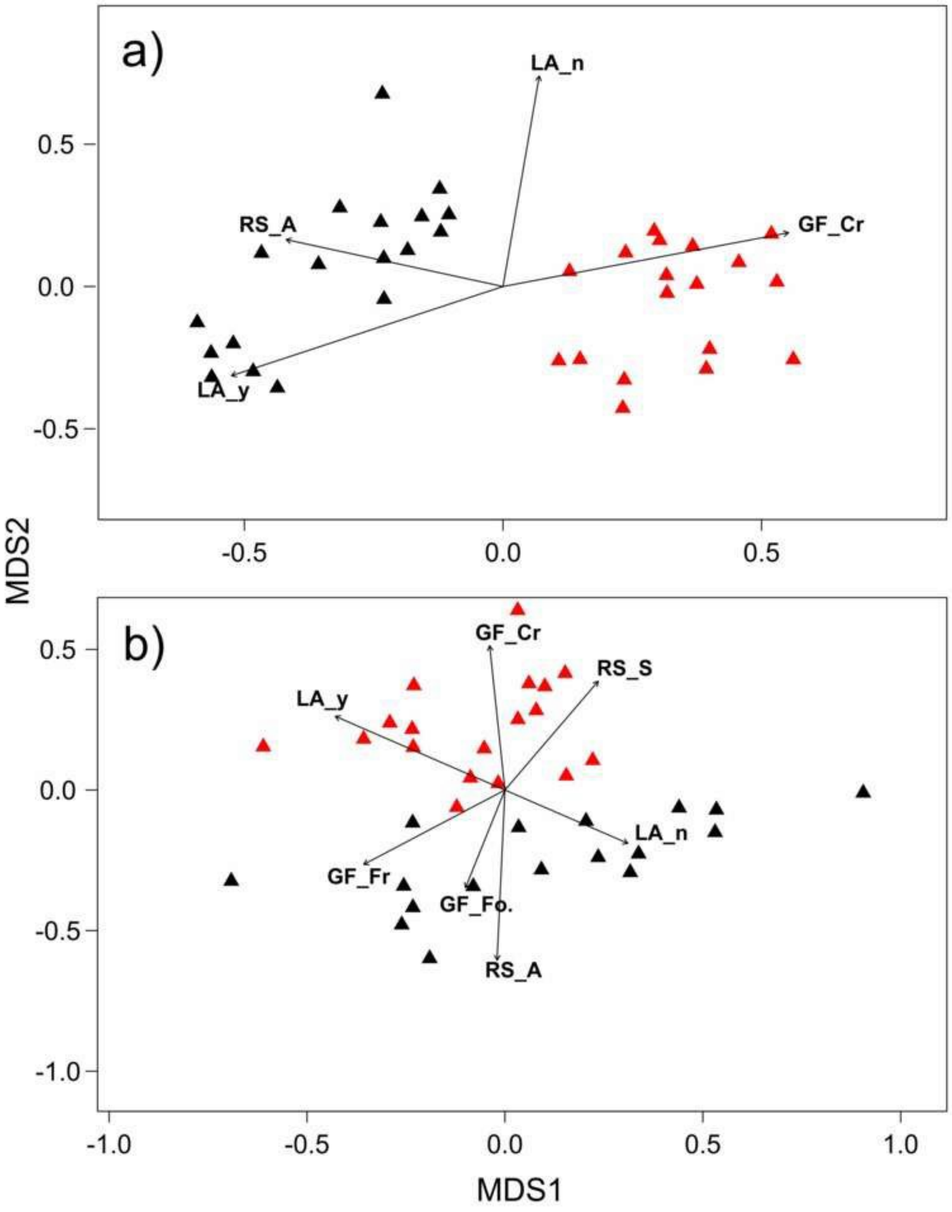

Figure 2. Non-Metric Multidimensional Scaling plot of tree lichen composition sample dissimilarities in Abetone (a) and Campigna (a), according to ant presence: black symbols, tree within the ant range; red symbols, control trees. The significant functional traits are plotted as vectors: Growth form: fruticose (GF_F), foliose (GF_Fo), crustose (GF_Cr). Reproduction strategy: asexual (RS_A), sexual (RS_S). Presence of lichenic acids: presence (LA_y), absence (LA_n). The orientation of vectors represents the correlation direction with ant presence.

CWM values varied according to ant presence (Table 1, Fig. 2). At Abetone, the crustose growth form (GF_Cr) was negatively associated with RWA presence, while the asexual reproductive strategy (RS_A) and the presence of lichenic acids (LA_y) were positively associated with ant. This was also evident in the nMDS plot, where their vectors pointed toward the RWA-sites (Fig 2a). In Campigna (Fig. 2b), the sites with ants were related with a higher abundance of lichen species with an absence of lichenic acids (LA_n), asexual reproductive strategy (RS_A) and a foliose (GF_Fo) and fruticose (GF_Fr) growth form. On the contrary, these sites were negatively related to crustose growth form (GF_Cr), presence of lichenic acids (LA_y), and sexual reproductive strategy (RS_S). β-diversity results (Fig. 3) indicated that plots with RWA were more variable, with a higher value of species replacement (R = 37.4) and lower values of similarity (S = 41.5) and relative richness difference (D = 20.9) compared to control areas (R = 29.1, S = 43.8, D = 27.1).

Site	**Trait**	**r^2**	***P***	**Direction**
Abetone				
	GF_Cr	0.3706	0.001	-
	GF_Fo	0.0422	0.514	
	GF_Fr	0.0001	0.998	
	RS_A	0.2208	0.021	+
	RS_S	0.0100	0.859	
	LA_n	0.6009	0.001	n
	LA_y	0.4068	0.001	+
Campigna				
	GF_Cr	0.3658	0.002	-
	GF_Fo	0.1805	0.041	+
	GF_Fr	0.2723	0.004	+
	RS_A	0.5062	0.001	+
	RS_S	0.2833	0.006	-
	LA_n	0.1829	0.035	+
	LA_y	0.3484	0.004	-

Table 1. Correlation values between Community Weighted Mean of each functional trait and ordination axes at both sites. For traits significantly correlated with axes, the type of effect summarizes whether RWA presence has a positive, negative or no influence on the trait. Acronyms for traits: Growth form: fruticose (GF_F), foliose (GF_Fo), crustose (GF_Cr). Reproductive

strategy: asexual (RS_A), sexual (RS_S). Presence of lichenic acids: presence (LA_y), absence (LA_n).

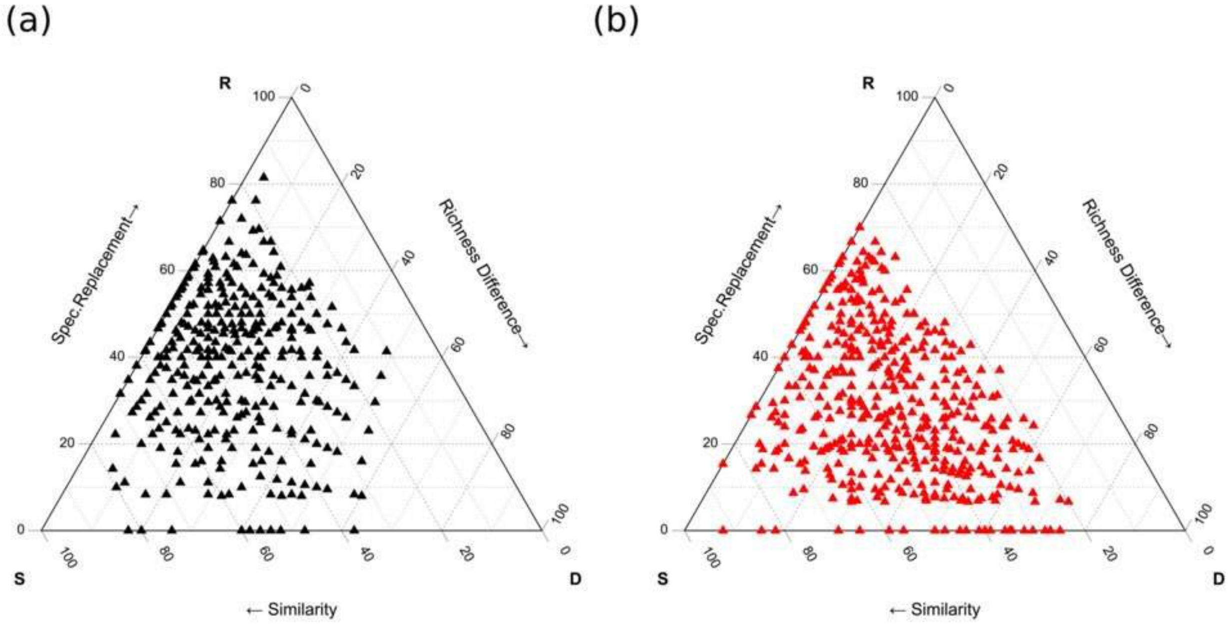

Figure 3. Three β-diversity components estimated in the areas with (a) and without *F. paralugubris* (b). R, relative species replacement; D, relative richness difference; S, similarity.

Discussion

Overall, our results showed that the lichen epiphytic communities significantly varied according to RWA presence in the two surveyed areas. Ants affected lichen communities in terms of species composition. In particular, they seemed to affect lichen functional traits, favouring species with asexual reproduction. One possible explanation is that they unintentionally transport lichen propagules during their movements, acting as a vector for lichen asexual dispersion in surrounding trees (Lorentsson & Mattsson 1999).

In our sites, lichen richness was not affected by ant presence. Lichen epiphytic diversity, in terms of species number, has been shown to be influenced more by macroclimatic factors than specific habitat features (Matteucci et al. 2012). Thus, the overall epiphytic lichen diversity may not be a good proxy for assessing possible effects on lichen diversity carried out by ants. Our results differed from previous studies that found a negative influence of ant presence on lichen species richness (Lorentsson & Mattsson 1999; Thunes et al. 2018). However, these studies focused on both lichens growing on trees and on other substrates. Thus, ant effects may differ depending on which lichen community is considered (epiphytic vs. terricolous). Thalli fragments of terricolous lichen species were usually found in ant mounds, supporting this hypothesis (Heinken et al. 2007). Moreover, Thunes et al. (2018) considered *F. aquilonia*, which could have a distinct behavioural pattern compared to *F. paralugubris*, thus influencing cryptogam communities differently. *F. paralugubris* has been introduced in our forests, so we could also not exclude different behavioural patterns compared to the original ants' populations in the Alps or in other autochthonous areas of Europe.

The major influence of ants on lichen epiphytic communities seemed to be mediated by functional traits. Plots with ants displayed a greater presence of lichens with asexual reproduction as soredia and isidia and fewer crustose species. Previous research has reported that soredia could attach to ants when the latter pass over lichen thalli (Lorentsson & Mattsson 1999). Thus, our finding corroborates the hypothesis that ants could act as a dispersion vector of lichen soredia, transporting them onto other trees and enhancing their colonisation. To date, evidence of ant interaction with isidia is lacking, but we argue that continuous movements over lichen thalli could enhance the detachment of these propagules, favouring their dispersion. Wood ants establish complex networks of foraging routes that radiate to nearby trees and connect different nests (Ellis & Robinson 2016). These trail networks change dynamically to track temporal changes in resource availability, mainly aphid colonies, and allow an efficient and almost uniform exploitation of the area surrounding the colony (Buhl et al. 2009). As a consequence, isidia and soredia could travel through this network, potentially dispersing to other trees in the area surrounding a nest. In this way, ants may affect lichen species composition due to temporal changes of the foraging trees according to aphid availability.

Regarding the growth form, control sites had a greater presence of crustose lichen species. Epiphytic lichens are often predated by invertebrates such as snails (Benesperi & Tretiach 2004), and RWA presence may negatively affect invertebrate assemblages. Frizzi et al. (2018) have previously found in the same forest of this study that *F. paralugubris* negatively impacts invertebrates' presence, describing a clear decrease in their abundance in colonised areas. This could suggest that the decreased abundance of invertebrates feeding on lichens may positively influence the presence of foliose and fruticose lichens that may be more likely predated (Gauslaa et al. 2006).

It is also worth reporting that the geographic position seemed to have an effect in influencing lichen communities. Several studies have shown that epiphytic lichen species richness and composition are mainly influenced by microscale factors such as bark pH, roughness, water holding capacity, and tree species, and macroscale factors such as temperature (e.g., Marini et al. 2011). Moreover, the difference in lichen species could be affected by the wide variety of growth forms, reproductive strategies, and photobionts that could allow the replacement of lost species. In our work, we tried to avoid the effect of these factors by comparing sites with similar pedoclimatic conditions. Additionally, the influence of the phorophyte was not an issue here, as only one tree species was present in both sites. However, it is evident that some microscale factors (i.e. at the plot scale) could not be controlled for, despite our efforts.

Our study also revealed a significant difference in β-diversity between sites with and without RWA. β-diversity was higher where RWA were present, and all its components (i.e., replacement, similarity and richness) were affected. The specific mechanism behind this pattern are not known and surely deserve further research, but in principle, the same mechanisms discussed to explain the observed differences in lichen functional diversity may apply here.

Conclusion

Our study revealed a significant effect of the presence of *F. paralugubris* on lichen communities and extended our knowledge on the impact of this introduced species on autochthonous communities. Our results suggest both a direct effect, with a physical dispersal of lichen propagules by ant workers, and an indirect effect, by ant predatory pressure on lichen herbivores. Our study is the first attempt to unveil the possible effects of RWA on lichen epiphytic diversity using a functional approach. We stressed that possible effects of the presence of RWA could be concentrated on species with specific functional traits. Nevertheless, further studies are needed to clarify the mechanisms involved in the interactions between RWA and lichen communities.

Author contributions

RB and GS conceived and designed the experiments. LDN, EB, RB, AM, FF, GS, MBC, FM, YS, CV and PB collected the data. FF, LDN and AM performed data analysis. LDN and AM wrote the first draft and contribute equally. All authors contributed extensively to the ideas, writing and discussions. The work was funded by grants of the University of Florence to GS and RB.

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A comparative study of the fauna associated with nest mounds of native and introduced populations of the red wood ant *Formica paralugubris*

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Abstract

In the second half of the twentieth century, many red wood ant populations were transferred from the Alps to the Apennines as biological control agents. Since the introduction involved the relocation of entire nest mounds, it is presumable that the associated fauna was also relocated. While the introduction of these ants has raised several concerns about their ecological impact, there has been no attempt to investigate the introduction of other nest associated species. In this study, we collected samples of soil and nest material from three populations of the red wood ant Formica paralugubris, one Alpine native and two imported into the Apennines. We aimed to confirm that nest mounds are hotspots for soil fauna, detect the occurrence of new myrmecophilous species, and compare the nest-associated fauna among sites, to test the hypothesis of mass species relocation. We focused our analyses mainly on two taxa, springtails and oribatid mites, two highly representative groups of the mesofauna inhabiting nest mounds. The results showed higher richness and diversity in nests than soil for oribatids but not for springtails. We found 17 myrmecophilous oribatid species, but only two springtail species. Finally, native and imported sites shared only a few oribatid and springtail species, suggesting that massive relocation did not occur with nest transplants or it was likely limited. Additionally, we found some species never before collected in Italy.

Keywords: Red wood ants, Myrmecophily, Soil fauna, *Formica paralugubris*, Oribatid mites, Springtails, New species

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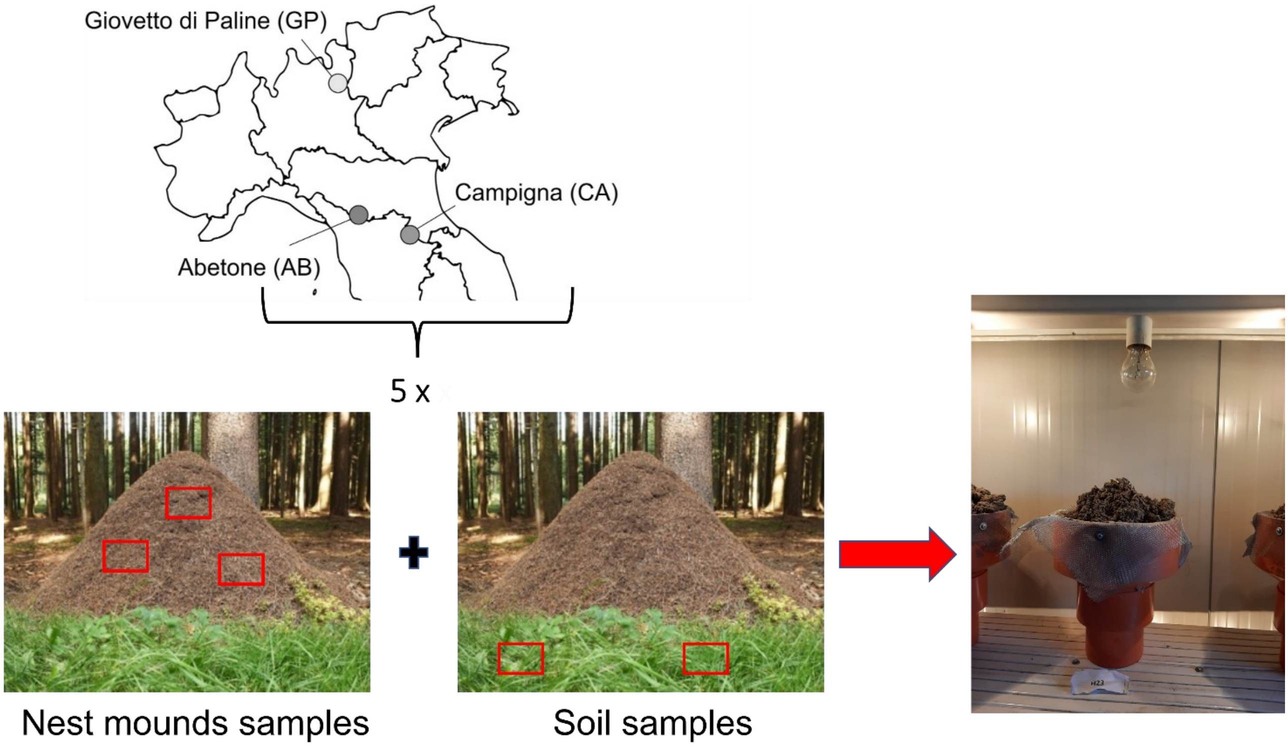

Introduction

Red wood ants (RWA) are typical in Central and Northern Europe, where seven species of *Formica* s.s. or the *F. rufa* group are known [1]. In contrast to other ants, these species are mostly associated with coniferous trees and require cold climates [2]. In the southern portion of their distribution area, their presence is limited to higher altitudes [3–6]. In Italy, the species of *Formica s.s.* are widespread along the Alpine chain, and only the more thermophilic *Formica pratensis* Retzius, 1783 naturally occurs at more southern locations in the Apennine mountains [7], although its exact distribution is still unknown. In the mid-twentieth century, nests of several alpine RWA species, such as *F. paralugubris* Seifert, 1996, *F. polyctena* Förster, 1850, and *F. aquilonia* Yarrow, 1955, were repeatedly introduced to the Apennines as biocontrol agents for forest insect pests, and in some cases, viable populations established and started to spread [8–10]. Their impact on the arthropod fauna inhabiting the newly occupied area has been demonstrated [9]. All RWA species are known to profoundly affect local communities, from plants to vertebrates, due to their predatory ability, dominant status, and capacity to modify the physical properties of the habitats they colonize [1,11–13]. The large aboveground nest mound is one distinguishing characteristic of these ants. They are formed to maintain stable humidity and temperature in the nest chambers, and often contrast with harsh external conditions [14–16]. For example, in *F. polyctena* mounds, the difference between the nest and air temperature during the winter can exceed 20 °C [17]. In cold habitats, this microclimatic stability may turn the RWA mounds into "warm islands", which can be attractive for a wide array of organisms [18,19]. More in general, that ant nests can provide shelter, food and favorable physical conditions for many litter animals has been established for several ant species [e.g. 20,21,22,23,24].

The invertebrate fauna hosted in RWA nest mounds, assessed in multiple studies, includes a broad range of taxa: isopods, spiders, mites, nematodes, springtails and a long list of insects, from flies to several beetle species [25–28]. More than 120 obligate myrmecophilous species have been found in RWA nest mounds, as well as several other species that occasionally inhabit nests because of their combined and often favorable moisture, pH and temperature conditions [19]. Myrmecophily can be defined as the partial or complete dependence on ant colonies by non-ant species [29]. This relationship can be based upon parasitic, commensal or even mutualistic interactions [30,31]. Thus far, more than ten thousand myrmecophilous arthropods are known, and they have evolved numerous strategies to inhabit or have access to ant nests [32–34], such as chemical camouflage, morphological mimicry and pheromonal attractors [35–37]. In this study, we analyze the invertebrate fauna hosted in the nest mounds of *F. paralugubris*, a highly polydomous and polygynous RWA species [38]. Since this species was one of the most often introduced into the

Italian peninsula [10], we focus our study on the comparison of the nest myrmecophiles from native and introduced populations. Ant translocation involved the movement of entire nest mounds [8], and this likely translocated all the associated fauna as well. The main aims of this study are: i) to confirm that nest mounds can be attractive for the soil fauna, both in the native and imported populations, by comparing assemblages inhabiting nest mounds and soil; ii) to identify potentially myrmecophilous species not previously described in the literature; iii) to compare soil and nest fauna across sites and evaluate if this difference supports the hypothesis of species relocation. We focused on springtails (Hexapoda, Collembola) and oribatid mites (Acari, Oribatida), which were used in previous studies as common representative taxa of the mesofauna inhabiting RWA nest mounds [39–42]. Among microarthropods, springtails and oribatid mites constitute two of the most species-rich taxa of the soil ecosystem [43]. Although quite distant phylogenetically, the two groups share several ecological and behavioral features because of their adaptation to similar ecological niches. Both groups feed on litter and soil micro-organisms and graze on fungi, and thereby affect the dispersion of saprophytic and mycorrhizal species and control fungal populations [44–47]. Their community structure is modulated by several natural factors, including plant diversity, litter quality and meta-population dynamics. However, these two key groups are also sensitive to anthropogenic factors such as land use, soil tillage, environmental pollution, physical disturbance and fire [48–51].

Materials and methods

Study area and sampling design

The sampling was carried out between June and August 2017. Samples of nest material and soil were collected from three sites, one in the Alps in the Giovetto di Paline Nature Reserve (abbreviated as GP, 45° 57′57″ N, 10° 7′48″ E), and two in the Apennines, in the Abetone forest (abbreviated as AB, 44° 08′50″ N, 10° 40′24″ E) and the Campigna Biogenetic Nature Reserve (abbreviated as CA, within the Foreste Casentinesi, Monte Falterona e Campigna National Park, 43° 52′00″ N, 11° 44′14″ E) (Fig. 1). The Alpine site, GP, is the site of origin, where nests later imported to both Apennine sites were collected [52]. Nests used for sampling in the Campigna Biogenetic Nature Reserve were imported in 1958 [9,52], whereas the exact year of transplants is not available for the Abetone forest. However, the period was probably similar (Groppali, personal communication). The habitats of the three sites are similar, a mixed forest composed of a dominant conifer species and beech (*Fagus sylvatica* L. 1753). The dominant species in the Alpine site is the red fir (*Picea abies* (L.) H. Karst, 1881), while in the Apennine sites, it is the white fir (*Abies alba* Mill, 1759). The geographic position of all ant nests—including sampled nests—was recorded in 5 ha per site by a GPS locator (Garmin eTrex® 10, accuracy ~ 3 m), and the density per hectare was evaluated.

Five nests of *F. paralugubris* of similar size (height range, 1.3–1.5 m; diameter at the base of the mound, 2.3–2.6 m) were chosen in each area. Nests were spaced at least 15 m apart to ensure independent sampling of the nest fauna. Moreover, we preliminarily checked for the presence of ant trails connecting the selected nests. From each mound, we collected three 1-dm^3 samples of nest material (total number of samples = 45, 15 per site) and two 1-dm^3 samples of soil 3 m from each nest (as control) utilizing a soil corer (15 cm diameter, 50 cm height). Nest material was collected at 40 cm depth from the surface of the mound. Each sample was separately stored in a plastic bag. All samples were transferred to the laboratory within 6 h from the time of collection. The material was gently stirred and homogenized in a plastic basin and then placed into a Berlese funnel. Samples were left in the funnels for 5 days, following Parisi et al. [53]. The specimens collected were examined using a stereomicroscope. Oribatids and springtails were identified to the species level, while most of the other organisms were identified to a higher taxonomic level. All samples were stored in pure ethanol for further analysis.

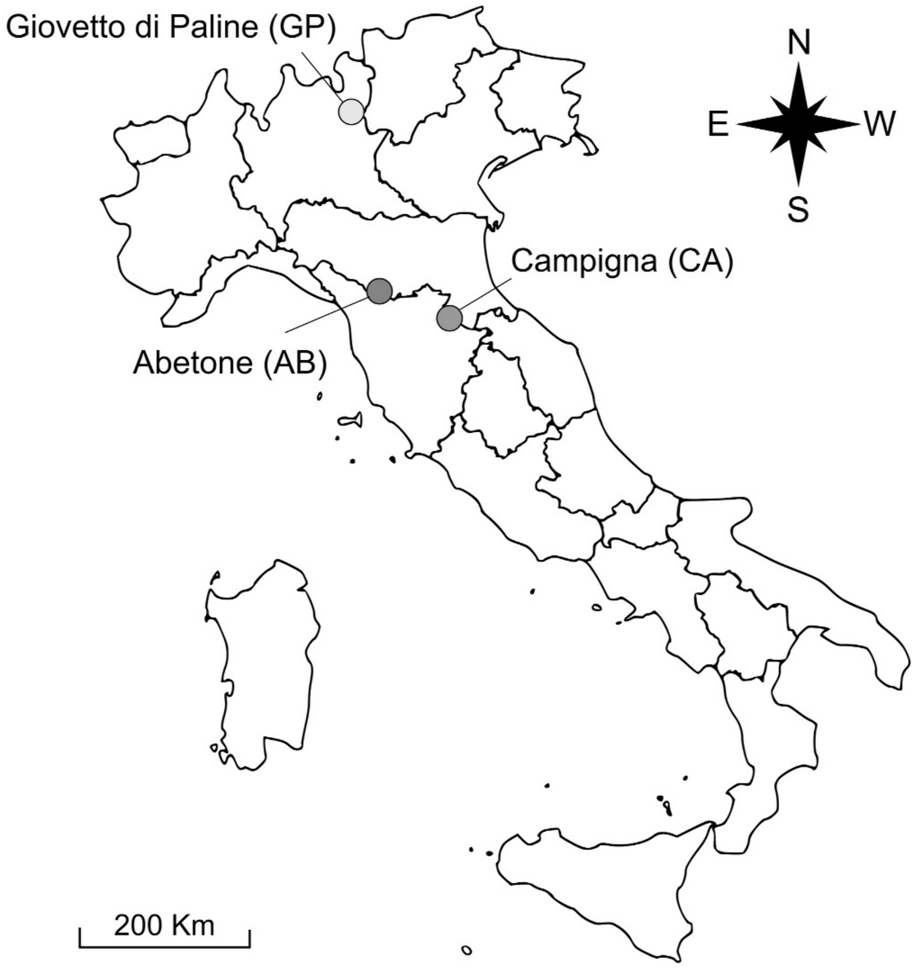

Figure 1. Map of the three sampling sites. The light grey circle is the alpine site of the original population, and dark grey circles are the two Apennine sites of the two introduced populations.

Statistical analyses

Species diversity at the three sites was estimated following Chao et al. [54], using as input the abundance data for oribatids and presence/absence for springtails. The use of presence/absence for springtails is motivated by the strongly aggregated distribution of several species of this taxon [55,56], which may hamper diversity estimates [54]. The method for evaluating diversity is based on the estimation of Hill numbers, qD, and yields estimates of total (rarefied and extrapolated) species richness ($q = 0$), and the exponential values of the Shannon diversity ($q = 1$) and Simpson diversity ($q = 2$) indices. The 95% confidence intervals were obtained from bootstrapping, based on 4999 replications of the reference sample set. Finally, differences in observed species density among sites (defined as the number of species captured in each sample) were assessed using mixed-effect modelling with Poisson error distribution, including the nest as a random variable [57]. A Tukey post-hoc test was then performed to assess the differences in pairs.

Association of a species with either nests (i.e., myrmecophily) or soil was evaluated by analyzing the probability of occurrence in the respective material. Generalized Linear Mixed Models (GLMM) were used for this analysis, with the type of habitat (nest or soil) as the main factor. For springtails, we used binomial distribution (presence/absence data) in the model, whereas for oribatids, given the high difference in abundance between samples, we log-transformed data and used a Gaussian distribution. For springtails, we analyzed only species that occurred in at least three samples in each site in which they were present. For oribatids, following Elo et al. [42], we tested only species that occurred in at least three samples and with at least ten individuals per site. Sites in which the species did not occur were omitted from the analysis.

Compositional differences among sites were investigated using multivariate techniques. Rare species (those with less than three observations) and empty samples with no specimens were omitted from the following analyses [58]. The multivariate distances among samples were computed with the Bray-Curtis dissimilarity index after log transformation of the data, and the resulting distance matrix was analyzed by non-metric multidimensional scaling (nMDS) according to Clarke and Warwick [58]. Species composition differences were tested with a permutation-based non-parametric multivariate analysis of variance (PERMANOVA), using the factor “site” (fixed) and “nest” (random, nested) to account for non-independence of observations. β-diversity was computed following Anderson et al. [59], and permuting model residuals were compared to generate a permutation distribution of F under the null hypothesis of no difference in dispersion between groups. All analyses were conducted using the R software package (ver.3.6) using the libraries "vegan", "iNEXT" and "ecodist".

Results

The estimate of nest density was similar among sites (AB ~ 13 nests/ ha, CA ~ 12 nests/ha, GP ~ 12 nests/ha). We collected a total of 32 springtail species (11 in GP, 21 in CA and 16 in AB) belonging to 21 genera, 122 oribatids (49 in GP, 39 in CA and 51 in AB) belonging to 62 genera, 53 morphospecies of other arthropods, 2 morphospecies of Annelida (Haplotaxida) belonging to the Enchytraeidae and Lumbricidae families, and one nematode morphospecies. Of the 53 morphospecies of arthropods, 39 were insects, subdivided into Coleoptera (23), Diptera (6), Hemiptera (4), Lepidoptera (2), Hymenoptera (2), and one each of Thysanoptera and Psocoptera. Other arthropods included four Diplopoda, three Chilopoda, three Arachnida, one Protura, one Diplura and one Symphila. Detailed lists of the focus groups (Collembola and Oribatida) and other morphospecies collected, with their abundance at each site and type of habitat (nest or soil), are reported in Supplementary materials S1 and S2, respectively.

Except for springtails and oribatids, the most represented group was that of beetles. Overall, most coleopteran morphospecies were found in nests (20 out of 23, 17 of them exclusively in nests), whereas only seven out of 23 morphospecies were collected in soil samples (three of them exclusively in soil). Among Coleoptera, Staphylinidae was the most represented taxon, with ten total morphospecies collected. Diptera (6 morphospecies) were collected only in the soil samples. All three spider morphospecies collected were associated with nests only. None of the other groups with more than one morphospecies was exclusive for either of the two types of samples.

We found three oribatid species new to Italy: *Damaeus selgae* P´erez- ´I˜nigo, 1966 and *Scheloribates tuberculatus* Pérez-Íñigo jr., Herrero and Pérez-Íñigo, 1987 only known from Spain [60], and *Dissorhina ornata peloponnesiaca* Mahunka, 1974, recorded in Spain [61], Greece [62] and recently, Poland [63]. Moreover, the myrmecophilous beetle *Monotoma conicicollis* Chevrolat, 1837 (Monotomidae), previously recorded only in the Alps, was recently recorded for the first time in the Apennines [64]. Several species of insects, previously known only in the Alps, have often been overlooked, and their populations only recently recorded in the northern Apennines and surrounding areas [65,66].

In total, we found 19 species that preferred ant nests (2 springtails and 17 oribatids), whereas seven species preferred soil (2 springtails and 5 oribatids) (Supplementary material S1). Pooling all sites, no difference in the number of springtail species in soil and nests was found (Fisher exact test $p = 0.805$), whereas oribatid species richness was significantly higher in nests than soil (Fisher exact test $p = 0.022$). The number of species shared between soil and nests differed among sites for springtails (Fisher exact test $p = 0.022$, Fig. 2), but not for oribatids (Fisher exact test $p = 0.310$). The number of species shared among sites is summarized in Table 1. There was no difference between the proportion of shared species both in soils and in nests with respect to the total number of species in that habitat (Mantel-Haenszel chi-square test with continuity correction: $\chi^2 = 2.1, p = 0.147$).

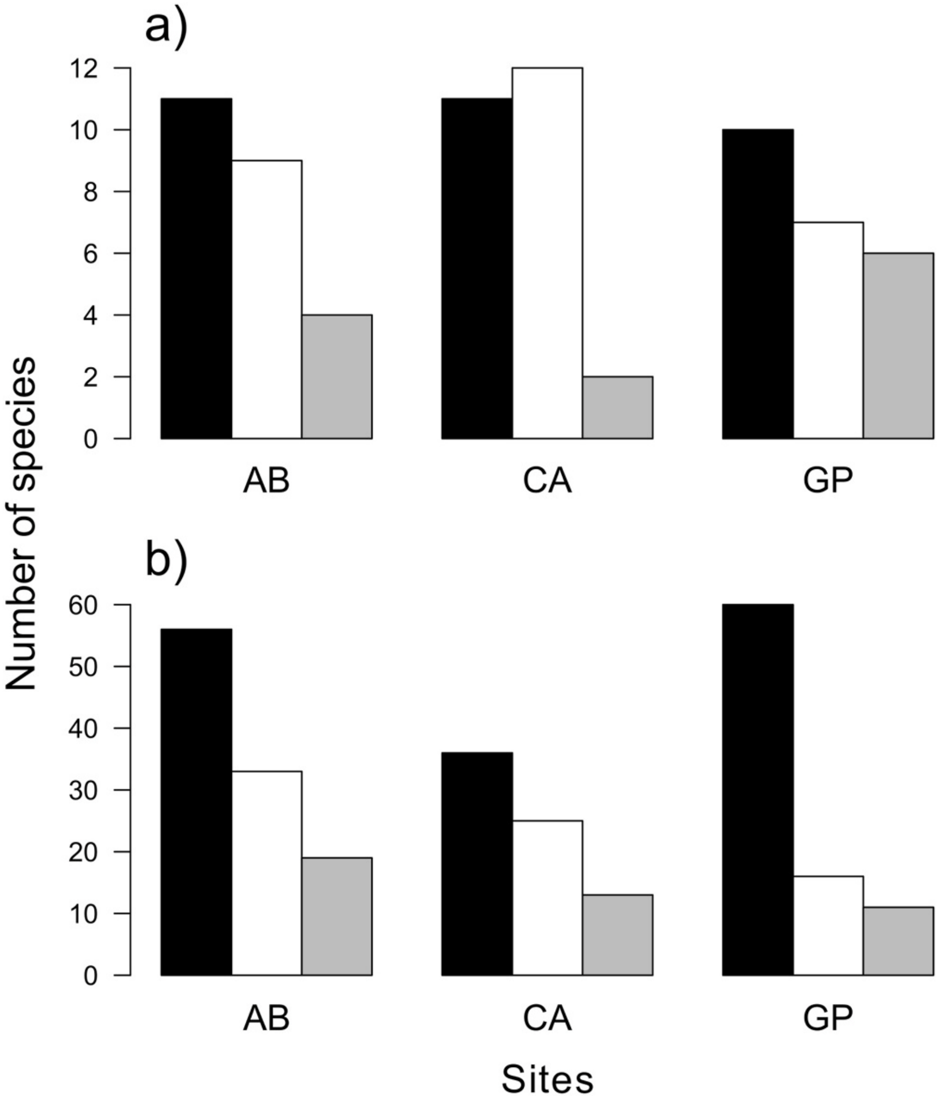

Figure 2. Cumulative number of species observed at the three study sites for a) springtails and b) oribatids. Black bars = nests, white bars = soil, grey bars = species shared between soils and nests. Sites: AB = Abetone (Apennines), CA = Campigna (Apennines), GP = Giovetto di Paline (Alps).

	Springtails				Oribatids			
	soil		nests		soil		nests	
Group	shared	total	shared	total	shared	total	shared	total
AB-CA	3	18	5	17	13	45	19	73
AB-GP	2	14	4	17	4	45	22	94
CA-GP	0	19	3	18	6	35	21	75
AB-CA-GP	0	23	2	22	3	54	12	102

Table 1. Number of species shared between sites, both for soils and nests. Sites: AB = Abetone (Apennines), CA = Campigna (Apennines), GP = Giovetto di Paline (Alps).

Focusing on nest samples, CA generally had slightly higher springtail species richness and diversity than either AB or GP, although the confidence intervals of the three sites overlapped widely (Fig. 3). Species density did not differ among sites for springtails (Type III Wald test: χ^2 = 1.727, df = 2, p = 0.422). For oribatids, CA had lower α-diversity than AB and GP, while species density was significantly lower than at AB and GP (χ^2 = 18.943, df = 2, p < 0.001; multiple comparisons: CA vs AB: z = -3.572, p < 0.001; CA vs GP: z = -4.078, p < 0.001; AB vs GP: z = -0.525, p = 0.859).

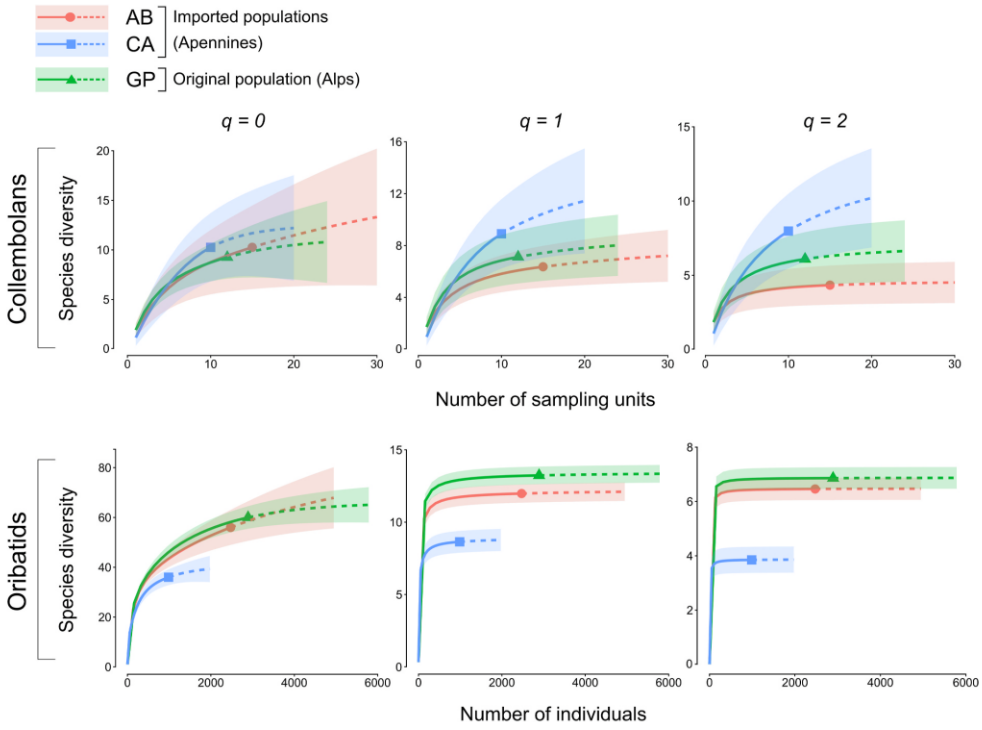

Figure 3. Species diversity in the three sites (red = Abetone, Apennines; blue = Campigna, Apennines; green = Giovetto di Paline, Alps). Sample-based plots with 95% confidence intervals for species richness (q = 0), Shannon (q = 1), and Simpson (q = 2) diversity indexes. Continuous lines = observed values; dashed lines = extrapolated values.

The nMDS ordination plot for springtails (Fig. 4a, stress = 0.06) showed a separation among groups, whereas nest samples were partially separated by site. The PERMANOVA (Table 2) revealed a significant effect of the factor site, but also significant variability among replicate nests within each site. A clearer picture emerged from the nMDS ordination plot of oribatids (Fig. 4b, stress = 0.17). In this case, the three sites clearly had different soil assemblages, but the two introduction sites AB and CA were closer to each other (i.e., more similar) than to GP. The data points of the former two were located on opposite sides of the plot of the latter one. Nest assemblages were also fairly distinct by site but were more similar than soil samples. All soil samples were located in the central part of the plot, in close connection to each other. Notably, while the soil samples from the Apennine sites were clearly separated by those from the Alps, the nest samples were more similar. Even in this case, PERMANOVA revealed significant differences in nest assemblages among sites, both also significant within-site variability (Table 2). Finally, no significant difference in β-diversity among sites was found for either springtails (p = 0.054) or oribatids (p = 0.431).

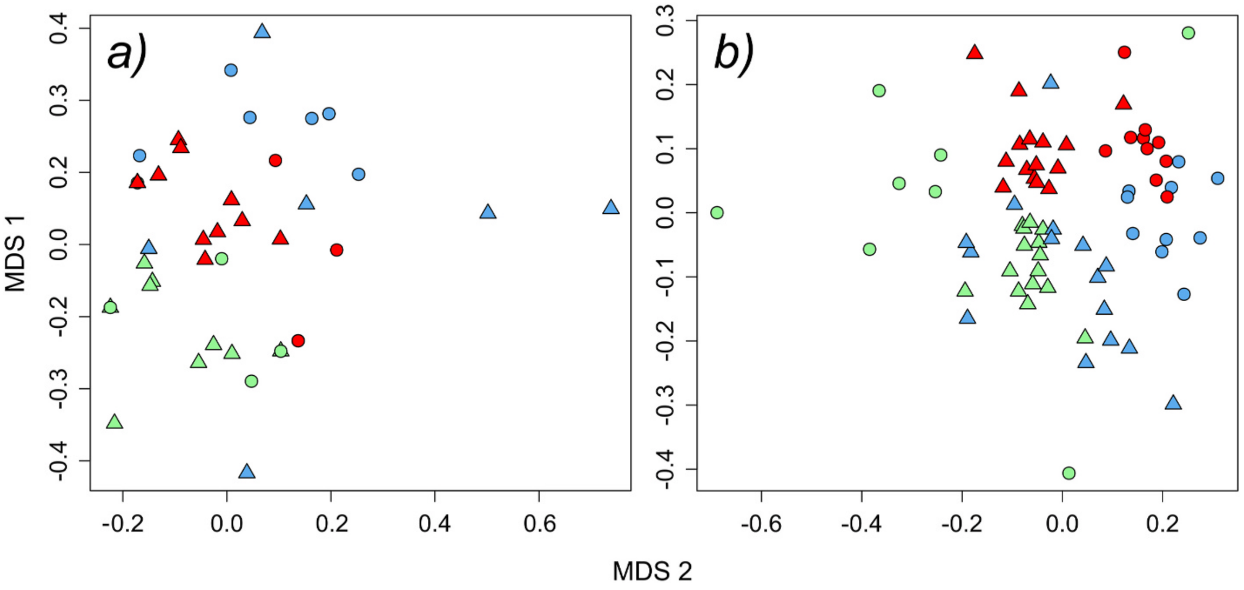

Figure 4. nMDS ordination plots for a) springtail and b) oribatid datasets. Colors indicate the sites (red = Abetone, Apennines; blue = Campigna, Apennines; green = Giovetto di Paline, Alps), symbols indicate sample types (triangles = samples from nests; circles = samples from soils).

	Springtails				Oribatids			
Factor	df	SS	F	*P*	df	SS	F	*P*
Site	2	2.300	2.35	< 0.001***	2	3.669	4.04	< 0.0001***
Nest [Site]	12	5.864	3.12	< 0.0001***	12	5.451	2.24	< 0.0001***
Residuals	21	3.288			30	6.076		

Table 2. Results of PERMANOVA tests.

Discussion

Richness and diversity of soil and nest fauna

The predictions that higher species richness would be found in nest mounds of *F. paralugubris* than in the surrounding soil was confirmed for oribatids, but not for springtails. As for this latter group, the presence of red wood ants is known not to be a determining factor affecting their occurrence (see Lenoir et al. [40] for a study with *F. polyctena*). However, little is known about the difference in richness and diversity between ant nests and the surrounding soil. Conversely, for oribatids, our finding is consistent with Laakso and Setälä [18], who found higher mite richness in nests of *F. aquilonia* than in the nearby soil.

Our results contrast with those of Elo et al. [42], who found different species but comparable species richness in the nest mounds of *F. polyctena* and the surrounding soil. As no other replicate studies on the same ant species are available, it is not possible to know if these differences represent specific features of the three species assessed, or, instead, they are driven by local environmental determinants, as suggested by Elo et al. [42]. These authors hypothesized that this incongruence could be due to differences in the quality of the leaf litter, which is assumed to be poorer in coniferous stands than in broadleaf forests. This relative lack of litter might compel mites to aggregate within nest mounds more strongly in the former than in the latter. A second suggested factor could be nest density: the higher density may drive species to be more distributed and less concentrated among ant nests. In our study, the hypothesis regarding leaf litter quality might be supported, because all three sites were dominated by coniferous trees (*Abies alba* and *Picea abies*), as in Laakso and Setälä [18], although a denser understory is present in the Alpine site. Nest density may have an effect as well, but we do not have any reference to verify whether the density in our sites is relatively high or low, and therefore how it might influence the aggregative behavior of nest symbionts. However, density was very similar among sites; hence this factor should similarly influence all three populations.

Myrmecophilous or soil-preferring species

Springtails and oribatid mites showed different degrees of potential myrmecophily. Only two springtail species, *Cyphoderus albinus* Nicolet, 1842 and *Lepidocyrtus cyaneus* Tullberg, 1871 selectively chose ant nests, and both are known myrmecophilous inhabitants of RWA nest mounds [19,26], and other ant species (e.g., *C. albinus* in *Lasius neglectus* Van Loon, Boomsma & Andrasfalvy, 1990 nests [67]). In contrast, two species, *Folsomia penicula* Bagnall, 1939 and *Pseudosinella apuanica* Dallai, 1970*,* both known to occur in Italy [68], preferred soil habitats, even though in both cases, the preference was not absolute, as they also occurred in some ant nest samples, though to a lesser extent.

Conversely, a higher number of oribatid species (17 out of 122) were found to be potentially myrmecophilous. Four of these, *Carabodes labyrinthicus* Michael, 1879, *Carabodes ornatus* Stork´an, 1925, *Liacarus coracinus* Koch, 1841, and *Pergalumna nervosa* Berlese 1914, have previously been described as preferring the nest habitat [42]. Most of the species collected are quite common, such as the ones belonging to the *Carabodes* genus, which can be found both in European and American forests, mainly in tree bark where lichens, mosses, and fungi are present [69,70]. Since all these latter organisms (or parts of them) can be found within nest mounds, either alive or as nesting material [71,72], it is possible that nest habitat is suitable for some *Carabodes* species. Two *Pergalumna* species were recognized as potentially myrmecophilous, *P. nevosa* and *P. altera*. Both species have previously been collected in central Italy [73,74]. The species within this genus are known predators of nematodes [75,76], which, in turn, may find the particular nest conditions favorable for proliferation [77]. Unfortunately, the Berlese funnel is not an accurate tool for detecting nematodes, so we cannot provide reliable information on nematode communities.

Regarding the linkage between *Phauloppia lucorum* Koch 1841 and ant nests, usually, most species of the *Phauloppia* genus are known to inhabit and feed on lichens [78–80]. Thunes et al. [81] found that the occurrence of the RWA *F. aquilonia* negatively impacted the lichen community, probably because of the chemical changes in the environment or by the direct collection of thallus fragments, which can be found within RWA nest mounds [72]. However, to our knowledge, it is hitherto unclear whether those lichen fragments came from active harvesting by ants or merely from the collection of detached lichen fragments. In any case, *P. lucorum* may feed on the lichens transported by ants into the nests, making the nest mound a suitable habitat for this species as well.

Only a few species, such as two *Oppiella* species and *Minunthozetes pseudofusiger* Schweizer, 1922 showed a preference for soils. For the latter, the preference for soil instead of other habitats, such as grass, roots or litter, is known [82], although studies on their avoidance of ant nests are not available. The generalist and eurytopic *Oppiella* species are known to occur in harsh habitats, such as newly burned soils [83], suggesting that in this context, they prefer to occupy habitats with more extreme temperatures and moisture conditions [84].

Comparison of assemblages among sites and species relocation hypothesis

We detected differences in species richness and diversity across sites only for oribatids but not for springtails. Lower oribatid diversity was found at Campigna, whereas the native site of Giovetto di Paline and Abetone were very similar. This result is quite surprising, because the two imported populations, Campigna and Abetone, are geographically close and very similar in their general features (fir-dominated forests, altitude and climate). The Alpine site, although similar in dominant tree composition, shows denser understory (Frizzi, personal observation), which may potentially affect both springtails and mite diversity [85,86]. However, many factors may influence diversity, such as altitude, climate conditions, and geographic location [2]. Moreover, metapopulation rules apply to the nest mound fauna of RWA populations, and inter-nest distances may affect diversity [87]. Although the mean nest density was similar among sites, the distances between sampled nests with neighboring ones were not measured in a precise manner, and a different exchange of mesofauna between nests among sites cannot be ruled out. Thus, the reasons behind this apparent incongruence are not yet determined and could be a subject for future studies.

According to the multivariate analysis, the three sites had different springtail and oribatid assemblages, both for soil and nest communities. No springtail species and only three oribatid species were shared among the three soil sites. The difference in the soil assemblages probably reflects the geographic distance, which is shorter between the two imported populations than between native and imported sites. On the other hand, notably, the oribatid fauna was more similar among nests than among soils, irrespective of the site, which may suggest a wider geographic diffusion of some nest-preferring species than of the soil fauna. Indeed, the proportion of shared species was nearly twice among nest than soil samples (12 out of 102 in nests, 3 out of 54 in the soil). To our knowledge, this is a novel finding and a targeted genetic analysis of these taxa in soils and nest mounds could shed light on the effective dispersion history of the species [88,89].

Nonetheless, the number of species sampled in nests and shared among sites (2 springtails and 12 oribatids) was rather low compared to the total number of species collected in all nest samples (22 springtails and 102 oribatids). This result may suggest that the number of species transferred from the Alps and established in the Apennines was not high or that some of the species already occurred in central Italy. For example, the two springtail species, *C. albinus* and *L. cyaneus,* are known myrmecophilous species in many parts of Europe [26,67]. Hence it is likely that they spontaneously occur at all sites independent of introductions. Thus, possible relocation with nest material can be hypothesized only for a few species. For example, we collected a few specimens of the oribatid mite *Jugatala angulata* Koch, 1839 in the nests of all three sites, even though this species has never been recorded in Italy except for the Alps [90,91]. Similarly, *Cymbaeremaeus cymba* Nicolet, 1855 was found only in nest samples in both introduction sites, although this species has already been recorded in the Italian peninsula, but only in northern and southern sites and not in the central regions [73]. For the other nest-preferring species, it is challenging to hypothesize a relocation process, because some of them were also recorded in soil samples (*Adoristes ovatus* Koch, 1839, *C. labyrinthicus*, *Hermannia gibba* Koch, 1839, *Scheloribates pallidulus* Koch, 1841). It cannot be excluded that individuals of some species—not strictly dependent on ants to survive—shifted toward inhabiting the soil.

Conclusions

In conclusion, this is the first study to include an introduced population in a comparative analysis of the nest-associate fauna in red wood ants. We confirmed that RWA nests are hotspots for arthropod biodiversity, particularly for oribatid mites. We found several potentially myrmecophilous species that preferred the nest habitat instead of soil, though the symbiotic relationships with ants should be further investigated to verify myrmecophily. We used for the first time a comparative approach in the analysis of the nest fauna, assessing the differences between a native Alpine population and two introduced Apennine populations of the RWA *F. paralugubris*. Although we might expect some clear traces of a large species relocation given the massive transport of nest material during introductions, we found only a few species that potentially could have been transferred, suggesting that it probably did not occur. More comparative studies are recommended, e.g. on RWA imported populations, since the fate of most of them—and the fauna inhabiting their nests—are hitherto almost wholly unknown.

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Chapter 2

Impact of introduced *Formica paralugubris* on the community

Plasticity in the trophic niche of an invasive ant explains establishment success and long-term coexistence

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Abstract

Invasive species are one of the main threats to biodiversity worldwide and the processes enabling their establishment and persistence remain poorly understood. In generalist consumers, plasticity in diet and trophic niche may play a crucial role in invasion success. There is growing evidence that invasive ants, in particular, occupy lower trophic levels in their introduced range compared to the native one, but evidences remain fragmented. We conducted stable isotope analysis at five locations distributed on two continents to infer the trophic position of the invasive ant *Formica paralugubris* in the native and introduced part of the range. This species forms large colonies and can be a voracious predator while feeding on sugar-based resources as well. Whereas native populations had trophic positions comparable to that of an omnivore, the introduced populations varied from being honeydew specialists to top predators, or omnivore. Where other ant species co-occurred, there was no overlap in their trophic niches, and *F. paralugubris* occupied the lower position, suggesting that trophic displacement may enable the coexistence of different ant species. Taken together, our results suggest that shifts in diet associated with changes in the trophic niche of introduced species might mediate invasion success and enable long-term coexistence with native species.

Keywords: ants, impacts, invasive species, niche plasticity, stable isotopes, trophic level

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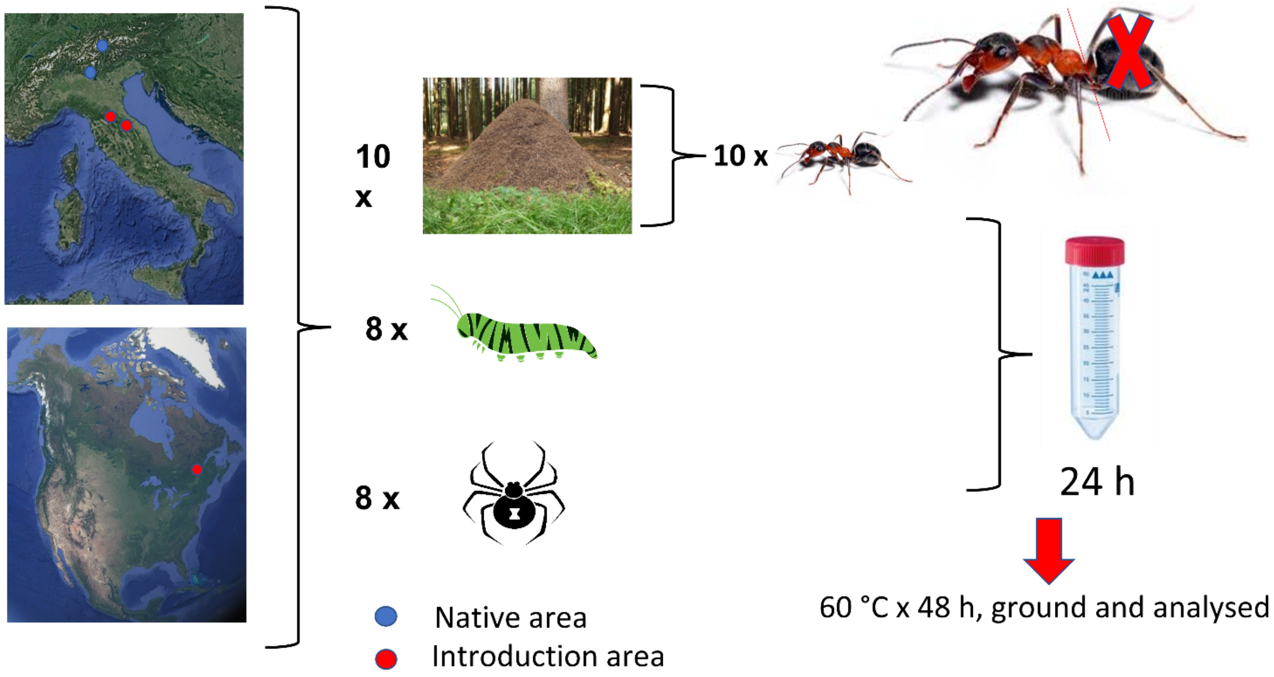

Introduction

Invasive species are one of the primary threats to biodiversity and ecosystem functioning, due to their adverse effects on native communities (Bohan et al. 2017). One central question in invasion biology is whether some characteristics of introduced species might increase the probability they will successfully establish, spread and impact native communities (Ricciardi et al. 2017). In particular, the ability of species to adapt to the new conditions they encounter in their introduced range might be a key trait facilitating invasion. Indeed, shifts in the ecological niche occupied in the introduced range relative to the native range have been widely documented (Da Mata et al. 2010, Wei et al. 2017).

The ability of invasive species to shift their trophic niche is a fundamental aspect of invasion success, and there is evidence that trophic plasticity allows overcoming resource availability as an establishment constraint (Loureiro et al. 2019). Previous work indeed suggest that invasive species can shift their trophic niche in order to avoid interspecific competition and/or to adjust their diet to match resource availability in the invaded habitat (Grey and Jackson 2012, Jackson et al. 2012). As an example, the ecological success of invasive ants seems to be promoted by the association with honeydew producing Hemiptera in the introduced range (Styrsky and Eubanks 2007). This phenomenon was demonstrated in some globally invasive ants, like the red imported fire ant *Solenopsis invicta* (Wilder et al. 2011) and probably the yellow crazy ant *Anoplolepis gracilipes* (Wittman et al. 2018), that behaved like predators in their native range but became predominantly honeydew-consumers – thus becoming functionally herbivores – in their introduced range (Helms 2013). Also the Argentine ant *Linepithema humile* followed this pattern, though a certain variability in its trophic position was detected in different introduced areas (Tillberg et al. 2007).

In this study we investigate the trophic ecology of a red wood ant *Formica paralugubris* native to the Italian Alps and introduced to other parts of Italy and in Canada as biological control agent (Seifert 2016, Frizzi et al. 2020). Red wood ants (RWA) are a group of dominant ant species, widespread at high latitudes and altitudes in the Northern Hemisphere (Stockan et al. 2016). Recent investigations revealed that introduced populations of *F. paralugubris* are now becoming invasive (Seifert 2016, Frizzi et al. 2018).

We therefore aimed to 1) test whether the trophic positioning of *F. paralugubris* differs between its native and introduced range; 2) compare its trophic niche between its native and introduced range; 3) compare the trophic niche of *F. paralugubris* to that of other co-occurring ants. We expected the trophic positioning of *F. paralugubris* to be lower, and its trophic niche width to be smaller, in its introduced range compared to its native range. We also expected low overlap between the trophic niche of *F. paralugubris* and that of co-occurring species.

Materials and Methods

We conducted this study at five different sites: two in the Alps (Swiss National Park, Switzerland – SNP; Giovetto di Paline, Italy – GP), representing the native area, two in the Apennines (Abetone and Campigna, Italy – AB and CA) and one in Canada (Valcartier, Quebec – VC), representing the introduction areas (Supporting information).

Sampling was performed during summer 2017 (2018 for SNP). In each site, we randomly selected 10 *Formica paralugubris* nests, allowing a distance greater than 25 m from each other, from which we collected 10 workers from the surface of nest mounds. To have reference points for ants' trophic positioning, at each site, we also collected herbivorous (caterpillars/grasshoppers, $5 < n < 32$) and predator (spiders, $8 < n < 20$) arthropods, samples of leaves of the dominant tree species and the most abundant herbaceous plant ($6 < n < 10$). Also, we collected workers of all other ant species we encountered. At CA and GP, we did not find any other ant in the area dominated by *F. paralugubris*, and samples were collected at the border of its population.

To remove potential biases due to ingested food, we removed gasters from ants and kept all the other arthropods alive for 24 h to let them empty their gut, before being killed by freezing. All samples were stored without any chemical at −20°C until they were dried in oven at 60°C for 48 h and ground to fine powder. Approximately 0.5 mg (for animals) and 2.0 mg (for plants) of each sample were packed into tin capsules and analysed using an isotope ratio mass spectrometer connected to an elemental analyser. One pool of 10 workers was analysed for each nest for each site.

Within each site, we obtained the trophic positioning of *F. paralugubris* by comparing its $\delta^{15}N$ values with those of herbivores and predators using Welch-corrected ANOVA, followed by Holm-corrected t-tests as multiple comparisons, where necessary. We calculated Layman's metrics (Layman et al. 2007) to compare intra-specific niche width: $\delta^{15}N$ range (NR) and $\delta^{13}C$ range (CR) as the differences between the most enriched and the most depleted individual, the total convex hull area encompassed by all individuals (TA), the mean Euclidean distance of each individual from the centroid of the $\delta^{15}N$–$\delta^{13}C$ values (CD), the mean nearest neighbour distance in the $\delta^{15}N$–$\delta^{13}C$ space (MNND), its standard deviation (SDNND) and the corrected standard ellipse area (SEAc), which is less biased from extreme values compared to TA (Jackson et al. 2011).

To test for differences among co-occurring ant species, we used PERMANOVA (Bray–Curtis dissimilarity, permutations = 4999), followed by t-tests with Benjamini–Hochberg p-value correction (Benjamini and Hochberg 1995) as multiple comparisons.

All statistical analyses were performed in R ver. 3.6 (<www.r-project.org>), using the packages SIAR (Parnell et al. 2010) and vegan (Oksanen et al. 2020).

Results

At all sites, we found a significant difference among the $\delta^{15}N$ of all groups (SNP: $F_{2,28.61} = 253.7$, $p < 0.001$; GP: $F_{2,12.18} = 53.50$, $p < 0.001$; AB: $F_{2,15.64} = 511.78$, $p < 0.001$; CA: $F_{2,17.33} = 82.81$, $p < 0.001$; VC: $F_{2,9.88} = 152.71$, $p < 0.001$; Fig. 1). More specifically, in both native areas, the $\delta^{15}N$ for *Formica paralugubris* were intermediate between those of herbivores and predators, and significantly different from both ($p < 0.001$). In the introduction areas, however, a more complex pattern emerged. At CA, similarly to native areas, *F. paralugubris* showed $\delta^{15}N$ intermediate between those of herbivores and predators, and distinct from both ($p < 0.001$). At AB, the $\delta^{15}N$ of *F. paralugubris* were significantly higher than that of herbivores ($p < 0.001$), but equal to that of spiders ($p = 0.20$). Finally, at VC, *F. paralugubris* $\delta^{15}N$ were significantly lower than that of spiders ($p < 0.001$), and lower, although marginally not significant ($p = 0.06$), than that of herbivores.

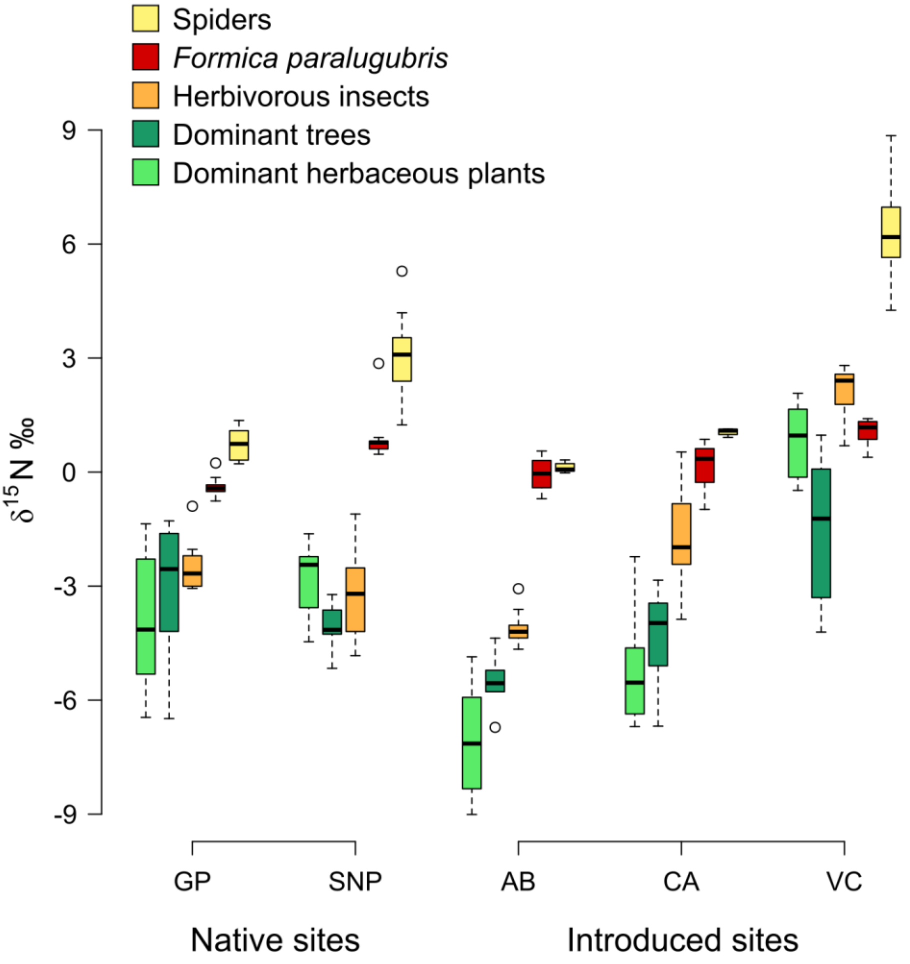

Figure 1. $\delta^{15}N$ (‰) of sampled groups in all sites. GP = Giovetto di Paline (Italy); SNP = Swiss National Park (Switzerland); AB = Abetone (Italy); CA = Campigna (Italy); VC = Valcartier (Canada).

We found no relationship between the intra-specific niche width and the autochthonous/introduced status of the populations, or their trophic positioning (Table 1). The nitrogen range was highest at SNP followed by Campigna, and lowest at Giovetto and Valcartier; the carbon range was highest in Abetone and lowest in Valcartier; the total area was highest in Abetone and Campigna, and lowest in Valcartier. Comparable values were obtained for centroid distance and mean nearest neighbour distance, while the standard deviation of the mean nearest neighbour distance was highest in Switzerland and lowest in Abetone and Valcartier. The corrected standard ellipse area confirmed the total area results, being highest in Abetone and lowest in Valcartier.

Site	NR	CR	TA	CD	MNND	SDNND	SEAc
Giovetto di Paline (GP)	1.0	1.0	0.5	0.4	0.2	0.1	0.3
Switzerland (SNP)	2.4	0.5	0.7	0.5	0.3	0.6	0.5
Abetone (AB)	1.3	1.3	0.8	0.5	0.2	0.1	0.5
Campigna (CA)	1.8	0.7	0.8	0.5	0.3	0.2	0.5
Valcartier (VC)	1.0	0.3	0.2	0.3	0.2	0.1	0.1

Table 1. Layman's metrics for *Formica paralugubris* populations. NR ($\delta^{15}N$ range) and CR ($\delta^{13}C$ range) are the differences between the most enriched and the most depleted individual; TA is the total convex hull area encompassed by all individuals; CD is the mean Euclidean distance of each individual from the centroid of the $\delta^{15}N$–$\delta^{13}C$ values; MNND and SDNND are the mean nearest neighbor distance in the $\delta^{15}N$–$\delta^{13}C$ space and its standard deviation; SEAc is the corrected standard ellipse area.

Other co-occurring ant species were found only at SNP and VC, while in all the other sites other ant species were only found at the edge of *F. paralugubris* dominated area. In Switzerland and Canada, *F. paralugubris* had an isotopic niche clearly segregated with respect to the native *Formica exsecta* ($F_{1,18} = 68.22$, $p < 0.001$) and *Formica glacialis* ($F_{1,12} = 121.15$, $p < 0.001$), respectively. Differences were evident in both sites for both $\delta^{13}C$ and $\delta^{15}N$ ($p < 0.001$ for each test), with *F. paralugubris* having lower $\delta^{15}N$ and higher $\delta^{13}C$ (Fig. 2). In Italy, ants were overall statistically differentiated at GP ($F_{4,15} = 4.25$, $p < 0.05$), but not at AB ($F_{3,14} = 2.26$, $p = 0.13$) and CA ($F_{6,30} = 1.95$, $p = 0.05$). In particular, in GP post-hoc test found a significant difference between *F.*

paralugubris and other ants of the genus *Formica*, *Myrmica* and *Lasius* ($p < 0.05$ for all), while no differences were found between *F. paralugubris* and *Camponotus* ($p = 0.09$) (Supporting information).

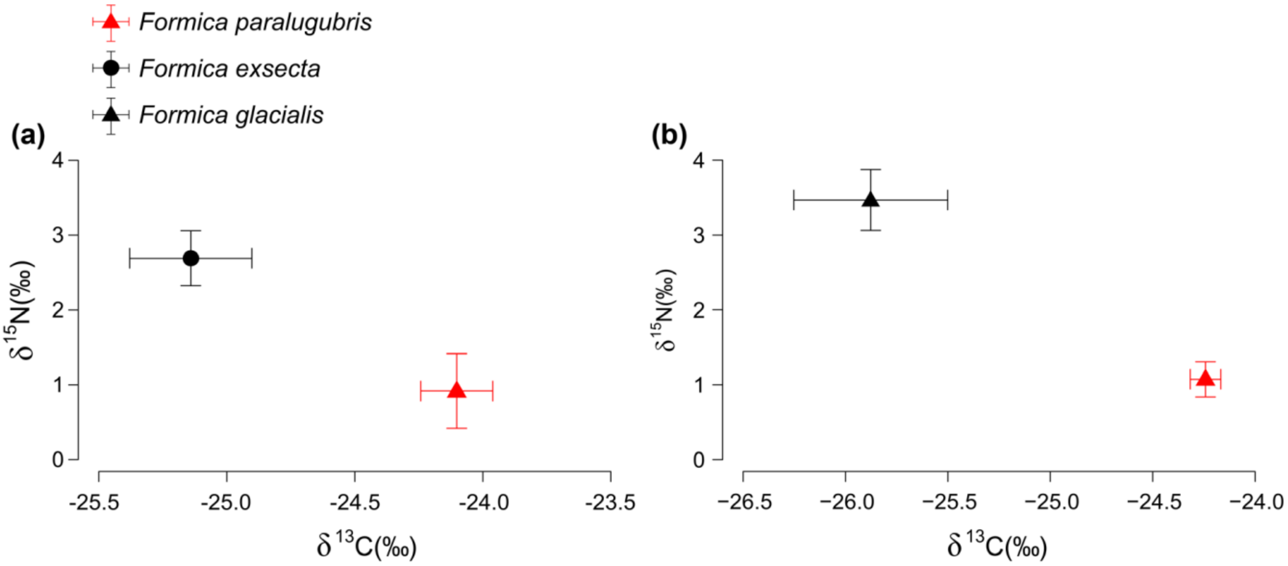

Figure 2. Isotopic biplot (mean $\delta^{15}N$ and $\delta^{13}C$ ± SE) for: (a) SNP (Swiss National Park, Switzerland) and (b) VC (Valcartier, Canada).

Discussion

Among several mechanisms determining the success of invasive species is the plasticity of their diet or trophic niche (Linzmaier et al. 2020, Rolla et al. 2020). We show that the trophic positioning of invasive ants can change in response to local conditions. We observed considerable inter-population variability in trophic positioning of *Formica paralugubris* suggesting trophic niche plasticity. Our results suggest that invasive species can adapt to resource availability and/or interactions with other co-occurring species in the introduced range by switching diet.

Within its native range, *F. paralugubris* behaved like an omnivore, supporting the literature demonstrating that RWA rely on both animal preys and aphid honeydew (Domisch et al. 2016), even if one species was found to be a first-order predator (Iakovlev et al. 2017), suggesting that generalizations are hardly possible for this group.

Isotopic values of introduced populations of *F. paralugubris* suggest that invasive ants can adopt behaviours ranging from being omnivore to being specialized predators or herbivores. In line with our expectations, the Canadian population occupied a trophic positioning of an exudate-feeding herbivore, indicating an almost complete reliance on aphid honeydew (Brewitt et al. 2015).

Indeed, invasive ants are known to form association with honeydew-producing insects in their introduced range which lower their trophic position. Introduced *L. humile* decreased their trophic level compared with native populations, with a shift from predatory habits to greater consumption of plant-derived sources (Tillberg et al. 2007). This result was confirmed for *S. invicta*, whose colonies supplied with aphids' honeydew were also significantly denser than controls (Wilder et al. 2011). Similar results were found also for *A. gracilipes*, whose $\delta^{15}N$ in invasive populations was negatively correlated with ant density (Wittman et al. 2018).

Two explanations have been postulated to explain the decrease in trophic position in invasive ants: the resource preference hypothesis (RPH) and the resource limitation hypothesis (RLH). According to RPH, invasive ants feed on their preferred source (carbohydrates) due to the lack of strong competitors. According to RLH, the shift is a consequence of the reduced availability of other preferred preys (Shik and Silverman 2013). *Solenopsis invicta*, which in the invaded range monopolized sugar-rich resources, seems to support the RPH (Wilder et al. 2011), while *L. humile*, which lowered its trophic level during its invasion history as a result of prey selection, seems to confirm the RLH (Tillberg et al. 2007). Whether the observed shift in the Canadian population of *F. paralugubris* follows the former or the latter hypothesis is not clear, even if evidence in favour of RPH is stronger. Indeed, in Valcartier *F. paralugubris* coexists with the native *F. glacialis*, which relies more on animal preys as indicated by its higher $\delta^{15}N$ values, suggesting that prey availability may not be an issue here. Moreover, aphid populations markedly increased after the introduction of *F. paralugubris* to Valcartier (McNeil et al. 1977).

The other introduced populations showed varying behaviours. The Campigna population exhibited omnivorous habits, while the Abetone population exhibited predatory habits. At Campigna, no other ant species coexist with *F. paralugubris*, making interspecific competition unlikely, and arthropod populations are depleted by ant predation (Frizzi et al. 2018). Though no information on the status of arthropod assemblages is available for Abetone, competitive exclusion of other ants seems to be less stringent, because in this site some small ant nests occur where *F. paralugubris* density is lower. However, competition with these sparse and small colonies cannot explain the lower reliance on aphid exudates. This variability in the trophic positioning of introduced populations reflects that found for *L. humile*, which showed herbivorous, omnivorous and predatory habits in different invaded sites (Tillberg et al. 2007).

The analysis of the trophic niche did not reveal any clear pattern related to the autochthonous/introduced status nor the trophic positioning of the populations. This was true for the metrics resuming either the carbon and nitrogen variability (i.e. carbon range and nitrogen range) as well as the isotopic niche area (e.g. total area and corrected standard ellipse area). In Valcartier, we recorded the highest degree of trophic specialization of *F. paralugubris* (expressed by the narrowest nitrogen range, carbon range, total area and corrected standard ellipse area). The narrow nitrogen range suggests the consumption of a single N source, and the narrow carbon range suggests feeding on a few aphid species on a limited number of plant species, since honeydew reflects phloem isotopic signature (Sagers and Goggin 2007). This specialization could reflect the exclusion of native ants from carbohydrate resources, supporting previous results (Tillberg et al. 2007, Wilder et al. 2011). In contrast, the Swiss population showed the wider nitrogen range values, suggesting the consumption of preys belonging to different trophic levels. Interestingly, some inter-colonial differences in trophic behaviour were found in the native range, as found in *S. invicta* (Roeder and Kaspari 2017).

We found coexistence with other species only at a few sites. At Valcartier and in Switzerland, *F. paralugubris* coexisted with *F. glacialis* and *F. exsecta*, respectively. These species had isotopic values completely separated, suggesting niche partitioning and likely avoiding exploitative competition. At both sites, *F. paralugubris* had a lower trophic positioning than the native species and fed on different carbon sources. At all the other sites, *F. paralugubris* apparently excluded most of the other ants, at least from the areas where its population density is higher (Frizzi et al. 2018). An overall trophic niche segregation was found in Giovetto, while in the Apennines all other ants had overlapping niches with *F. paralugubris*. However, interspecific competition is likely reduced by spatial segregation.

In conclusion, the expected decrease in trophic position of the introduced populations of *F. paralugubris* is not always supported by our findings. Instead, this species demonstrated considerable trophic plasticity, being able to adapt to different ecological scenarios, supporting the importance of trophic plasticity as a key feature of invasive ants (Suehiro et al. 2017) and other species (Jackson et al. 2017). Our results show that generalizations on trophic shift in invasive species are much more difficult than previously thought. As such, further investigation on a wider range of taxa are required to investigate whether the absence of clear directional trends is a feature of this particular species or is a more common but neglected phenomenon among invasive species. Moreover, evaluating whether species exhibiting dietary plasticity are more likely to become invasive and impact several trophic levels in the invaded communities through competition and predation will be a promising research avenue. Lastly, it will be interesting to expand this line of research to other ecosystems and look for spatiotemporal variations in invasive species' trophic plasticity, for example across the invasion stages.

Data availability statement

Data available from the OSF repository: <www.osf.io.yjpcw> (Balzani et al. 2021).

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Conflicts of interest

The authors declare no conflicts of interest.

Author contributions

Paride Balzani: Conceptualization (equal); Formal analysis (lead); Investigation (equal); Writing – original draft (lead). **Salvatrice Vizzini**: Resources (equal); Writing – review and editing (equal). **Filippo Frizzi**: Investigation (equal); Writing – review and editing (equal). **Alberto Masoni**: Investigation (equal); Writing – review and editing (equal). **Jean-Philippe Lessard**: Investigation (equal); Writing – review and editing (equal). **Christian Bernasconi**: Investigation (equal); Writing – review and editing (equal). **André Francoeur**: Investigation (equal); Writing – review and editing (equal). **Javier Ibarra-Isassi**: Investigation (equal); Writing – review and editing (equal). **François Brassard**: Investigation (equal); Writing – review and editing (equal). **Daniel Cherix**: Investigation (equal); Writing – review and editing (equal). **Giacomo Santini**: Conceptualization (equal); Investigation (equal); Supervision (lead); Writing – review and editing (equal).

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Chapter 3

Impacts of introduced *Formica paralugubris* on the forest ecosystem

CO_2 biogeochemical investigation and microbial characterization of red wood ant mounds in a Southern Europe montane forest

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Abstract

Red wood ants are ecologically important species in Europe that form large colonies. Their nest mounds are characterized by stable microclimatic conditions, that are favourable to the development of rich invertebrate and microbial communities. Through their respiration processes, all these inhabitants contribute to the total gas emissions of the mounds. Quantifications of red wood ant mounds CO_2 production are only available from Northern and Central Europe, and the Alps, where these ants are common. During the second half of the last century some species were transplanted from the Alps to southernmost sites, where they were not present, to be employed as biocontrol agents. No information on the contribution of these low-latitudes populations to the local forest CO_2 production is available. The microbial communities living within red wood ant mounds are also poorly known. In this study, we investigated the CO_2 gas emissions and the microbiome of the mounds of an introduced population of the red wood ant *Formica paralugubris* in a Southern Europe montane forest. We found that ant mounds produced more CO_2 than the forest soil, and that their CO_2 efflux as well as internal concentration were higher during summer than winter, with a lighter CO_2 carbon isotopic signature in summer than winter, likely due to an increased ant activity. Moreover, the top part of the mound was characterised by higher CO_2 efflux and lower CO_2 internal concentration compared to the bottom, probably due to its internal structure and conditions. The isotopic signature of the mound material was similar between summer and winter, suggesting a metabolic similarity of the microbial communities. Also, we estimated the ants' relative contribution to the total mound CO_2 production to be 83%, whereas the microbiota CO_2 contribution was estimated at 17%. Finally, the mound microbiome composition varied between summer and winter, though no seasonal difference in the diversity indexes or β-diversity was found. Our results demonstrate the impacts of the introduced red wood ants on the carbon dynamics of the recipient ecosystem.

Keywords: carbon dioxide, carbon stable isotopes, greenhouse gases, coniferous forest, introduced ants, microbiome

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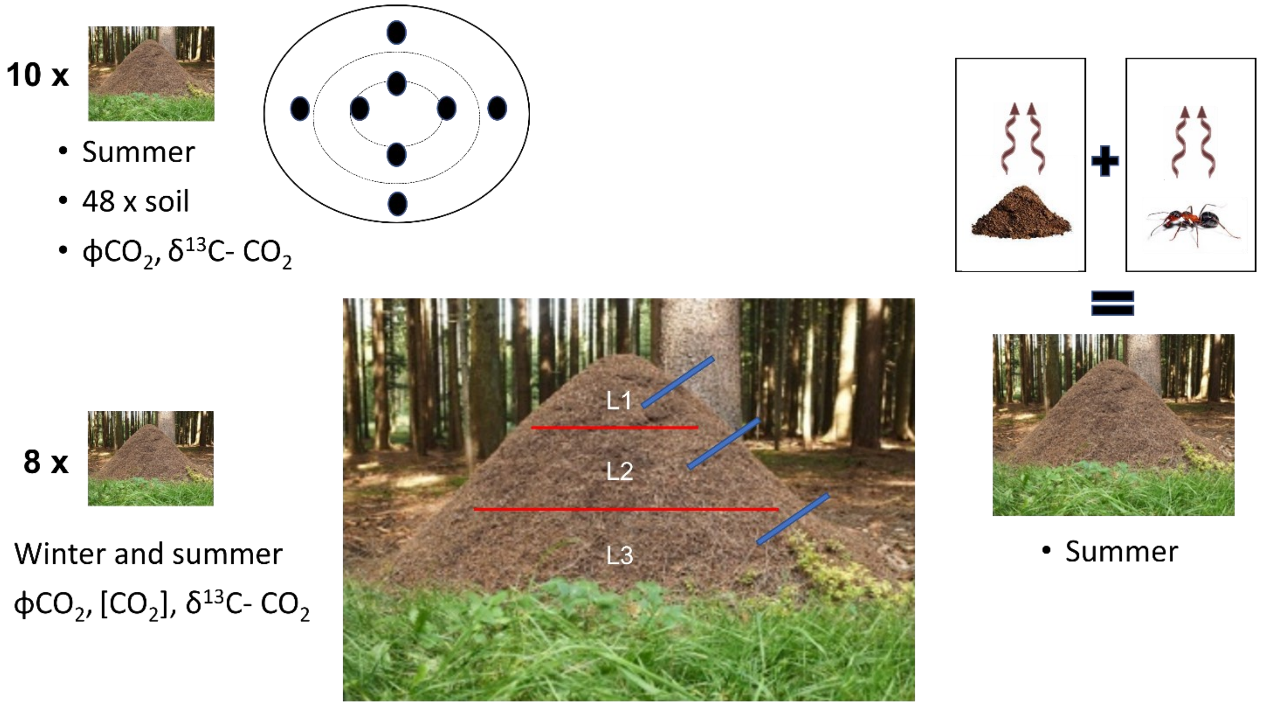

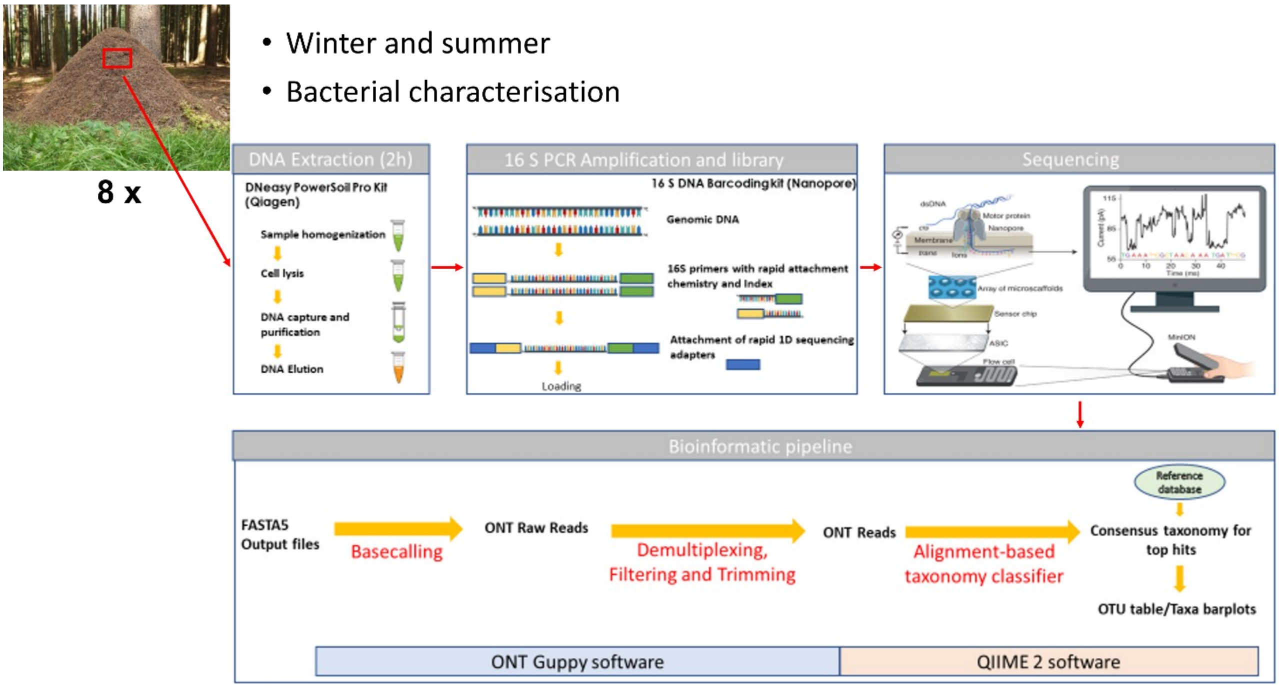
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Winter and summer
Bacterial characterisation
DNA Extraction (2h)
DNeasy PowerSoil Pro Kit (Qiagen)
Sample homogenization
Cell lysis
DNA capture and purification
DNA Elution
16 S PCR Amplification and library
16 S DNA Barcoding kit (Nanopore)
Genomic DNA
16S primers with rapid attachment chemistry and Index
Attachment of rapid 1D sequencing adapters
Loading
Sequencing
dsDNA
Motor protein
Nanopore
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Ions
Array of microscaffolds
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Bioinformatic pipeline
FASTA5 Output files
Basecalling
ONT Raw Reads
Demultiplexing, Filtering and Trimming
ONT Reads
Alignment-based taxonomy classifier
Reference database
Consensus taxonomy for top hits
OTU table/Taxa barplots
ONT Guppy software
QIIME 2 software

Introduction

It is well known that all living organisms produce carbon-bearing gases through respiration processes; therefore, the greater the biomass of a group, the greater its gas production. Among several high-biomass taxa, large colonies formed by social insects are recognized as important sources of both CH_4 and CO_2. It is estimated that termite mounds can contribute 1–4%, and up to 2% of the CH_4 and CO_2 global budgets, respectively (Sanderson, 1996; Jamali et al., 2011; Nauer et al., 2018). The contribution of ants to greenhouse gases production is less studied, although there is growing consensus that in some circumstances these organisms are important emitters due to their abundance and biomass in the majority of terrestrial ecosystems (Bender and Wood, 2003; Wu et al., 2013, 2015). In Neotropical areas, the most striking example is provided by leaf-cutting ants (Sousa-Souto et al., 2012; Fernandez-Bou et al., 2019, 2020), whereas in colder climates, red wood ants (RWA) may play a significant role (Ohashi et al., 2005).

Red wood ants (*Formica rufa* group) are abundant and ecologically dominant species in boreal and Alpine forests in the Holarctic region (Stockan et al., 2016). All the species of this group build large aboveground mounds collecting plant material and different types of debris (Risch et al., 2016), which allow maintaining relatively stable temperature and humidity to buffer the harsh environmental conditions faced during winter (Rosengren et al., 1987; Jones and Oldroyd, 2006; Kadochová and Frouz, 2014). The temperature within the mound is indeed higher than that of the external air, and it is often kept above 0 °C in winter (Frouz and Finér, 2007; Jílková et al., 2015a). These “hot islands” are also nutrient hotspots, as ant workers carry inside considerable amounts of organic matter (Frouz et al., 2005, 2016; Jílková et al., 2015b). Warm temperature and nutrient availability fuel complex trophic chains that include many invertebrate taxa (Parmentier et al., 2014; Robinson et al., 2016; Frizzi et al., 2020), but also rich microbial and fungal communities (Laakso and Setälä, 1997; Frouz, 2000; Jílková et al., 2015b; Sorvari and Hartikainen, 2021), which are supposed to actively contribute to heat production and resource metabolization (Jílková and Frouz, 2014). Therefore, all these communities contribute to the total metabolism of the nest, adding to the gas emissions from ants. Moreover, while invertebrate assemblages associated with the mounds are well studied (Robinson et al., 2016), the associated microorganisms are poorly known. Available studies evidenced that fungi are more abundant than bacteria in autumn and spring whereas bacteria prevail over fungi in summer (Jílková et al., 2015b), and that methanotrophic and nitrogen-fixing microorganisms can be found in RWA mounds (Frouz et al., 1997; Jílková et al., 2016). However, only one study characterized such communities in nests of *Formica polyctena* (Kaczmarczyk-Ziemba et al., 2020).

Since RWA mounds host large biomass, they are regarded as hotspots of CO_2 emissions, producing more gases than the surrounding soil (Ohashi et al., 2005; Risch et al., 2005a; Domisch et al., 2006). Gas emissions from the mounds have multiple sources, as all the organisms living within them contribute through respiration processes. Disentangling the contribution of each component to the total gas emission is tricky; however, previous studies demonstrated that gas release predominantly originates from the activity of ants and microbes within the mound (Jílková and Frouz, 2014). A single mound can indeed contain up to a few million workers (Rosengren et al., 1987). On the other hand, microbial respiration in the mound material produces significantly higher carbon dioxide quantities than the forest floor (Jílková et al., 2016). Moreover, mound respiration rate is influenced by temperature variations, water and carbon content (Jílková et al., 2013). Respiration rate is indeed temperature-dependent for both ants and mound material (Jílková et al., 2015b), and wet mounds are known to respire more than dry mounds (Jílková and Frouz, 2014). Consequently, seasonal and diurnal changes of mound CO_2 efflux may occur; emissions peak during summer and drastically reduce in winter, following the changes in activity and metabolism of the ants and other organisms (Domisch et al., 2006; Jílková et al., 2015a). Similarly, emissions can be higher during nighttime than during daytime, probably due to the increased number of workers within the nest (Risch et al., 2005b; Ohashi et al., 2007). Disentangling the fine-scale dynamics of metabolism of RWA mounds can increase the knowledge of carbon balance on a broader scale (i.e. the forest scale). Such data are only available for Northern and Central Europe and the Alps, but comparatively less is known for populations at lower latitudes.

In this paper, we focused on the metabolic emissions and the mound-associated microbial community of *Formica paralugubris* (Seifert, 1996), a RWA species native to the Western Alps. This ant was repeatedly introduced out of its native range to some southernmost regions in Italy but also in Canada as biocontrol agent against forest pests (Pavan, 1959; Storer et al., 2008; Seifert, 2016; Masoni et al., 2019). Some of the introduced populations are still extant and are experiencing an expansion phase, causing negative ecological impacts on other arthropods (Frizzi et al., 2018; Balzani et al., 2021). Previous studies quantified RWA mounds' effluxes in boreal, temperate and subalpine forests (Risch et al., 2005a; Domisch et al., 2006; Jílková et al., 2015a), but to the best of our knowledge no studies have been carried out South of the Alps, nor on introduced populations. Moreover, no studies have measured the carbon isotopic analysis of CO_2 produced by these RWA mounds, even though this methodology can provide interesting insights on the gas sources (Venturi et al., 2019; Balzani et al., 2020). Further, investigations on microbes associated with ants' nest environments have recently been growing (Kellner et al., 2015). The MinION™ (Oxford Nanopore) platform represents a promising portable sequencing technology capable to produce long sequences

(>10 kb in length) in real-time, using nanopores to sequence a single DNA molecule per pore with an accuracy of 98.9–99.6% (Kasianowicz et al., 1996; Sahlin and Medvedev, 2021). This powerful technology was recently tested for the full 16S rRNA gene (about 1500 bp) analysis, showing promising results (Mitsuhashi et al., 2017; Shin et al., 2018; Matsuo et al., 2021). Therefore, applying a multimethodological approach to investigate the gas production and the composition of microbial communities in this population may help fill a gap in the literature and provide novel insights. The specific aims of this study were to i) measure daily and seasonal variation in CO_2 production of the nest mounds; ii) assess whether CO_2 emission varies according to the mound topology; iii) estimate the relative contribution of ants and the microbial communities associated with the mound material to the overall CO_2 emission of mounds; and iv) characterize and test for seasonal differences in the taxonomic composition of the mound microbial community, which represent the main living component of the mound material.

Materials and methods

The study site (43°52′20″ N, 11°44′41″ E) is a white fir (*Abies alba*) montane forest within the Foreste Casentinesi, Monte Falterona, and Campigna National Park, at the border between Tuscany and Emilia-Romagna (central-northern Apennines). Sampling was performed during the summer (July 2018 and 2019) and the winter (February 2019). The two seasons (winter and summer) were chosen as they are the extremes of temperature variation and ant activity, with spring and autumn probably showing a pattern intermediate between these extremes. Within the study site (7.9 ha), the nest mounds were randomly selected so that they were at least 30 m apart one from the other, to ensure independence and guarantee sufficient spatial variation. The first survey (July 2018) was devoted to compare the overall CO_2 efflux and isotopic ($^{13}C/^{12}C$ ratios) signature from the surface of 10 ant mounds, and the surrounding soil. CO_2 efflux measurements and gas samples from the soil were collected at 48 points spaced 2 m apart corresponding to the nodes of a regular 6 × 8 grid centred on each sampled mound.

The second (February 2019) and third (July 2019) surveys were devoted to measure the seasonal variations of gas emissions, and if these differ in different parts of the mound. To this purpose, we randomly chose eight *F. paralugubris* nests of comparable sizes (mean height: 160 cm). From each of these mounds, CO_2 efflux from the surface was measured, and air samples from the inner part of each mound at three different height levels (L1: top; L2: middle; L3: bottom) were collected. Sampling was carried out in the central part of the day (from 13:00 to 15:00 h), but for two randomly selected nests, all the measurements were also repeated 1 h after sunrise and 1 h before sunset to acquire more information about the daily trends of gas emission.

In the field, fluxes were measured using the accumulation chamber (AC) method (Chiodini et al., 1998; Elío et al., 2016). The chamber, consisting in a cylindrical pot (basal area: 200 cm^2; inner volume: 3060 cm^3) with a rubber edge, was placed firmly on either the mound or soil surface, avoiding outside air entering the AC. The gas accumulating in the chamber was continuously pumped through a low-flux pump (20 ml/s) to an Infra-Red (IR) spectrophotometer Licor® Li-820 (measurement range: 0–20,000 ppm; accuracy: 4%) and reinjected into the chamber to minimize gas flux disturbance. The CO_2 flux was determined from the measured increase in time of CO_2 concentration inside the chamber (dC/dt) considering the geometry of the measuring equipment and the air temperature and pressure, as described in Venturi et al. (2019). CO_2 efflux measurements in the field can be affected by the different porosity and openings density on the mound surface, resulting in different gas releases. To reduce this bias, replicate measures were taken from eight points across the two main axes of each mound, and these values were subsequently averaged for subsequent statistical analyses (Risch et al., 2005a).

Gas samples for carbon isotopic analysis of CO_2 released from soil were taken placing a PVC static closed chamber (SCC; basal area: 177 cm^2; inner volume: 4415 cm^3) on each sampling point (Tassi et al., 2015), wherein gases were let to accumulate for 48 min before gas sample collection. Gas samples from the interior of the mounds were collected using a stainless-steel sampler (5 mm diameter) inserted into the mound at 80 cm depth. The sampler was equipped with a 3-way valve connected to a syringe that allows collecting 60 ml of gas into a 1 l Supelco's Tedlar® gas sampling bag equipped with a push/pull lock valve (Venturi et al., 2019). The stainless-steel samplers were positioned at the same inclination (ca. 30°) for each of the three sampled levels (L1, L2, L3). The carbon isotopic signature of CO_2 ($\delta^{13}C$–CO_2, expressed as ‰ vs. V-PDB) was determined using a Picarro G2201-i Analyzer, based on Cavity Ring-Down Spectroscopy (CRDS), by directly connecting the gas sampling bags to the instrument inlet line. According to the operative range of the Picarro G2201-i Analyzer, gas samples with $CO_2 > 2000$ ppm were diluted with high purity chromatographic air. Moreover, the instrument inlet line was equipped with a Drierite trap to avoid water vapor interferences (Venturi et al., 2019). Contextually, the mound internal temperature (surface, 20, 40, 60, 80, 90 cm depth) was measured throughout the day (morning, midday, afternoon). To estimate the water content, 50 ml of mixed (pooled) mound material samples at about 20 cm depth were collected from each mound. Samples were weighed using an electronic balance (Gilbertini E42, accuracy 0.01 mg), dried at 60 °C for 48 h, and then weighed again.

To characterize the isotopic signatures of the mound material (proxying the signature of the mound microbiome), we performed laboratory measurements on this component following the protocol described in Balzani et al. (2020). We collected 500 ml of mixed (pooled) mound material at about 20 cm depth from each mound during each season. After removing all the occurring invertebrates, a subsample of 100 ml of material from each mound was placed into a 3 l jar and acclimated for 12 h at constant temperature (resembling that recorded in the field, namely 4 °C in February and 19 °C in July). Four empty jars were used as controls. The jars were then closed with a screw lid having a 3-way valve sealed and kept in the dark at constant temperature (4 or 19 °C for February and July, respectively) to reproduce the within-mound conditions. The air in the jars was sampled at the beginning of the experiment (h0), and after 6 and 24 h (h6, h24). Before sampling, the air in the jar was mixed and 50 ml of air were collected using a 60 ml syringe connected to the valve. Samples were stored into 1 l plastic gas bags (Supelco's Tedlar®) and analysed for CO_2 concentration and $\delta^{13}C$–CO_2 value by CRDS, as described in Balzani et al. (2020).

The same experimental setup was used to estimate the relative proportion of CO_2 produced by ants and the mound microbiome. Measurements from ants were only possible during the summer (July 2019), since in winter the ants moved into the underground chambers and mounds could not be excavated. For ants, 50 workers were collected from each nest and transferred to each jar. The jar walls were coated with Fluon® in the upper part to avoid ants escaping and a wet cotton wad was inserted to maintain humidity (Balzani et al., 2020). The CO_2 in the respiration jars was a mixture of gas produced by ants or mound material during the experiment and atmospheric air already present in the jar. To estimate the signature of the CO_2 produced, Keeling plots (Keeling, 1958, 1961; Carleton et al., 2004) were used separately for each jar. Keeling plots are biplots of time-repeated measurements with the $\delta^{13}C$ values on the y-axis and $1/[CO_2]$ on the x-axis. In the biplot, the y-intercept of the regression line between time-repeated measures represents the case in which the CO_2 concentration is infinitely high and the ambient CO_2 is negligible (Balzani et al., 2020).

To identify the main components of respiration from mound material, the associated microbiome was characterized by collecting 50 ml of mixed (pooled) material from each mound in a sterile Falcon tube during each season and preserving it at −80 °C till laboratory analyses. Bacterial DNA was extracted from 0.5 g of homogenised material for each sample using the DNeasy PowerSoil Pro Kit (Quiagen), following manufacturer protocol, and then quantified with Qubit 4 Flourometer (ThermoFisher Scientific) using Qubit™ 1X dsDNA HS Assay Kit. The full-length 16S rRNA gene (~1500 bp) was amplified by PCR using two universal primers 27F and 1493R contained in the 16S Barcoding kit (SQK-RAB204, Oxford Nanopore) and using

LongAmp® Taq DNA Polymerase (New England BioLab). Each PCR was carried on a total volume of 50 µl, containing 5 µl 10x buffer, 5 µl dNTP mix, 2.5 µl of each forward and reverse primer, 0.25 µl Long Amp® Taq, 1 µl template DNA, and 33.75 µl nuclease free molecular grade water. PCR temperature conditions and cycles were set according to the manufacturer protocol. All PCR products were purified using Ampure XP beads (Beckman Coulter, Brea, CA, USA) and normalized to 45 µl containing 1 µg of purified PCR products. Primers with barcode index and 5' tags (which facilitate the ligase-free attachment of Rapid Sequencing Adapters), and all the reagents needed to generate the two sequencing libraries were contained in the 16S Barcoding kit (SQK-RAB204, Oxford Nanopore) that we used for the analysis. Two amplicon libraries were prepared according to the 16S Nanopore Barcoding recommendations, each contained 8 barcoded samples (4 samples for the summer and 4 for the winter of the same nest mounds). All sequencing runs were conducted using the MinION MIN-101B device and using a Nanopore FLO-MIN106D flow cell, according to the sequencing protocols developed by Oxford Nanopore. The two libraries were sequenced in two consecutive runs of 10 h, using the same flow cell, washed immediately following the completion of the previous sequencing run using the nanopore washing kit (EXP-WSH004).

All statistical analyses were performed using the software R (version 3.6; R Core Team, 2020). Log-transformed effluxes and CO_2 isotopic signatures from ant mounds and soils were compared using a linear model. To estimate CO_2 production at the forest scale, we estimated the mean efflux of 1 ha by summing the mean soil efflux with the mean mound efflux multiplied with the mean mound surface and nest density (4.50 m^2 and 12.27/ha, respectively, retrieved from Frizzi et al., 2018). We applied a linear mixed effect model to log-transformed CO^2 efflux, concentration and isotopic signature to test for differences among seasons, mound levels (L1, L2, L3) and their interaction, with nest as random effect. Significant factors were then tested using Tukey tests for multiple comparisons. Daily trends of CO_2 efflux, concentration and isotopic signature for the two monitored nests were plotted for the two seasons. For each season, the fixed effect of day period (morning, midday, afternoon), depth and their interaction were tested on temperature using a linear mixed effect model with nest as random effect. Linear mixed effect models with nest as random factor were used to test for seasonal differences in log-transformed mean mound temperature and water content. All log-transformations were performed using the natural logarithm to improve the normality of the data and homogeneity of variances.

For the respirometric chamber experiments, linear mixed effect models with time as fixed effect and jar as random effect were applied to test for differences in log-transformed CO_2 concentration and isotopic signature. Seasonal differences in the pure isotopic signature of mound material between seasons were tested with a linear mixed effect model with nest as random factor. For the experiments aiming to measure the relative amounts of CO_2 produced by ants and mound material, we applied a linear mixed effect model with time as fixed effect and jar as random effect to log-transformed CO_2 concentration and isotopic signature for the respirometric chambers. The isotopic composition of the air in the nest can be described by the following two-endpoint mixing model:

(1) $\delta^{13}C_{NEST} = p\ (\delta^{13}C_{ANTS}) + (1-p)\ (\delta^{13}C_{MATERIAL})$,

where $\delta^{13}C_{NEST}$ represents the mixed isotopic signature measured in the field, p is the proportion of CO_2 produced and $\delta^{13}C_{ANTS}$ and $\delta^{13}C_{MATERIAL}$ are the pure isotopic signatures of ants and mound material, respectively. The latter two were obtained by averaging the pure isotopic signatures estimated by the intercepts in the Keeling plots, while the $\delta^{13}C_{NEST}$ value was obtained by averaging the isotopic signature measured at the three mound heights in the field. Solving the mixing model equation, we estimated the proportion p:

(2) $p = (\delta^{13}C_{NEST} - \delta^{13}C_{MATERIAL}) / (\delta^{13}C_{ANTS} - \delta^{13}C_{MATERIAL})$.

For microbiota analysis, we followed the Meta-barcoding pipeline for analysing ONT data in QIIME 2 framework, emulating EPI2ME 16S workflow (Oxford Nanopore) developed by Maestri et al. (2019) and Maestri (2021). The raw FAST5 files obtained for each sequencing run were base-called and converted to pass reads in FASTQ format for downstream analysis using GUPPY toolkit (Oxford Nanopore) with high accuracy base calling algorithm. Primer sequences and adapter were trimmed with the same software while reads demultiplexing and filtering were carried out using QIIME 2 package (Bolyen et al., 2019). Sequences with ambiguities, homopolymers (greater than six nucleotides in length), chimeric sequences and those with an average quality score <7 were removed. Operational taxonomic unit (OTU) picking was conducted via SILVA ver. 132 (Quast et al., 2012) as reference database and VSEARCH aligner, first clustering the sequences into de novo operational taxonomic units (OTUs) at 97% similarity and then assigning them a microbial taxonomic classification (confidence threshold of 0.8). The taxonomic composition of the microbial community of each mound across the two seasons was represented with barplots at phylum level using QIIME 2 package. We focused our analysis at genus level because, although the whole 16S was amplified and sequenced, taxonomic identification of many OTUs stopped at this level without species assignment. Seasonal differences in microbial community composition were represented with a non-metric multidimensional scaling (nMDS) and tested with a permutational analysis of variance (PERMANOVA) using Jaccard similarity index and 9999 permutations on presence/absence data at the genus level of OTUs. The β-diversity was computed according to

Anderson et al. (2006) and permuting model residuals were compared to generate a permutation distribution of F under the null hypothesis of no difference in dispersion between the two seasons (9999 permutations). Moreover, OTU diversity at the two seasons was estimated following Chao et al. (2014), using the presence/absence data as input. The method for evaluating diversity is based on the estimation of Hill numbers (qD) and yields estimates of total (rarefied and extrapolated) species richness (q = 0), and the exponential values of the Shannon diversity (q = 1) and Simpson diversity (q = 2) indices. The 95% confidence intervals were obtained from bootstrapping, based on 9999 replications of the reference sample set.

Results

CO_2 efflux (measured in the first sampling) was significantly higher in mounds than soil ($F_{1,56} = 532.15$, $P < 0.001$; Fig. 1A), as well as the CO_2 isotopic signature ($F_{1,56} = 25.62$, $P < 0.001$; Fig. 1B). The mean soil efflux amounted to 21.15 g m^{-2} day^{-1}, while the mean mound efflux was 820 g m^{-2} day^{-1}. The estimated CO_2 efflux of one forest hectare was 256,670.3 g ha^{-1} day^{-1}, corresponding to 69,985.08 kg C ha^{-1} day^{-1}.

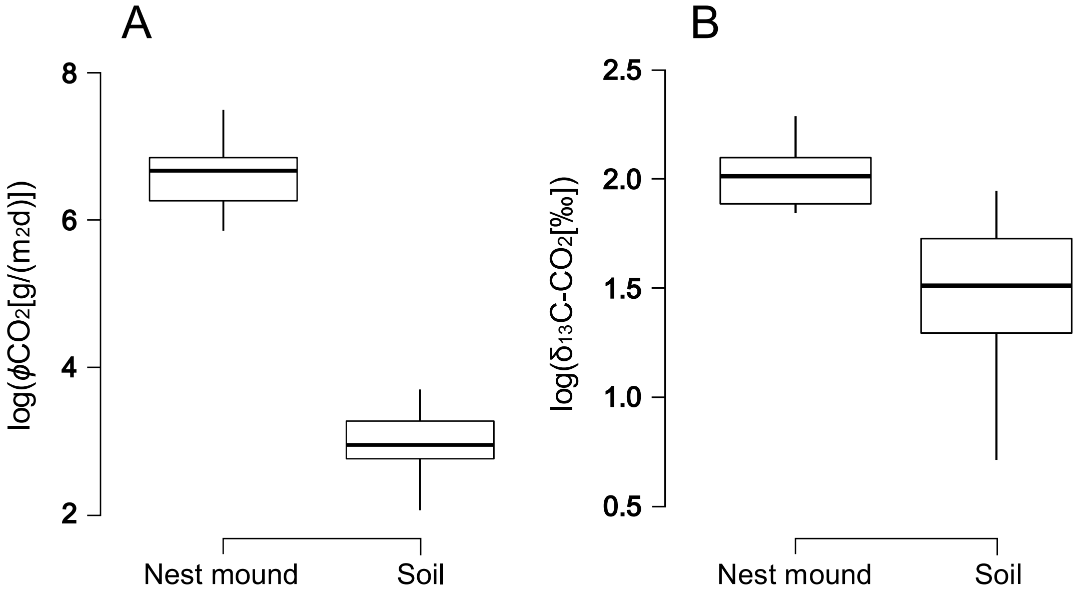

Figure 1. Overall CO_2 efflux (A) and CO_2 carbon isotopic signature (B) values for nest mounds and soils measured during the first sampling survey (summer 2018).

The fitted models (Table 1) showed a significant effect of the season on CO_2 efflux, concentration and isotopic signature ($P < 0.001$ for all), and a significant effect of the mound level for both the CO_2 efflux and concentration ($P < 0.01$ and $P < 0.001$, respectively). In particular, CO_2 efflux and concentration were higher in summer than in winter (Fig. 2A and B), and the CO_2 isotopic signature was higher during winter than the summer (Fig. 2C). A consistent pattern among mound levels occurred in both seasons, with CO_2 efflux higher at the top (L1) than the bottom (L3) of the mound ($P < 0.05$; Fig. 2A) and CO_2 concentration higher at the bottom (L3) than the top (L1) of the mound ($P < 0.01$; Fig. 2B). On the contrary, CO_2 isotopic signatures did not significantly vary within the mound (Fig. 2C).

Response variable	**Fixed effect**	**Chisq**	**df**	**P**
CO_2 efflux	season	52.85	1	< 0.001
	level	11.53	2	< 0.01
	season:level	0.50	2	0.78
CO_2 concentration	season	90.66	1	< 0.001
	level	15.27	2	< 0.001
	season:level	0.80	2	0.67
$\delta^{13}C$-CO_2	season	28.57	1	< 0.001
	level	3.40	2	0.18
	season:level	2.14	2	0.34

Table 1. Analysis of Deviance Table (Type II Wald chi-square tests) for linear mixed effect models with log-transformed CO_2 efflux, concentration and isotopic signature as response variables and season, mound level and their interaction as predictors, with ant nest as random effect.

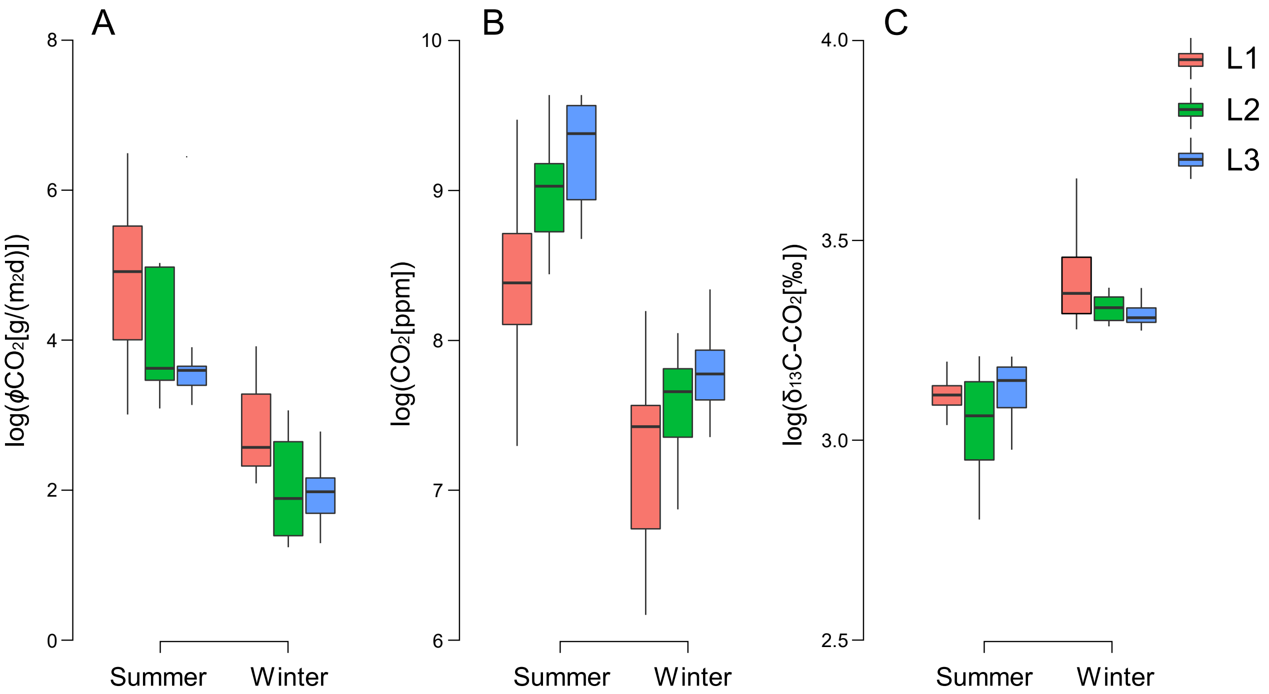

Figure 2. CO$_2$ efflux (A), concentration (B), and carbon isotopic signature (C) measured for ant mounds at three mound levels (L1, L2, L3) in the two seasons (winter and summer 2019).

Daily trends of CO$_2$ efflux, concentrations and isotopic signatures did not show any general clear pattern, except for the CO$_2$ efflux from the top level (L1) that decreased during the day (Figure S1). Mound temperature changed with depth and day period in both winter and summer (Fig. 3; Table 2). The temperature increased with increasing depth and overall from the morning to the afternoon. Seasonal differences were found in mean mound temperature ($F_{1,7}$ = 1099.6, $P < 0.001$) but not in water content ($F_{1,7}$ = 0.13, $P = 0.73$).

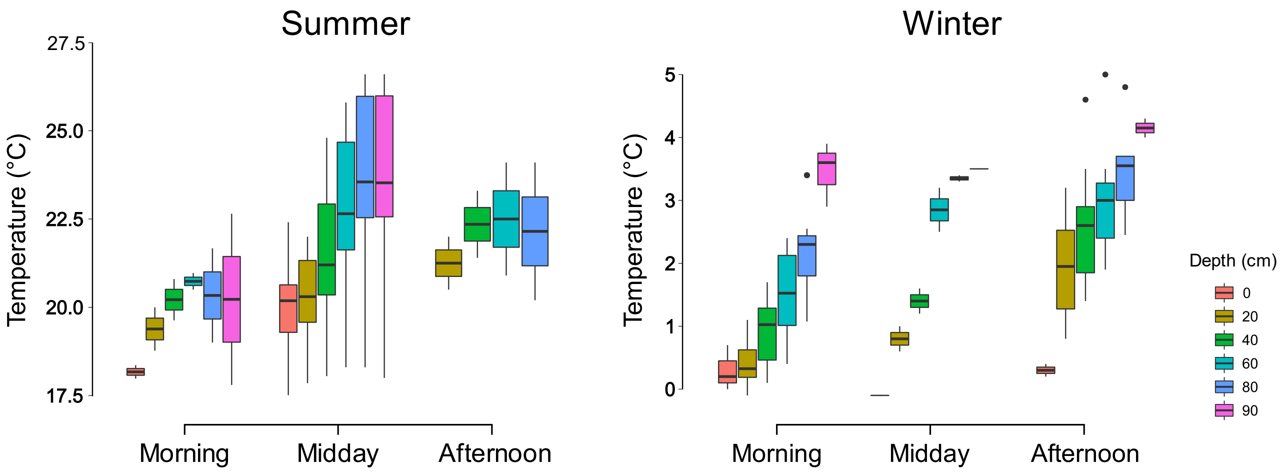

Figure 3. Ant mound internal temperatures measured at different depths from the mound surface in three different period of the day (morning, midday, and afternoon) in the two seasons (summer and winter).

Response variable	season	Fixed effect	Chisq	df	P
temperature	winter	day period	166.24	2	< 0.001
		depth	326.41	5	< 0.001
		day period:depth	17.95	10	0.06
	summer	day period	14.35	2	< 0.001
		depth	55.19	5	< 0.001
		day period:depth	7.24	8	< 0.001

Table 2. Analysis of Deviance Table (Type II Wald chisquare tests) for linear mixed effect models with temperature as response variable and day period (morning, midday, afternoon), depth and their interaction as predictors, with ant nest as random effect.

In the laboratory experiments, during the winter, controls did not show any significant trend in CO_2 concentration ($F_{1,7} = 1.37$, $P = 0.28$) nor isotopic signature ($F_{1,7} = 1.08$, $P = 0.33$), whereas significant temporal changes in mound material were observed in both CO_2 concentration ($F_{2,14} = 5.66$, $P < 0.05$) and isotopic signature ($F_{2,14} = 22.01$, $P < 0.001$). Similarly, no differences in terms of CO_2 concentration ($F_{1,10} = 0.26$, $P = 0.62$) and isotopic signature ($F_{1,10} = 0.17$, $P = 0.69$) were recorded in summer controls, whereas significant variations were found across time in the mound material for both CO_2 concentration ($F_{2,14} = 74.14$, $P < 0.001$) and isotopic signature ($F_{2,21} = 46.93$, $P < 0.001$). The mean pure isotopic signature of mound material was found to be −29.6 and −26.4‰ in winter and summer, respectively, and this difference was not significant ($F_{1,7} = 4.16$, $P = 0.08$).

As for the ant experimental setup, significant differences across time were found for both CO_2 concentration ($F_{2,14} = 19.15$, $P < 0.001$) and isotopic signature ($F_{2,14} = 7.51$, $P < 0.01$). The mean pure isotopic signature for ants in summer was found to be −29.2‰ and that estimated for mound material was −26.4‰. The mean isotopic signature of gas sampled from mounds (in the field) was −28.7‰. Therefore, in summer, the proportion of CO_2 produced by ants (p) was calculated to be 83%, whereas the proportion of CO_2 produced by the microbial community (1-p) was computed at 17%.

The bacterial dataset contained 3,154,908 good quality reads, 98% of them with a length of 1551 nucleotides. More than 150,000 reads were produced and analysed for each sample identifying 28 bacterial phyla in total (Fig. 4). Overall, the most frequent phylum was Proteobacteria, followed by Acidobacteria, Bacteroidetes, Planctomycetes, Actinobacteria, and Firmicutes. The microbial community composition was significantly different between the two seasons (Fig. 5; $F_{1,14} = 2.63$, $P < 0.001$). No significant differences were found in β-diversity values between the two seasons ($F_{1,14} = 0.13$, $P = 0.71$). Moreover, there were no differences in the number of OTUs nor in the diversity indices between the two seasons (Figure S2).

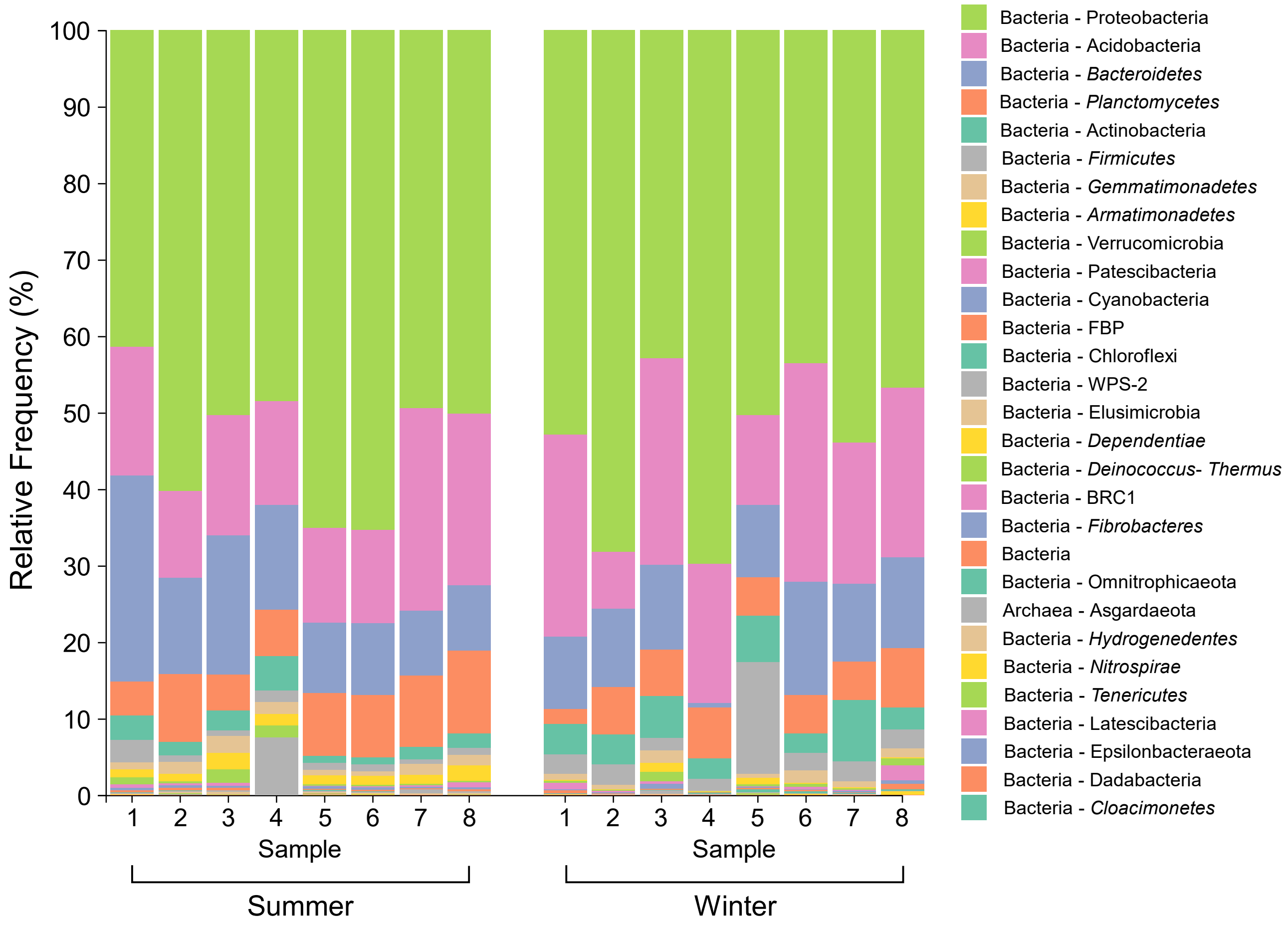

Figure 4. Relative frequency of microbial phyla for each ant mound in the two seasons (summer and winter). The legend is shown following a decreasing frequency order, from the top to the bottom.

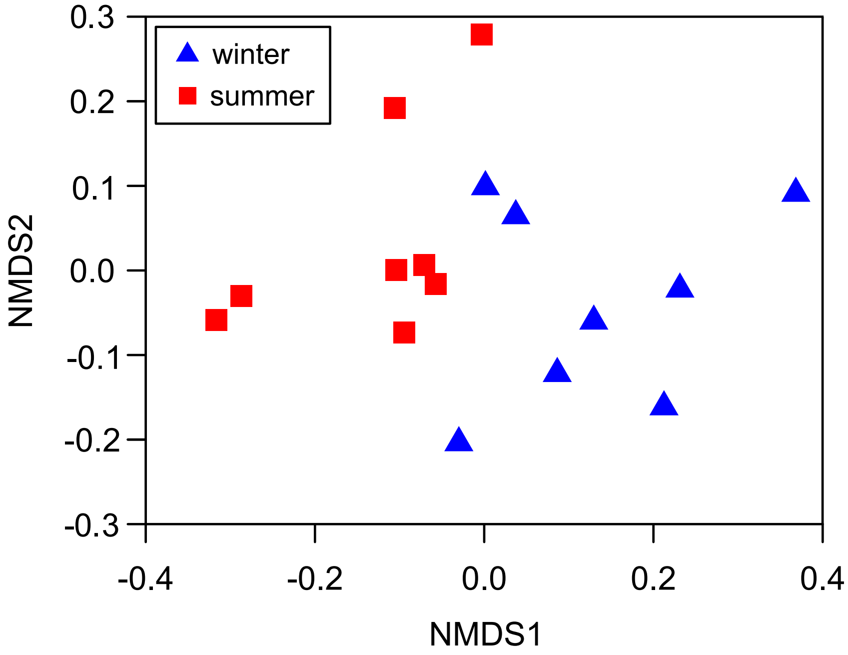

Figure 5. nMDS of nest mound microbial community composition at the genus level in the two seasons (blue triangles: winter; red squares: summer). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Discussion

Soil respiration accounts for 50–95% of the total ecosystem respiration (Yuste et al., 2005). In particular, CO_2 efflux from forest soils is an important process in the forest C cycle, accounting for almost 80% of the total respiration in temperate boreal forests (Law et al., 1999; Janssens et al., 2001; Etzold et al., 2011). Red wood ant emissions contribute up to almost 1% to the total CO_2 emissions of forest ecosystems (Ohashi et al., 2005; Domisch et al., 2006). In this study, as expected, the mounds were found to be CO_2 hotspots during the ants' active season, producing on average 39 times the carbon dioxide produced by the forest soil, thus significantly contributing to the local heterogeneity. However, comparing our results with those from previous studies revealed that while the soil efflux was comparable to those found in the same season for some regions (Finland 27.12 g m^{-2} day^{-1}, Domisch et al., 2006) but higher than others (Finland: 13.68 g m^{-2}

day^{-1}, Ohashi et al., 2005; Czech Republic: 2.69 g m^{-2} day^{-1}, Jílková et al., 2015a), the mean mound efflux was considerably higher. Indeed, studies carried out in Finland reported mound efflux values ranging from 13.68 to 168 g m^{-2} day^{-1} in summer (Ohashi et al., 2005, 2007; Domisch et al., 2006), and similar values were reported for the same season in the Czech Republic (14.45 g m^{-2} day^{-1}; Jílková et al., 2015a) and Switzerland (144 g m^{-2} day^{-1}; Risch et al., 2005 b). The mean mound efflux observed in our study (820 g m^{-2} day^{-1}) was almost five times the highest reported value, showing that the studied population could contribute much more to the global carbon budget in the studied forest. Whether this is due to the southernmost position of our study site or to the peculiar ant population studied is not known, but probably both factors contribute to the final result.

Seasonal differences in CO_2 production were found in ant mounds, supporting previous findings (Risch et al., 2005a; Ohashi et al., 2007; Jílková et al., 2015a). This could be due to either a higher metabolic activity of the nest mound inhabitants or a higher number of workers in summertime. Within the mound, the top level emitted more CO_2 than the medium and bottom profiles, in agreement with previous data on respiration from a Finnish birch forest (Jílková et al., 2013) and a Swiss mixed coniferous forest (Risch et al., 2005a). On the contrary, Jílková et al. (2013) found significant differences only in a birch forest but not in another forest type (pine). They explained the differences found in the birch forest with the top part of the mound having a higher moisture, while the lack of differences was explained with similar moisture and a higher C:N ratio in the top compared to the bottom of the mound. Unfortunately, such data were not available for this work, but a combination of these and possibly other, not measured environmental variables together with the internal structure of the mound can better explain these differences on a local scale.

Although no clear pattern was evident in within-mound daily trends, this may be due to the low number of monitored nests ($n = 2$). Previous investigations, indeed, found lower CO_2 emission in the middle of the day, likely due to a higher number of workers outside of the nest (Risch et al., 2005b; Ohashi et al., 2007). Moreover, in contrast to Ohashi et al. (2007), a significant relationship between mound temperature and day period was recorded, suggesting that some temperature fluctuation can occur within the nest mound in both seasons. Interestingly, both these fluctuations and the inter-mound variability in temperature were much smaller during winter, highlighting the insulating properties of the mound. On the contrary, the higher variability in the mound temperature recorded during summer was probably due to the different sun exposure of mounds.

Ants majorly contributed to the overall nest CO_2 production. While this was already hypothesized for RWA (Lenoir et al., 2001), the sole other experimental quantification has been only recently performed (Jílková and Frouz, 2014). These authors estimated that the ants' proportion of CO_2 produced was about 75%, while the associated microbes produced about 25%. Our results suggest an even more important role of ants in the overall nest metabolism. This could be due to several factors. First, Jílková and Frouz (2014) studied a different species, *F. polyctena*. Second, our study site is climatically warmer, possibly leading to higher metabolic activity of ants. Third, it is known that large ant mounds respire more than the smaller ones, probably due to a high workers' number (Risch et al., 2005a; Ohashi et al., 2012). Though our nest mounds did not differ in their dimensions and presumably age, some could be bigger than those from the previous study. Finally, we cannot exclude the possibility that the ants' higher relative contribution may have resulted from a lower microbes' metabolic activity, possibly due to the low water content registered in the mounds. Nonetheless, it should be stressed that these results refer to the summer season. During the cold season, all ant workers gather in the underground part of the nest, reducing their metabolism and relying on energy reserves stored during the active season. Therefore, it is possible that their isotopic signature as well as their relative contribution to the overall CO_2 production of the mound would change in this period.

The study of the microbial community composition associated with the ant nests is rapidly developing (Ramalho et al., 2017; Lindström et al., 2018; Di Salvo et al., 2019). While previous works functionally described the microbiota of RWA mounds (Frouz et al., 1997; Jílková et al., 2016), this study is one of the few investigations (Kaczmarczyk-Ziemba et al., 2020) aimed at also characterizing the taxonomic composition of such communities, to identify the main contributors to CO_2 production from mound material. The overall taxonomic composition of mound microbiota is in line with that of the nest mounds of the congeneric species *F. polyctena* (Kaczmarczyk-Ziemba et al., 2020) and *Formica exsecta* (Lindström et al., 2019, 2021). Some of the phyla recorded in all three studies, like Acidobacteria, Actinobacteria, and Proteobacteria, typically occur in the soils of coniferous forests (Baldrian et al., 2012). We found some genera that are commonly associated with ants: *Pseudonocardia*, *Burkholderia*, *Methylobacterium*, *Streptomyces*, and *Brevundimonas* (Jaffe et al., 2001; van Borm et al., 2002; Kost et al., 2007; Folgarait et al., 2011). These genera were also recognized in the mound material of *F. exsecta* and were supposed to form symbiotic relationships with the ants (Lindström et al., 2019), as they were recorded as core indicators of ant nests (Lindström et al., 2021). In particular, the genera *Burkholderia*, *Streptomyces*, and *Pseudonocardia* produce antifungal compounds and were reported in the nest of leaf-cutting ants (Santos et al., 2004; Haeder et al., 2009; Barke et al., 2010). On the other hand, other genera such as *Frondihabitans* and *Methylobacterium*, also found in *F. exsecta* mounds, are frequently associated with coniferous needles, thus their presence can simply be due to the nest mound material

(Lindström et al., 2019). In this study, other bacterial genera, previously recorded with other ant species, were found, including: *Opitutus* (Kautz et al., 2013), *Candidatus* and *Sodalis* (Ramalho et al., 2017), *Conexibacter*, *Rhizomicrobium*, *Caulobacter*, *Phenylobacterium*, *Sphingomonas*, and *Mycobacterium* (Lindström et al., 2021), *Neokomagataea*, *Asaia*, *Gluconobacter*, and *Acetobacter* (Chua et al., 2018), *Erwinia*, *Vibrio*, *Inquilinus*, *Citrobacter*, and *Hydrogenophaga* (Di Salvo et al., 2019).

Potential pathogenic bacteria also occurred as reported in other ant species, like *Pseudomonas* (Johansson et al., 2013), the endoparasites *Rickettsia* (Eilmus and Heil, 2009; Kaczmarczyk-Ziemba et al., 2020), *Rickettsiella* (Di Salvo et al., 2019) and *Spiroplasma* (Funaro et al., 2011; Kautz et al., 2013), as well as endosymbiont bacteria, such as *Wolbachia* (Wenseleers et al., 1998; Eilmus and Heil, 2009; Russell, 2012; Kaczmarczyk-Ziemba et al., 2020), *Saccharibacter* and *Entomoplasma* (Johansson et al., 2013). This latter genus seems to be more frequent in predatory ants, as well as the phylum *Firmicutes* (Funaro et al., 2011). Interestingly, some nitrogen-fixing taxa previously reported to be associated with other ant species (van Borm et al., 2002; Eilmus and Heil, 2009; Pinto-Tomás et al., 2009), like the phylum Cyanobacteria, the orders Rhizobiales, Burkholderiales, and Pseudomonadales, and the genera *Klebsiella*, *Azospirillum*, *Serratia* and *Pantoea*, were found. This supports the findings of previous studies, since nitrogen-fixing (Frouz et al., 1997) and type II – but not type I – methanotrophic bacteria (Jílková et al., 2016) have been found in RWA mounds. Noteworthy, *Serratia*, that was present in all mounds except one, is thought to be involved in the chemical mimicry of myrmecophilous hosts (Di Salvo et al., 2019).

Our results also highlight an interesting insight into the temporal dynamics of the mound microbiota. While Lindström et al. (2021) found that the microbiota in *F. exsecta* mounds is temporally stable across months and years within the same season (summer), we found that it differs between extreme seasons (summer and winter) within the same year. On the other hand, the pure isotopic signatures of CO_2 produced by mound material was found to be similar across the two seasons, suggesting that, despite taxonomic differences, the microbial communities associated with the mound material were metabolically similar.

Our results can contribute to the debate on the ecosystem-level effects of invasive species. The studied population of *F. paralugubris* can be considered locally invasive due to its recognized effects on several autochthonous taxa (Frizzi et al., 2018; Di Nuzzo et al. in press). In addition to direct impacts on other organisms, invasive species can also affect ecosystem functioning (Crooks, 2002), especially when they are also ecosystem engineers (Frelich et al., 2006; Jochum et al., 2021).

Recent investigations on a few terrestrial invasive engineers have demonstrated their effect on the aboveground carbon cycle (Papier et al., 2019; O'Bryan et al., 2021). Ants are indisputably important ecosystem engineers (Lenoir, 2001) and targeted studies on the effect of their invasive populations on the ecosystem carbon balance are only now beginning (Milligan et al., 2021).

Conclusions

The nest mounds of the introduced population of *F. paralugubris* were demonstrated to be hotspots of CO_2 emission, significantly contributing to the overall forest gas emissions. The gas release was mainly produced by ants, but there was an important contribution by the hosted microbiota. This is the southernmost population studied to quantify the gas release by ants in Europe and the first one aiming at a fine taxonomic characterization of the bacteria associated with mounds of *F. paralugubris*. Further studies are nevertheless needed to increase the knowledge on gas emissions by ant nests. Many ant species can form large colonies, thus contributing to the soil gas production. Last, the functional role of the bacteria associated with RWA mound seems to be a promising research area for future studies.

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Chapter 4

Red wood ant protection status

Red wood ants in Europe – trends, local paradoxes, and a call to action

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Abstract

Red wood ants (RWA) are a group of keystone species widespread in temperate and boreal forests of the Northern Hemisphere. Despite this, there is increasing evidence of local declines and extinctions. Here, we review the IUCN threat classification and give an overview of the current protection status of RWA throughout Europe. Only some RWA species have been assessed at a global scale, while not all national red lists of the countries where RWA are present include these species. Moreover, different assessment criteria and risk categories are used in different countries, and data deficiency is frequent. The legislative protection is even more complex, with some countries protecting RWA implicitly together with the wildlife fauna, while others explicitly protect the whole group or particular species. This complexity often extends within countries, for example in Italy, where, outside of the Alps, only the introduced species are protected, while the native ones in decline are not. Therefore, an international, coordinated framework is needed for the protection of RWA. However, this firstly requires that the conservation target should be defined. Due to the similar morphology, complex taxonomy and frequent hybridization, protecting the whole RWA group seems a more efficient strategy than protecting single species, though with a distinction between autochthonous and introduced species. Second, an update of the current distribution of RWA species is needed, especially in the southern countries where this information is scarcer. Third, a protecting law cannot be effective without the collaboration of forest managers, whose activity influences RWA habitat. Finally, we stress that RWA nest mounds offer a peculiar microhabitat, hosting a multitude of taxa, some of which are obligate myrmecophilous species listed in the IUCN Red List. Therefore, RWAs' role as umbrella species could facilitate their protection if they are considered not only as target species but also as providers of species-rich microhabitats.

Keywords: insects, red wood ants, *Formica rufa* group, forest diversity.

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Introduction

With at least 13 species described in the Palearctic and up to 19 species reported in North America, red wood ants (RWA, i.e. species belonging to the *Formica rufa* group) are ecologically dominant species (Stockan et al., 2016). RWA are considered to be keystone species in temperate and boreal forests of Eurasia. Due to their large and long-lasting nests they impact functioning of mainly forest ecosystems in many ways and across several trophic levels, e.g. by controlling forest pest species (Trigos-Peral et al., 2021). Although these species are still abundant in many parts of their distribution range, their conservation raises increasing concerns (Dekoninck et al., 2010; Cherix et al., 2012; Breen, 2014; Mabelis & Korczyńska, 2016). Indeed, there is evidence of local decline or even extinction. For example, *F. uralensis* went extinct in Switzerland (Cherix & Maddalena-Feller, 1986), while the scattered relict populations of this species in France, Germany and Poland are facing high extinction risks (Stankiewicz et al., 2005; Wegnez & Mourey, 2016). Moreover, local information is scattered and sometimes contradictory. For example, *F. pratensis* is reported as extinct in mainland Britain since at least 1988 (Nicholson, 1997). However, its presumed extinction is frequently erroneously dated to 2005, the year of the last update for this species on the Bees Wasps and Ants Recording Society (BWARS, www.bwars.com), although the page clearly reports that “The last known nest, near Wareham, died out in 1987”.

The main threats for these species have already been discussed in detail by Sorvari (2016). However, it is worth stressing that the relative importance of these threats varies considerably in different parts of their Palearctic distribution range. In the southernmost countries RWA are restricted to mountain areas, whereas at northernmost sites they also occur at lower altitudes (Stockan et al., 2016), and threatening factors may thus differ. Additionally, their problematic taxonomy, with some species identifiable only through molecular analysis (Bernasconi et al., 2010), the presence of cryptic species (Bernasconi et al., 2011; Seifert, 1996, 2021) and widespread hybridization (Seifert et al., 2010; Beresford et al., 2017), makes it difficult to efficiently assess population size and distribution.

Despite their ecological importance and widespread distribution, Hymenoptera, with the exception of wild bees (Kleijn et al., 2015; Drossart & Gérard, 2020), lag behind other insect taxa, like Lepidoptera or Coleoptera, as conservation targets (Leandro et al., 2017). Ants (particularly RWA) were an early group to be defined as vulnerable and worthy of protection (Wells et al., 1983). Given the importance of RWA in forest pest management, already in 1965 the European Council recommended all the member states to adopt legal provisions for protecting these species, highlighting their decline and the need for conservation (Pavan, 1981). However, more than 50 years later there is no unique legal framework, and contradictory measures are sometimes taken. The importance of the focus on RWA protection extends beyond the conservation of these species per se. Indeed, they are important ecosystem engineers and umbrella species, so their conservation is relevant also for a wide range of other taxa. Moreover, RWA are perfect flagship species, providing an important example for the establishment of a supranational scheme aimed at the conservation of an invertebrate group. In this paper, we review the legal aspect of RWA protection and discuss how conserving these species must have support in national laws in Europe.

We will briefly review their position in the IUCN red list, then give an overview of their protection at the European level and, finally, we provide examples representative of the many contradictions and paradoxes that characterize the protection of these species. It is not the aim of this paper to list all the laws in all European countries, but instead to provide a wide overview with some in-depth analysis of specific cases, the importance of which extends beyond their specific limitations, as they can be paradigmatic of the difficulties encountered in the protection of many other invertebrate taxa.

RWA: a neglected target for conservation

According to the IUCN Red List (accessed 8th October 2021), RWA species are classified as Near Threatened at a global level, but only some species (*F. rufa, F. lugubris, F. polyctena, F. aquilonia*, *F. pratensis,* and *F. uralensis*) have been assessed. Previous assessments (from 1983 to 1994) classified all the above RWA species as Vulnerable except *F. uralensis* that was classified as Indeterminate (from 1986 to 1994).

RWA (and in general, ants) are not included in the European Red List (https://ec.europa.eu/environment/nature/conservation/species/redlist/index_en.htm, accessed 11th October 2021). On the national or regional level, the situation is more complex. Not all European countries include ants, or even insects, in national red lists (https://www.nationalredlist.org/, accessed 11th October 2021). For example, in Ireland, no red list has been produced that covers ants at all, even though all RWA species present are in urgent need of local protection (Breen, 2014). Moreover, when RWA are considered, there is no consensus across different national red lists on which species to include, assessment criteria differ, some risk categories are not fully comparable, and data deficiency is frequent (Appendix S1). In addition, it is unclear how hybrids, an often-occurring phenomenon in RWA, should be treated. For example, only the provisional Red List of the ants of Flanders explicitly assessed hybrids (*F. rufa x polyctena;* Dekoninck et al., 2003, 2005).

The lack of RWA risk assessments is not surprising, as only 149 out of the approximately 14,000 globally known living ant species (https://www.antweb.org/statsPage.do, accessed 4th January 2022) are listed in the IUCN Red List, and for all of them, the last official assessment dates back to 1996, and needs therefore to be updated. RWA currently face increasing threats throughout their distribution range, but the available information on both threats and distributions is highly variable (Sorvari, 2016). The situation may be particularly critical in the countries at the southern margin of their distribution (Italy, Greece, Turkey), where the effects of climate change are probably stronger (Rebetez & Reinhard, 2008), and information limited (Kovats et al., 2014). Since in these regions RWA are restricted to high elevations, the upward shift of populations will progressively be limited by a lower habitat availability. Moreover, only species included in official Red Lists (following the IUCN criteria) can be protected by law in some countries (e.g. Belgium). Despite their ecological importance, RWA protection receives limited attention, and no effort has been made to standardize protection measures at least in Europe. The complexity of the legal status between and within countries, and the diversity of protection measures taken by different States necessitate the development of broad-scale conservation actions and the deployment of common, coordinated strategies.

RWA protection

Several countries protect RWA (Figure 1; Appendix S2). Some of them, such as Austria, implicitly protect them by protecting all the wildlife fauna, while others explicitly mention RWA, at least as a group. For example, in Estonia and Poland, all RWA are protected species, and in Hungary RWA are protected and their nests assigned a monetary value. In Switzerland, RWA are listed as protected since 1966 and all species are explicitly included in the Annex 3 of protected species in the Ordinance on the Protection of Nature and Landscape of the Swiss Federal Council. In Germany, besides being protected by the Federal Law for the protection of nature (Bundesnaturschutzgesetz, BNatSchG) like all wildlife, all mound-building RWA are additionally listed as especially protected in Germany (like all wild bees and a few wasp species) under the Federal Ordinance on species protection (Bundesartenschutzverordnung, BArtSchV), which includes a list of protected species. It is thus prohibited to disturb or destroy their nests or remove workers or other life-stages. Moreover, *F. polyctena* x *rufa* hybrids are implicitly protected as well since the parental species are protected. In Belgium, all RWA species were protected by a law of 1980. Later, Belgium became a federal state and in 2009 the governments of the Flanders and Wallonia published a law in which 3 (for Flanders) and 2 (for Wallonia) species were protected, whereas Brussels protects only one species (*F. polyctena*). Finally, some other countries explicitly prioritize the protection of particular RWA species. In Bulgaria, some RWA species have been protected since 1959, though the obsolete scientific names included have never been updated, and *F. rufa* is protected by the 2002 Bulgarian Biodiversity Act. In the United Kingdom, *F. pratensis* is a UK British Action Plan (BAP) priority species, i.e. those species “that were identified as being the most threatened and requiring conservation action”, being also listed in the Species of Principal Importance in England. Also, *F. aquilonia* is included in the Northern Ireland priority species list.

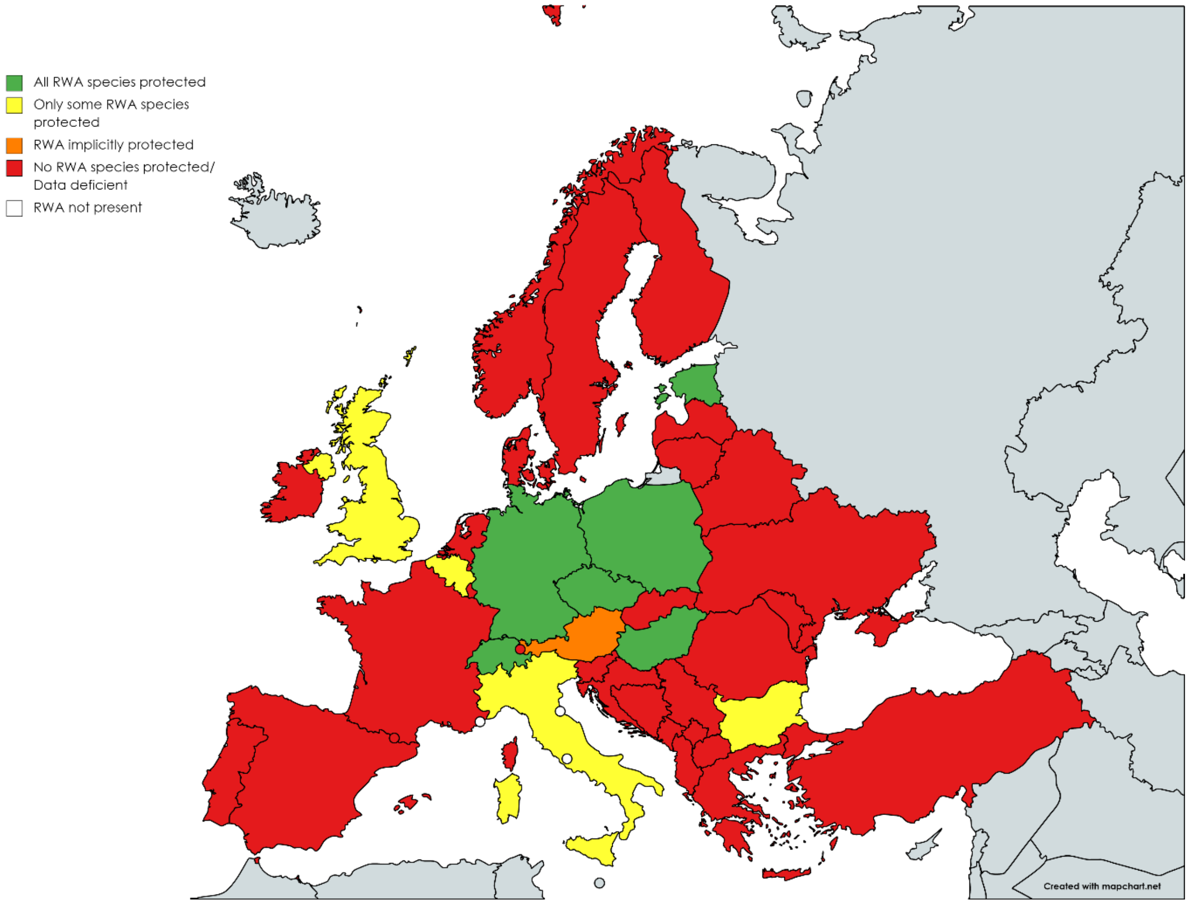

Figure 1. Map of red wood ant protection status across European countries.

The Italian paradox

Italy is paradigmatic of what happens in the countries at the southern limit of RWA distribution, where less information is available, and public awareness is lower. In Italy, these species are typical dwellers of the Alps (Pavan et al., 1971), where they occur at elevations between 450 and 2000 meters, with some species-specific differences. However, this information dates back to several decades ago and it is to be taken cautiously, since a shift of the distribution area towards higher elevations due to warming as documented in other insect taxa (Hagen et al., 2007; Moret et al., 2016) is possible. Further south, the situation is more complex. The only autochthonous species outside of the Alps is *Formica pratensis*, occurring also in the Apennine mountains. This species is the most thermophilic of RWA, forms smaller colonies than the other species, occurs at lower elevations, and prefers open sunny habitats at the margin of wooded areas (Seifert, 2018). Its actual distribution and abundance are however unknown, and the few existing reports are outdated publications (Pavan et al., 1971), personal observations, and sparse, often unconfirmed notes on platforms (e.g. iNaturalist). It is clear, however, that some of the Apennine populations have recently disappeared or significantly decreased in number (G. Santini personal observation). This declining trend is in line with the tendency observed in other countries, such as Switzerland, Belgium, Romania and Turkey (Dekoninck et al., 2003; Freitag et al., 2008; Kiss & Kobori, 2010; Çamlıtepe & Aksoy, 2019), as well as the British mainland (Nicholson, 1997).

This situation is further complicated by the fact that since the 1950s to 1980s, several introductions were carried out by transplanting entire RWA nests from the Alps to the Apennine mountains as biological control agents (Pavan, 1959). Most of these introduced populations belonged to the species *F. paralugubris* (Masoni et al., 2019). These introductions had varying success. Some populations introduced in Southern and warmer areas (e.g., Sicily and Elba Island) are now extinct (Ronchetti & Groppali, 1995), some lack updated information (e.g. *F. aquilonia* and *F. rufa* in Sardinia; Ronchetti & Groppali, 1995), while others like *F. paralugubris* in the Apennines have grown considerably, are developing traits of invasiveness, and have harmful effects on the native fauna (Frizzi et al., 2018; Balzani et al., 2021).

In Italy, no national law protects RWA (nor any other ant), despite an aborted attempt to include the whole group in a law in 2001 (N. 5013 – Rules for the protection of the heterotherm fauna), which was not approved. Instead, each local Authority (Region) legislates on the matter and imposes the protection of particular species. Several Regions grant some type of protection (Appendix S3) either by generally protecting ant nests, mentioning the "*Fomica rufa* group", or specifying the names of some species (sometimes with misspelled names). Some other Regions protected the group in the past with laws that were abrogated and substituted by a law that does not mention RWA. Interestingly, one of these new regional laws currently grants protection to other ant species, including *Formicoxenus nitidulus*, an obligate myrmecophilous ant listed as Vulnerable at a Global level (IUCN Red List) cohabiting within the nests of various RWA species (Härkönen & Sorvari, 2017). Similarly, in the UK, *F. nitidulus* is a BAP priority species for conservation, but its wood ant hosts are not protected. How to protect an obligate myrmecophile without protecting its host ant is unfortunately not specified.

The most peculiar situation occurs in the Regions straddling the Tuscan-Emilian Apennine, where both the native *F. pratensis* and the introduced *F. paralugubris* occur, the former declining and the latter spreading. Quite surprisingly, protection laws were formulated for the introduced species, and protection started soon after the first introductions in the 1950s (Pavia, Prefectoral Decree 6th April 1956). Moreover, efforts to increase public awareness of the introduced species have been done, whereas the declining *F. pratensis* did not receive comparable attention.

What to protect?

One key decision point is whether to focus conservation efforts on single species or to consider the entire group as a target. Protecting single species has the great advantage of allowing for individually tailored protection policies based on the specific needs of species or local populations. This approach, however, has the associated cost of the harmonization of legal frameworks across Countries and requires considerable and informed expertise to support the legal actions. The examples provided here suggest that this is not always the case and that establishing legal protection to the entire group is by far a much simpler task. Moreover, protection at the species level also faces the many difficulties stemming from the taxonomy of these species, starting from the fact that species identification may prove difficult. Furthermore, should we protect hybrids? Hybridization occurs frequently in RWA and is probably one of the mechanisms promoting speciation (Bernasconi et al., 2011). As pointed out by Robinson and Stockan (2016), conservation measures should allow the preservation of evolutionary processes like this, but how to translate it into laws? Targeting the group could be an easier way to cope with such problems, although care should be taken into distinguishing between autochthonous and introduced species, as the case *F. pratensis* – *F. paralugubris* in Italy shows.

The need for updated information on distribution

Establishing a common and unambiguous legal framework is, however, only the first step toward the effective protection of RWA. One of the main difficulties in achieving effective conservation strategies is the non-systematic, and sometimes anecdotal information on their distribution, making it impossible to monitor populations over time. In turn, the lack of such data hinders the compilation of Red Lists based on the IUCN criteria. Moreover, habitat requirements are often recorded at a local scale from presence-only recording, running into false absence biases (Vandegehuchte et al., 2017). Switzerland is an important exception, as a mapping of RWA mounds (especially *Formica lugubris* and *F. paralugubris*) was carried out within the fourth National Forest Inventory (https://www.waldwissen.net/en/forest-ecology/forest-fauna/insects-invertebrates/red-wood-ants-in-switzerland#c97108). However, these data are incomplete, as the sampling design - oriented to trees - did not allow the obtaining of suitable data for less frequent species such as *F. rufa* and *F. polyctena*, or species living outside forests such as *F. pratensis*. Of course, public engagement and citizen science projects contribute greatly to mapping efforts in particular because RWA nests are usually conspicuous. Successful cases are the Swiss “Ameisenzeit” (https://www.ameisenzeit.ch/) and “Opération fourmis” (Avril et al., 2019; Freitag et al., 2020), the activities of amateur associations such as the Ameisenschutzwarte (https://www.ameisenschutzwarte.de/) in Germany, Nest Quest (https://www.buglife.org.uk/get-involved/surveys/nest-quest/) in the UK, and the results obtained by Sorvari (2021) in Finland. However, to enable a European-level risk assessment a common, standardized international monitoring strategy for RWA would be vital and would allow the collection of data on RWA habitat requirements in each country. Furthermore, such a scientifically coordinated monitoring scheme would allow reducing the inevitable bias related to any survey involving lay organizations. Indeed, RWA occurrence correlates with many environmental features (e.g. Berberich et al., 2016; Vandegehuchte et al., 2017). This will finally allow the determination of whether common protection strategies can be applied, or more fine-grained strategies are needed (e.g. between Northern and Southern countries).

How to protect

The existence of a law protecting RWA does not guarantee effective protection. In Switzerland, for example, the application of the law depends on each of the 23 cantons, which does not facilitate the ants' protection. It is also often difficult to define what the right protective measures are or should be. When nests are located in areas where work is to be carried out (road widening, new construction, etc.), the ant nests are usually moved. Unfortunately, the success rate of these translocations is often low (Serttaş et al., 2020). Forestry practices must also be considered. Even if nests are not directly destroyed during logging, their survival is often reduced due to the major disruption of the environment (Sorvari & Hakkarainen, 2007; Sorvari, 2016), although this effect depends on the species and there is evidence of RWA tolerance towards anthropogenic habitat disturbances (Fitzpatrick et al., 2021). On the other side, the natural closure of the forest canopy can eliminate the habitat suitable for RWA species (Vandegehuchte et al., 2017; Fitzpatrick et al., 2021). Viable solutions must therefore be proposed to foresters to reconcile logging and the protection of the RWA. In particular, to achieve effective conservation results, there is the need to train foresters to apply ecologically-sound management plans that take into account specific RWA needs on a local base. Examples are the creation of forest gaps and clearings where canopy closure is excessive or, at the other extreme, reducing the extensions of clearcut areas to facilitate the recolonization of disturbed sites. Also, RWA colony foundation can sometimes rely on temporary social parasitism over colonies of species belonging to the subgenus *Serviformica* (Maeder et al., 2016). The protection of these species could, therefore, facilitate the successful establishment of new RWA colonies.

RWA as umbrella species

RWA host many myrmecophiles that thrive within their nest mounds, some of which are obligate mutualists and cannot survive outside RWA nests (Robinson et al., 2016). Some of these obligate guest species are listed in the IUCN Red List. Clearly, conserving RWA is integral to protecting these organisms, most of which belong to invertebrate groups even less likely to have been assessed for conservation than the Hymenoptera (Parmentier et al., 2014; Robinson et al., 2016). Since the conservation of a species strongly depends on the conservation of its habitat, a thorough revision of the conservation status of myrmecophilous species could be very useful in updating the conservation status of RWA. Considering RWA not only as target species but also as providers of species-rich microhabitats might prove a key strategy to conserve not only them, but all their associated guest species.

We hope with this work to ignite the construction of an international network aimed at the conservation of this important group, at least at the European level.

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Conclusions

General conclusions

The overall aim of this thesis was to deepen the comprehension of the ecological importance of RWA in general, investigating their effects on various other taxa, and particularly to investigate the ecological role and impacts of introduced RWA populations on the recipient community and ecosystem using a multi-methodological approach.

The results supported the previous literature on RWA and demonstrated that the impacts of their introduced populations of are diverse and manifold, acting at multiple levels, from single taxa to communities to ecosystems. Specifically, I found that *Formica paralugubris*: i) affect the composition and structure of various communities; ii) their nest mounds act as biodiversity hotspots; iii) they occupy key trophic positions in the community; iv) they significantly contribute to the carbon dioxide dynamics of the forest ecosystem they live in; v) their nest mounds host complex and diverse microbial communities. These findings confirmed the role of RWA as keystone species (Wardle et al. 2011; Robinson et al. 2016) and ecosystem engineers (Lenoir 2001) and contributed to a deeper understanding of their ecology.

The main result of Chapter 1 is that *F. paralugubris* is a key element in the structuring of other assemblages. Indeed, the presence of this species significantly drove the lichen epiphytic community composition as well as its functional structure (Chapter 1.1). This could be the result either of a direct influence on lichen propagules dispersal by ant workers, or an indirect effect on lichens' consumers (i.e. herbivorous invertebrates). This study is also relevant as only another study previously investigated the impact of an invasive animal on this taxon. Indeed, Gheza et al. (2018) found that the invasive rabbit *Sylvilagus floridanus* negatively impacts the lichen richness in dry grasslands. On the other hand, *F. paralugubris* mounds hosted a wide variety of myrmecophiles (*sensu lato*), as discussed in Chapter 1.2. Of these species, some could have been translocated when their host ant was introduced in the Apennines, as they were not previously recorded there. However, the conclusion of accidental introductions of other taxa – and thus the incurrence of new allochthonous species – has to be considered with caution because the local myrmecophile fauna, like the pedofauna, is generally scarcely studied (e.g. von Beeren et al. 2021).

In Chapter 2, I demonstrated that *F. paralugubris* is capable of great trophic plasticity, being able to adapt its diet depending on the local environment where it occurs. The factors that probably drive this behavioral plasticity are resource availability and the presence of competitors. I found that *F. paralugubris* spatially excluded other ant species, as it is common with RWA (Mabelis 1983; Savolainen & Vepsäläinen 1988, 1989; Savolainen et al. 1989), or had separated trophic niches, suggesting niche partitioning. The results also question the commonplace that RWA mainly rely on aphid honeydew (Wellenstein 1952; Rosengren & Sundström 1991; Finér et al. 2013), since in only one site out of five they were found to occupy such a trophic positioning. Contrariwise, in the other sites, they behaved like omnivores, suggesting that the consumption of animal preys is much more important than previously thought. This result parallels the findings of Fiedler et al. (2007), who described, for eight *Formica* species (four of which RWA) in Central Europe, nitrogen stable isotope values – a proxy for the trophic position – between those of predatory species like *Myrmica* spp. and those of clearly herbivorous (i.e. trophobiotic) species like *Camponotus* spp. Moreover, in one site *F. paralugubris* occupied the same trophic positioning of predators, further supporting this idea. This is not the first study reporting a predatory behavior in RWA, at least in some species. Indeed, studying the isotopic composition of *F. aquilonia* in Siberia, Iakovlev et al. (2017) found this species to behave like a first-order predator. Moreover, with this study I questioned the validity of the paradigm according to which invasive ants lower their trophic level in the invaded areas compared to the native ones (Tillberg et al. 2007; Wilder et al. 2011), as a consequence of a greater reliance on aphid honeydew (Styrsky & Eubanks 2007; Helms 2013). Although the displacement or out competition of other ant species seem to take place, the results indicate that a preference for a plant-derived carbohydrates diet is not straightforward. I found evidence that invasive ants do not follow any deterministic pattern, nor their success is necessarily driven by specific mechanisms. Rather, they take advantage of their trophic plasticity to better exploit the locally available resources, as other invasive species do (Jackson et al. 2017; Linzmaier et al. 2020; Rolla et al. 2020).

In Chapter 3, *F. paralugubris* was found to significantly contribute to the forest carbon dioxide balance and to increase the heterogeneity of the forest soil, as found in other RWA species (Ohashi et al. 2005; Risch et al. 2005a; Domisch et al. 2006). The results are particularly relevant as they show a much higher CO_2 production by ant mounds compared to that reported from previous studies (Ohashi et al. 2005, 2007; Risch et al. 2005b; Domisch et al. 2006; Jílková et al. 2015). This outcome can result from the different ant species studied, but also from the geographical position of our study site. Indeed, it is important to underline that this is the southernmost study on ants' gaseous emissions, thus representing an important step towards a more complete understanding of the global carbon dynamics (Woodwell et al. 1978; Perruchoud et al. 1999; Amundson 2001; Mukhortova et al. 2015; Friedlingstein et al. 2020). This study is important also in the context of Invasion ecology, as I presented a quantification of the impacts of an invasive ant on the local carbon dioxide emissions. Although this is not the first study on this topic (Bender & Wood 2003), this subject has been only weakly stressed as a local impact of the introduced species to the recipient environment. Indeed, we are only now beginning addressing targeted studies on the effect of invasive ants on the ecosystem carbon balance (Milligan et al. 2021). However, this lack of information is not confined to ants, since only few terrestrial invasive species have been recently demonstrated to significantly affect the aboveground carbon cycle (Papier et al. 2019; O'Bryan et al. 2021). As pointed out by O'Bryan et al. (2021), we would like to underline that "we know little about the global carbon footprint of invasive species".

Finally, in Chapter 4, I outlined the complex scenario for the protection of RWA species. I highlighted the need for a framed and coordinated conservation program, but I also highlighted the difficulties that such programs could encounter. In particular, there is the operational need to choose the conservation unit that should be targeted, namely the whole group or the single species, given the complicated taxonomy of RWA. Also, a discrimination between autochthonous and introduced populations should be considered, to avoid paradoxical cases such as that happening in Italy, where the introduced species are protected by local authorities whereas the declining ones are not. Unfortunately, this is not the only case reported in the literature. Other invasive species are tolerated or even protected, especially when they represent an economic resource (Nuñez et al. 2012). In our case, the invasive populations of RWA are protected by antiquated laws as they represented a form of protection for trees, thus increasing timber production (Pavan 1961; Cotti et al. 1962). This is a clear example of discrepancy between the scientific results and policy makers and managers, probably due an obsolete point of view by these latter. Additionally, to effectively protect RWA species, there is an urgent need for updating the information on their distribution. Although not easy to achieve, RWA protection is very important, as they are umbrella species hosting a multitude of taxa within their mounds (e.g. Parmentier et al. 2014; Parmentier & Claus 2019), some of which are obligate myrmecophilous species listed in the IUCN Red List. Therefore, I proposed to consider RWA not only as species worth of conservation, but also as a peculiar microhabitat – provided by their mounds – that is vital for many other species.

***Formica paralugubris* as a peculiar invasive ant**

Invasive species are widely recognized as one of the primary threats to biodiversity and ecosystem functioning, due to their adverse effects on native communities (Bellard et al. 2016; David et al. 2017; Mollot et al. 2017). New alien species continue to be introduced and the negative impacts that some of them cause after becoming invasive are increasing (Seebens et al. 2017). Consequently, complete understanding the features facilitating the ecological success of invasive species, such as trophic plasticity, is of utmost importance in order to prevent new invasions (Fournier et al. 2019) and to control the spread and mitigate the adverse ecological effects of already established invaders

(Hoffmann et al. 2016; Ricciardi et al. 2017). Further, because the evolution of invasiveness also raises important questions in the realm of evolutionary ecology (Colautti et al. 2017; Bock et al. 2018), any progress in this field has a relevance that extends beyond the management of invasive species.

Among invasive species, ants deserve a special mention. Despite representing a small fraction of all known ant species, ants contribute 19 out of the 360 invasive species listed by the IUCN (Bertelsmeier et al. 2017), some of which are highly destructive invaders (Lach & Hooper-Bui 2010). At least five of them (the Argentine ant *Linepithema humile*, the yellow crazy ant *Anoplolepis gracilipes*, the little fire ant *Wasmannia auropunctata*, the red imported fire ant *Solenopsis invicta*, and the big-headed ant *Pheidole megacephala*) are currently listed among the world’s 100 worst invasive species (Bertelsmeier et al. 2016). Also, invasive ants cause considerable associated economic costs worldwide, though these are largely underestimated (Angulo et al. 2021). All the recognized invasive ant species share some ecological features that contribute to their invasion success. Among these, the ability to cope with unfavourable environmental conditions, and the possibility to switch to super- or unicoloniality are worth mentioning (Giraud et al. 2002; Tsutsui and Suarez 2003; Jackson 2007; Krushelnycky et al. 2010). Another important point is their capacity to adapt their feeding strategy to novel environmental features (Sagata and Lester 2009). Nevertheless, the underlying mechanisms that enable these species to become so dominant in their invasive range are not fully elucidated.

In this thesis, I demonstrated the multifaceted impacts of the introduced populations of a RWA species, that is ecologically dominant and has acquired strong invasivity features (Frizzi et al. 2018). Nonetheless, it is of utmost importance to stress that several of the ecological traits of *F. paralugubris* make it a peculiar invasive ant. First, as all RWA, it is a cold-adapted species and cannot tolerate warm habitats, thus limiting its spread (Vandegehuchte et al. 2017). Second, its dispersal ability is limited by its reproductive strategy, which mostly relies on colony budding rather than nuptial flights (Chapuisat et al. 1997). The expansion of *F. paralugubris* populations is therefore slow and somehow limited (Seifert 2016).

Future perspectives

These studies opened further research questions that need to be addressed by focused future studies.

Our findings highlighted the importance of extending and increasing the studies on the impacts of *F. paralugubris* on other taxa. First, the effect of *F. paralugubris* on the lichen community was tested only in invaded areas, while it could be very interesting to assess whether this strong effect occur also in the native range of this RWA species, as it was demonstrated for others (Heinken et al. 2007; Thunes et al. 2018). Second, to disentangle whether some species associated to *F. paralugubris* mounds were transplanted together with their nests, a comparative analysis of the myrmecophiles (*sensu lato*) occurring within the mound of the native *F. pratensis* in needed. Third, the overall impact of RWA on vertebrate taxa have been scarcely documented, except for birds (Aho et al. 1997,1999; Haemig 1996,1999; Jäntti et al. 2007). It would be very interesting to assess whether the communities of the lesser fauna, such as amphibians, reptiles, or micromammals, are affected by the presence of RWA. The introduced populations in Italy offer a great opportunity for this, by providing nearby occupied and unoccupied areas within the same sites and, consequently, identical habitat conditions.

Further, the trophic ecology of RWA should be reconsidered after these results, and more studies investigating their trophic niche are needed. In particular, it would be interesting to assess how the diet, and consequently the trophic position of RWA vary throughout the year according to the colony needs and brood presence (Mooney & Tillberg 2005; Dussutour and Simpson 2009; Platner et al. 2012). Indeed, seasonal changes in the isotopic signatures of RWA have been reported in *F. aquilonia* (Iakovlev et al. 2017), thus the impact of this species on the community might be not constant, but instead vary throughout the year. Additionally, I found a non-negligible variability in the trophic positioning of individual *F. paralugubris* colonies, so that it would be interesting to assess the degree of trophic specialization of each colony. Previous studies showed that such specializations could be a factor allowing *S. invicta* to reduce intercolonial competition and reach high abundances (Roeder & Kaspari 2017). The variability in the trophic positioning occupied by the invasive populations reopens the way for further research on the feeding ecology of other invasive species. Moreover, the trophic position of the autochthonous *F. pratensis* has never been assessed, while information on the trophic behavior of this species is needed to understand its role in the community. This is a very important point to be addressed, as *F. pratensis* populations are declining (Dekoninck et al. 2003; Freitag et al. 2008; Kiss & Kobori 2010; Çamlıtepe & Aksoy 2019), so that fully understanding its feeding ecology and requirements can help in disentangling the causes of this decline. Also, this knowledge could help identifying the areas where reintroduction efforts, that might be key to the protection of this species, could be concentrated or avoided.

In a biogeochemistry context, further investigations are needed to disentangle the fine dynamics of RWA mounds, and the functional role of the hosted microbial communities. For example, methane is one of the most important greenhouse gases and its natural production by insects such as termites was demonstrated to be relevant to the global CH_4 budget (Jamali et al. 2011; Nauer et al. 2018; Chakraborty et al. 2021). Given the biomass that RWA can reach within an ecosystem and the complex nature of RWA mounds, research on their methane emissions compared to the surrounding forest soil is needed. Furthermore, the presence of methanotroph as well as methanogenic bacteria within their mounds is determinant to shape the outcome of these tradeoffs, so a functional investigation on the microbiota role seems promising. Finally, also the gas emissions of *F. pratensis* mound should be measured to precisely quantify the change in CO_2 (and CH_4) production of the recipient forest ecosystem following the introduction of *F. paralugubris*. Indeed, the colonies *F. pratensis* do not reach the huge dimensions of those of the introduced species, they rather remain quite small (Pavan et al. 1971).

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Annexes

Supplementary material for Chapter 1.1

Supplementary material for Chapter 1.2

Supplementary material for Chapter 2

Supplementary material for Chapter 3

Supplementary material for Chapter 4

Supplementary material for "Red wood ants shape epiphytic lichen assemblages in montane white fir forests"

Table S1. Functional traits of lichen species. Growth form: Fruticose (Fr), Crustose (Cr), Foliose (Fol). Reproductive strategy: Sexual (S), Asexual (A). Presence of lichenic acids: presence (y), absence (n).

Species	Growth Form	Reproductive Strategy	Presence of lichenc acids
Acrocordia gemmata (Ach.) A. Massal. var. gemmata	Cr	S	n
Arthonia radiata (Pers.) Ach.	Cr	S	n
Arthopyrenia sp.	Cr	S	
Bacidia rosella (Pers.) De Not.	Cr	S	n
Buellia griseovirens (Sm.) Almb.	Cr	S	y
Candelariella reflexa (Nyl.) Lettau	Cr	A	y
Catillaria sp.	Cr	S	
Cladonia fimbriata (L.) Fr.	Fr	A	y
Cladonia sp.	Fr		
Dimerella sp.	Cr	S	
Coenogonium pineti (Ach.) Lücking & Lumbsch	Cr	S	n
Evernia prunastri (L.) Ach.	Fr	A	y
Flavoparmelia caperata (L.) Hale	Fo	A	y
Fuscidea stiriaca (A. Massal.) Hafellner	Cr	S	y
Hypogymnia physodes (L.) Nyl.	Fo	A	y
Hypogymnia tubulosa (Schaer.) Hav.	Fo	A	y
Lecania naegelii (Hepp) Diederich & van den Boom	Cr	S	n
Lecania sp.	Cr		
Lecanora albella (Pers.) Ach.	Cr	S	y
Lecanora argentata (Ach.) Malme	Cr	S	y
Lecanora carpinea (L.) Vain.	Cr	S	y
Lecanora chlarotera Nyl. subsp. chlarotera	Cr	S	y
Lecanora expallens Ach.	Cr	A	y
Lecanora pulicaris (Pers.) Ach.	Cr	S	y
Lecanora sp.	Cr	S	
Lecanora strobilina (Spreng.) Kieff.	Cr	S	y
Lecanora symmicta (Ach.) Ach.	Cr	S	y
Lecidella elaeochroma (Ach.) M. Choisy var. elaeochroma f. elaeochroma	Cr	S	y
Lepra albescens (Huds.) Hafellner	Cr	A	y
Lepra amara (Ach.) Hafellner	Cr	A	y
Lepraria sp.	Fo		
Melanelixia glabratula (Lamy) Sandler & Arup	Fo	A	y
Melanelixia subaurifera (Nyl.) O. Blanco, A. Crespo, Divakar, Essl., D. Hawksw. & Lumbsch	Fo	A	y
Micarea sp.	Cr	S	
Naetrocymbe punctiformis (Pers.) R.C. Harris	Fu	S	n
Naevia punctiformis (Ach.) A. Massal.	Cr	S	n
Normandina pulchella (Borrer) Nyl.	Sq	A	n

Ochrolechia parella (L.) A. Massal.	Cr	S	y
Ochrolechia sp.	Cr	S	
Parmelia saxatilis (L.) Ach.	Fo	A	y
Parmelia submontana Hale	Fo	A	y
Parmelia sulcata Taylor	Fo	A	y
Parmelina tiliacea (Hoffm.) Hale	Fo	A	y
Parmeliopsis ambigua (Hoffm.) Nyl.	Fo	A	y
Pertusaria coccodes (Ach.) Nyl.	Cr	A	y
Pertusaria flavida (DC.) J.R. Laundon	Cr	A	y
Pertusaria leioplaca (Ach.) DC.	Cr	S	y
Pertusaria pertusa (L.) Tuck. var. pertusa	Cr	S	y
Pertusaria sp.	Cr		
Pertusaria sp. 2	Cr		
Pheophyscia sp.	Fo		
Phlyctis agelaea (Ach.) Flot.	Cr	S	y
Phlyctis argena (Spreng.) Flot.	Cr	A	y
Physcia adscendens H. Olivier	Fo	A	y
Platismatia glauca (L.) W.L. Culb. & C.F. Culb.	Fo	A	y
Pleurosticta acetabulum (Neck.) Elix & Lumbsch	Fo	S	y
Pseudevernia furfuracea (L.) Zopf var. furfuracea	Fo	A	y
Punctelia subrudecta (Nyl.) Krog	Fo	A	y
Ramalina fastigiata (Pers.) Ach.	Fr	S	y
Ramalina fraxinea (L.) Ach.	Fr	S	y
Ramalina sp.	Fr	S	
Rinodina sp.	Cr	S	
Scoliciosporum umbrinum (Ach.) Arnold	Cr	S	n
Xanthoria parietina (L.) Th. Fr.	Fo	S	y

Supplementary material for "A comparative study of the fauna associated with nest mounds of native and introduced populations of the red wood ant *Formica paralugubris*"

Table S1. Species of springtails and oribatids collected, with their abundance and the estimated preference for nest or soil according to results of models. Pref = preference. Sites: AB = Abetone (Apennines), CA = Campigna (Apennines), GP = Giovetto di Paline (Alps).

Springtails	AB		CA		GP		
Species	Nest	Soil	Nest	Soil	Nest	Soil	Pref
Anurophorus atlanticus	1	-	-	-	-	-	
Ceratophysella armata	-	2	-	10	-	-	
Cyphoderus albinus	2277	-	24	-	438	2	Nest
Desoria sp.	1	-	9	1	-	-	
Entomobrya nivalis	-	-	-	-	4	-	
Entomobrya sp.	3	2	1	-	-	-	
Folsomia manolachei	6	-	-	12	-	-	
Folsomia penicula	5	39	-	-	32	25	Soil
Folsomia sp.	-	-	-	3	-	-	
Friesea mirabilis	24	-	6	-	-	-	
Hypogastrura viatica	-	-	-	-	1	-	
Isotomurus sp.	-	-	-	3	-	-	
Lathriopyga longiseta	-	-	3	-	-	-	
Lepidocyrtus violaceus	-	-	9	-	-	-	
Lepidocyrtus cyaneus	8	-	21	-	56	1	Nest
Lepidocyrtus lanuginosus	-	-	-	-	1	-	
Lepidocyrtus lignorum	-	-	-	-	1	1	
Mesaphorura sp.	-	-	-	-	-	1	

Paratullbergia callipygos	-	1	1	-	-	-	
Parisotoma notabilis	18	1	-	-	6	1	
Protaphorura armata	-	1	1	1	-	-	
Protaphorura campata	-	1	-	-	-	-	
Protaphorura cancellata	-	-	2	-	6	1	
Pseudachorutes dubius	-	-	-	2	-	-	
Pseudachorutes sp.	-	-	-	1	-	-	
Pseudosinella alba	-	-	-	1	-	-	
Pseudosinella apuanica	6	13	-	1	-	-	Soil
Tetracanthella pilosa	-	-	-	2	-	-	
Tomocerus minor	-	2	-	-	-	-	
Vertagopus arborea	-	-	2	-	-	-	
Xenylla maritima	-	-	-	-	4	-	
Xenylla schillei	29	-	-	1	-	-	

Oribatids	AB		CA		GP		
Species	Nest	Soil	Nest	Soil	Nest	Soil	Pref
Achipteria coleoptrata	-	-	25	7	19	-	
Achipteria italica	-	-	6	2	-	-	
Acrogalumna longipluma	55	-	32	-	-	-	Nest
Adoristes ovatus	19	-	64	2	111	1	Nest
Atropacarus clavigerus	-	-	-	-	19	-	
Atropacarus platakisi	-	-	-	-	3	-	
Banksinoma lanceolata	-	-	1	-	-	-	
Belba bartosi	-	-	-	-	1	-	

Berniniella (Berniniella) hauseri	3	-	-	-	-	-	
Berniniella bicarinata	7	1	-	14	-	-	
Berniniella coronata	-	-	-	-	-	1	
Berniniella (Hypogeoppia) dungeri	-	9	-	-	-	-	
Camisia horrida	-	-	-	-	2	-	
Camisia spinifer	4	-	-	-	-	-	
Carabodes areolatus	2	-	-	-	2	-	
Carabodes labyrinthicus	149	3	3	5	13	1	Nest
Carabodes manganoi	3	-	4	-	-	-	
Carabodes marginatus	-	-	4	-	5	-	
Carabodes ornatus	11	-	5	-	19	-	Nest
Carabodes palmifer	1	-	-	-	-	-	
Carabodes reticulatus	-	-	-	-	1	-	
Cepheus pegazzonae	1	-	-	-	-	-	
Cerachipteria minuscula	23	1	1	8	-	-	
Ceratozetes gracilis	-	-	59	21	2	-	
Ceratozetes minutissimus	-	-	-	-	2	-	
Ceratozetes peritus	2	13	-	-	-	-	Soil
Chamobates cuspidatus	-	1	-	-	1	-	
Chamobates pusillus	23	1	-	-	-	-	
Chamobates rastratus	-	-	-	-	271	-	
Chamobates voigsti	-	-	-	-	5	6	
Cymbaeremaeus cymba	9	-	3	-	1	3	
Damaeus selgae	-	-	-	-	60	-	

Dissorhina ornata peloponnesiaca	-	4	-	-	-	-	
Eniochthonius minutissimus	-	-	-	-	24	-	
Eremaeus cordiformis	-	-	4	-	5	-	
Eueremaeus oblongus	340	-	-	-	-	-	Nest
Eueremaeus silvestris	-	-	52	9	-	-	
Eueremaeus valkanovi	-	-	-	-	7	-	
Eulohmannia ribagai	-	-	-	1	-	-	
Eupelops acromios	3	-	-	-	3	-	
Eupelops affinis	1	-	-	-	-	-	
Eupelops torulosus	1	-	1	-	46	-	Nest
Euzetes globulus	-	1	-	-	-	-	
Fosseremus laciniatus	-	-	-	-	-	1	
Hemileius initialis	-	-	-	-	1	-	
Hermannia gibba	48	1	2	2	29	-	
Hypochthonius luteus	-	-	-	-	8	-	
Jugatala angulata	2	-	2	-	1	-	
Lepidozetes singularis	-	-	6	-	6	2	
Liacarus coracinus	30	1	-	-	197	3	Nest
Liacarus perezinigoi	-	-	-	-	1	-	
Liacarus subterraneus	-	-	4	-	-	-	
Liebstadia humerata	-	-	-	-	3	-	
Liebstadia longior	15	-	-	-	-	-	Nest
Medioppia pinsapi	-	-	-	-	-	2	
Metabelba parapulverosa	-	-	-	-	35	-	

Microppia minus	-	7	-	-	-	-	
Minunthozetes pseudofusiger	5	75	-	53	-	-	Soil
Multioppia glabra	-	-	-	-	5	-	
Nanhermannia nana	-	-	-	-	1	-	
Neotrichoppia (Confinoppia) confinis	20	9	-	3	84	1	
Nothrus anauniensis	-	1	-	1	-	-	
Nothrus silvestris	-	-	-	-	-	1	
Odontocepheus elongatus	11	-	-	-	2	-	
Ommatocepheus ocellatus	5	-	-	-	-	-	
Ophidiotrichus vindobonensis	-	-	-	-	3	-	
Oppiella (Moritzoppia) keilbachi	-	-	-	-	140	-	
Oppiella (Moritzoppia) unicarinata	-	-	37	-	4	-	
Oppiella (Oppiella) acuminata	96	-	-	-	-	-	
Oppiella (Oppiella) nova	30	39	-	10	25	-	
Oppiella (Rhinippia) fallax	1	2	-	-	-	-	
Oppiella (Rhinoppia) obsoleta	-	39	-	-	-	-	Soil
Oppiella (Rhinoppia) similifallax	-	1	-	-	-	-	
Oppiella (Rhinoppia) subpectinata	1	97	-	95	8	-	Soil
Oribatella brevipila	-	-	11	-	58	-	
Oribatella calcarata	-	-	-	-	4	-	
Oribatella euthricha	-	-	2	-	-	-	
Oribatella hungarica	15	-	-	-	-	-	
Oribatella quadricornuta	-	-	-	-	7	-	
Oribatella sexdentata	1	-	-	-	-	-	

Oribatula tibialis	4	-	8	3	2	1	
Pantelozetes paolii	-	1	-	-	2	-	
Parachipteria petiti	13	11	-	-	-	-	
Parachipteria punctata	1	-	-	-	-	-	
Pergalumna altera	39	-	4	-	63	-	Nest
Pergalumna nervosa	131	-	83	-	736	-	Nest
Phauloppia lucorum	17	-	-	-	-	-	Nest
Phauloppia rauschenensis	-	-	-	-	2	-	
Phthiracarus anonymus	1	-	-	-	8	-	
Phthiracarus clavatus	2	-	1	-	-	-	
Phthiracarus globosus	-	-	1	-	-	-	
Phthiracarus italicus	-	1	-	3	-	-	
Phthiracarus laevigatus	-	-	9	-	-	-	
Phthiracarus lentulus	1	1	-	-	-	-	
Phthiracarus nitens	1	2	5	1	-	-	
Phthiracarus peristomaticus	-	-	-	-	12	-	
Platynothrus peltifer	-	-	-	-	1	-	
Porobelba spinosa	551	-	121	-	-	-	Nest
Poroliodes farinosus	2	-	-	-	-	-	
Ramusella (Inscuptoppia) elliptica	1	22	-	-	-	-	Soil
Ramusella (Ramusella) assimilis	-	-	3	4	1	-	
Raphigneta numidiana	-	-	-	-	-	1	
Rhysotritia duplicata	-	-	10	1	109	1	Nest
Scheloribates laevigatus	1	-	-	-	-	-	

Scheloribates pallidulus	565	1	407	-	574	-	Nest
Scheloribates tuberculatus	-	-	-	-	108	3	
Serratoppia serrata	-	1	-	-	-	-	
Steganacarus (Steganacarus) applicatus	-	-	-	-	25	-	
Steganacarus (Steganacarus) donatoi	-	1	-	-	-	-	
Steganacarus (Steganacarus) hirsutus	19	1	-	-	-	-	Nest
Steganacarus (Tropacarus) carinatus	1	-	-	-	2	-	
Suctobelbella acutidens	-	1	-	-	-	-	
Suctobelbella alleonasuta	-	-	-	2	-	-	
Suctobelbella cf. *subcornigera*	1	-	-	-	-	-	
Suctobelbella opistodentata	-	-	-	2	-	-	
Suctobelbella sarekensis	-	1	-	2	-	-	
Tectocepheus sarekensis	9	1	-	5	1	1	
Tectocepheus velatus	10	-	2	1	2	-	
Xenillus clypeator	-	-	1	-	-	-	
Xenillus tegeocranus	22	-	-	-	-	-	Nest
Zygoribatula exilis	143	-	4	-	-	-	Nest
Zygoribatula propinqua	1	-	-	-	-	-	

Table S2. Species not belonging to springtails or oribatids collected, with their abundance. Sites: AB = Abetone (Apennines), CA = Campigna (Apennines), GP = Giovetto di Paline (Alps).

					GB		**AB**		**CA**	
Phylum	**Class**	**Order, Family (Subfamily)**	**Species**	**Additional note**	**Nests**	**Soil**	**Nests**	**Soil**	**Nests**	**Soil**
Arthropoda	Insecta	Lepidoptera		sp.1 - larva ('Microlepidoptera')	26	0	0	0	0	0
Arthropoda	Insecta	Lepidoptera		sp.2 - larva ('Microlepidoptera')	0	0	2	0	0	0
Arthropoda	Insecta	Coleoptera, Chrysomelidae (Cryptocephalinae)		larva	0	0	4	0	0	0
Arthropoda	Insecta	Coleoptera		larva	0	0	0	0	0	1
Arthropoda	Insecta	Coleoptera		larva	0	0	0	0	0	1
Arthropoda	Insecta	Coleoptera		larva	0	2	0	0	0	0
Arthropoda	Insecta	Coleoptera		larva	20	0	3	9	5	1
Arthropoda	Insecta	Coleoptera, Latridiidae	*Dienerella sp.*		35	0	10	0	6	0
Arthropoda	Insecta	Coleoptera, Latridiidae	*Corticaria sp.*		9	0	4	0	0	0
Arthropoda	Insecta	Coleoptera, Monotomidae (Monotominae)	*Monotoma conicicollis*		0	0	1	0	0	0
Arthropoda	Insecta	Coleoptera		larva Scarabaeoidea/ Chrisomelidae	1	0	0	0	0	0
Arthropoda	Insecta	Coleoptera, Staphylinidae (Aleocharinae)		cf. Leptusina	1	0	0	0	0	0
Arthropoda	Insecta	Coleoptera, Staphylinidae		cf. Xantholininae sp.1	0	0	0	2	1	0
Arthropoda	Insecta	Coleoptera, Staphylinidae		cf. Xantholininae sp.2	0	0	2	1	0	0
Arthropoda	Insecta	Coleoptera, Staphylinidae		cf. Xantholininae sp.3	0	0	4	2	2	0
Arthropoda	Insecta	Coleoptera, Staphylinidae		cf. Xantholininae sp.4	0	0	1	0	0	0
Arthropoda	Insecta	Coleoptera, Staphylinidae		cf. Xantholininae sp.5	0	0	5	0	3	0
Arthropoda	Insecta	Coleoptera, Staphylinidae		cf. Xantholininae sp.6	0	0	3	0	0	0
Arthropoda	Insecta	Coleoptera, Staphylinidae (Scydmaeninae)	*Stenichnus sp.*		0	0	0	0	6	0
Arthropoda	Insecta	Coleoptera, Staphylinidae (Scydmaeninae)	*Cephennium sp.*		0	0	0	0	4	0
Arthropoda	Insecta	Coleoptera, Staphylinidae		cf. Omaliinae	0	0	0	0	1	0
Arthropoda	Insecta	Coleoptera, Nitidulidae			2	0	0	0	1	0
Arthropoda	Insecta	Coleoptera, Scolytidae			0	0	0	0	1	0
Arthropoda	Insecta	Coleoptera, Endomychidae	*Mycetaea subterranea*		16	0	0	0	0	0
Arthropoda	Insecta	Coleoptera, Ptilidae	*Ptilium sp.*		0	0	5	0	5	0
Arthropoda	Insecta	Psocoptera			3	0	1	0	1	0
Arthropoda	Insecta	Hemiptera, Adelgidae			0	3	0	0	0	0

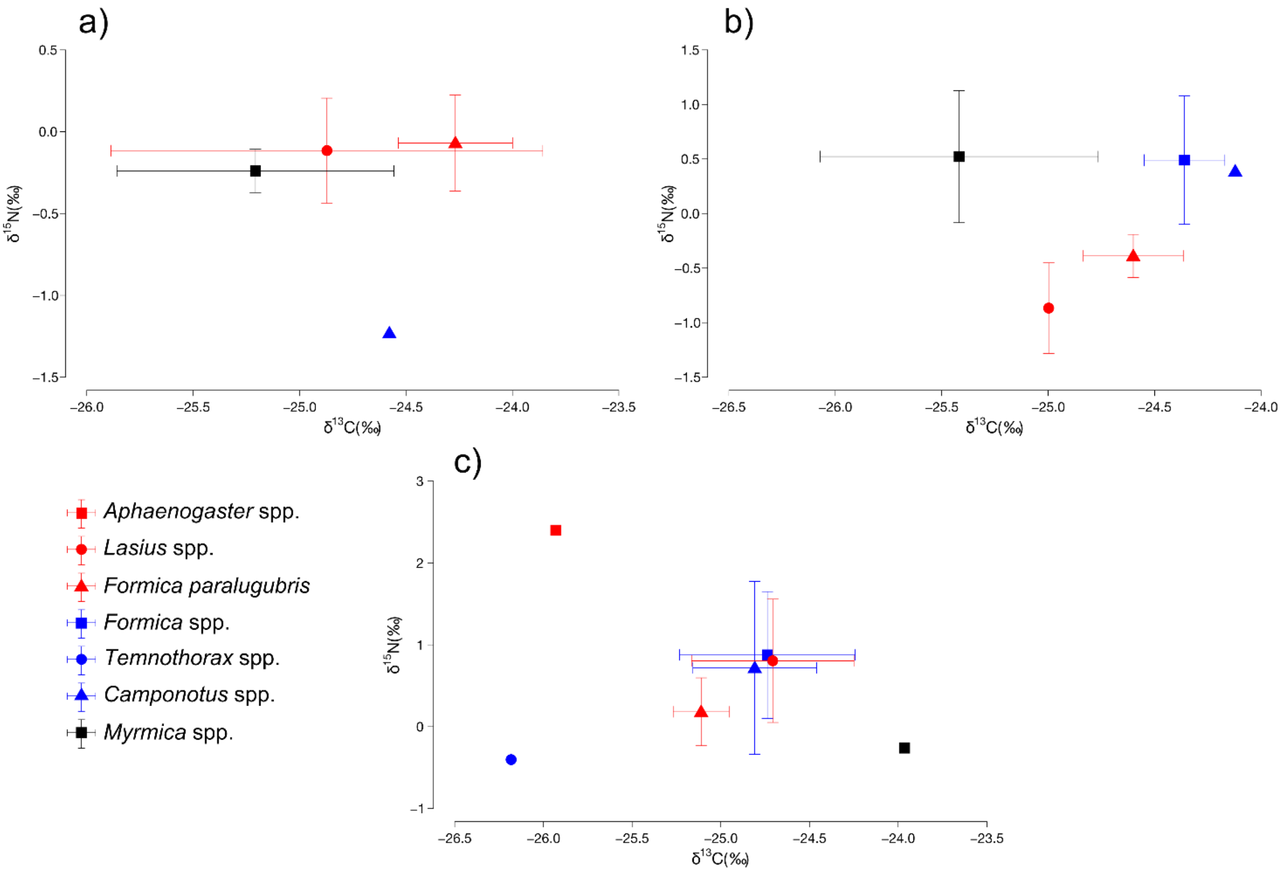
Arthropoda	Insecta	Hemiptera, Anthocoridae		0	0	2	0	0	0
Arthropoda	Insecta	Hemiptera	Sternorrhyncha	1	0	0	1	0	0
Arthropoda	Insecta	Hemiptera	Cicadoidea	0	0	0	0	1	0
Arthropoda	Insecta	Hymenoptera, Cynipidae		2	0	1	2	0	0
Arthropoda	Insecta	Hymenoptera	cf. Chrysidae	0	0	2	0	0	0
Arthropoda	Insecta	Diptera		0	1	0	0	0	0
Arthropoda	Insecta	Diptera		0	1	0	0	0	0
Arthropoda	Insecta	Diptera		0	0	0	1	0	0
Arthropoda	Insecta	Diptera	larva cf. Tipuloidea	0	0	0	1	0	0
Arthropoda	Insecta	Diptera	larva	0	2	0	0	0	6
Arthropoda	Insecta	Diptera	larva	0	0	0	0	0	1
Arthropoda	Insecta	Thysanoptera		0	1	1	0	0	0
Arthropoda	Diplopoda	Julida, Julidae		10	0	0	4	0	0
Arthropoda	Diplopoda			9	0	0	0	0	0
Arthropoda	Diplopoda			0	0	0	0	11	0
Arthropoda	Diplopoda		cf. Julidae	0	4	7	0	5	0
Arthropoda	Chilopoda			1	0	1	1	2	0
Arthropoda	Chilopoda		Lithobiomorpha	0	0	0	0	4	0
Arthropoda	Chilopoda		Geophilomorpha	0	0	0	0	7	2
Arthropoda	Symphyla			2	2	0	72	4	1
Arthropoda	Malacostraca	Isopoda		21	0	0	0	0	0
Arthropoda	Arachnida	Aranea		1	0	4	0	0	0
Arthropoda	Arachnida	Pseudoscorpiones	sp. 1	2	0	43	0	38	0
Arthropoda	Arachnida	Pseudoscorpiones	sp.2	0	0	0	1	0	0
Arthropoda		Protura		1	0	16	2	15	1
Arthropoda		Diplura		0	0	1	2	0	0
Nematoda				0	0	2	0	0	0
Annelida	Clitellata	Haplotaxida, Enchytraeidae		0	0	0	0	0	3
Annelida	Clitellata	Haplotaxida, Lumbricidae		0	0	0	0	0	1

Supplementary material for "Plasticity in the trophic niche of an invasive ant explains establishment success and long-term coexistence"

Supplementary Table. Plasticity in the trophic niche of an invasive ant explains establishment success and long-term coexistence.

Site	**Coordinates**	**Country**	**Native/Introduction area**	**Site area (ha)**	**Nest density (nests/ha)**	**Dominant tree species**
Swiss National Park (SNP)	46°40′17″N, 10°11′12″ E	Switzerland	Native	200	1.8	*Pinus mugo, Pinus cembra, Picea abies*
Giovetto di Paline (GP)	45°57′57″ N, 10°07′48″ E	Italy	Native	9	12	*Picea abies*
Abetone (AB)	44°08′50″ N, 10°40′24″ E	Italy	Introduction	8	13	*Abies alba*
Campigna (CA)	43°52′00″ N, 11°44′14″ E	Italy	Introduction	10	12	*Abies alba*
Valcartier (VC)	46°56'52" N, 71°29'55" W	Quebec, Canada	Introduction	4.6	11	*Pinus resinosa, Pinus strobus, Pinus banksiana, Picea glauca, Picea mariana, Larix laricina, Abies balsamea, Betula papyrifera*

Supplementary Figure. Isotopic biplot (mean $\delta^{15}N$ and $\delta^{13}C$ ± standard error) of ant species at the edge of the *Formica paralugubris* range for: a) Abetone (Italy, introduced); b) Giovetto di Paline (Italy, native); c) Campigna (Italy, introduced).



Supplementary material for “CO_2 biogeochemical investigation and microbial characterization of red wood ant mounds in a Southern Europe montane forest”

Figure S1. Daily trends of CO_2 concentration, efflux, and carbon isotopic signature (C) measured at three mound levels (L1, L2, L3) in the two ant mounds in the two seasons (summer and winter).

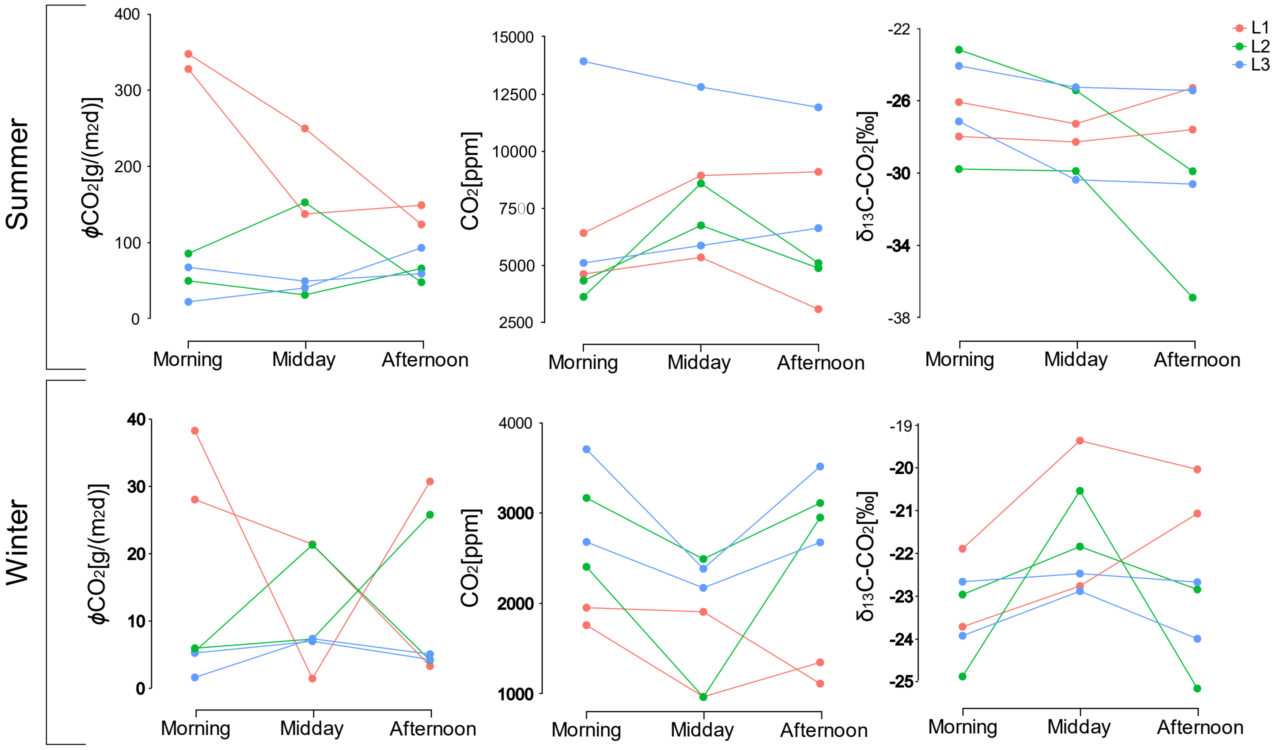
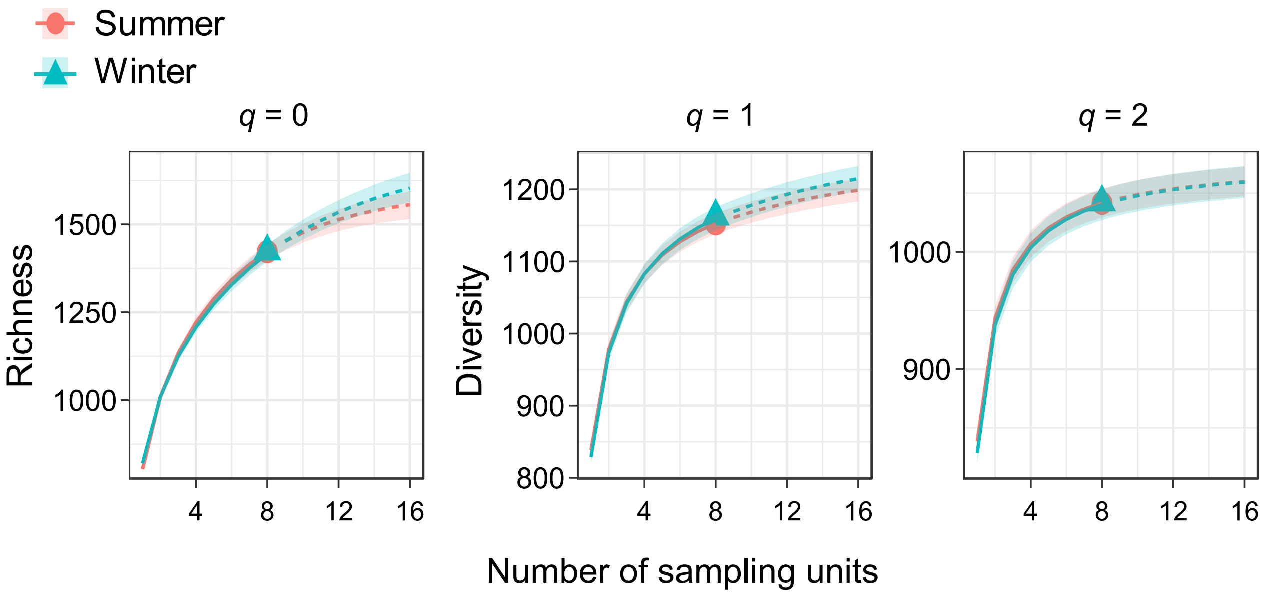


Figure S2. Sample-based estimates of ant mound microbial Hill's numbers in the two seasons (summer and winter) with the associated 95% confidence intervals: richness at the genus level (q = 0), Shannon diversity index (q = 1), and Simpson diversity index (q = 2). Continuous lines refer to actual sample numbers, while dashed lines to the extrapolated estimates.



Supplementary material for “Red wood ants in Europe: trends, local paradoxes, and a call to action”

Appendix S1. Threat status of red wood ant species in Europe according to National Red Lists and local sources.

Species	**Country (Region)**	**Regional status**	**Assessment year**	**Criterion**	**Reference**
F. rufa	Germany (Bayern)	endangered	2003	non IUCN	Schnittler et al. 1994
F. rufa	Poland	Near threatened	2002	IUCN	Glowacinski et al. 2002
F. rufa	Sweden	Least concern	2020	IUCN	SLU Artdatabanken. 2020
F. rufa	UK	Not a species of conservation concern	1991	modified IUCN	Falk 1991
F. rufa	Germany	Declining species	1998	non IUCN	Binot et al. 1998
F. rufa	Germany (Nordrhein-Westfalen)	Declining	2011	non IUCN	https://www.lanuv.nrw.de/
F. rufa	Switzerland	near threatened	1994	non IUCN	Agosti & Cherix 1994
F. lugubris	Poland	Vulnerable	2002	IUCN	Glowacinski et al. 2002
F. lugubris	Sweden	Least concern	2020	IUCN	SLU Artdatabanken. 2020
F. lugubris	Germany	Endangered	1998	non IUCN	Binot et al. 1998
F. lugubris	Germany (Bayern)	endangered	2003	non IUCN	Schnittler et al. 1994

F. aquilonia	Czech Republic	Endangered	2005	IUCN	Farkac et al. 2005
F. aquilonia	Poland	Vulnerable	2002	IUCN	Glowacinski et al. 2002
F. aquilonia	Sweden	Least concern	2020	IUCN	SLU Artdatabanken. 2020
F. aquilonia	Germany	endangered	1998	non IUCN	Binot et al. 1998
F. aquilonia	Germany (Bayern)	endangered	2003	non IUCN	Schnittler et al. 1994
F. pratensis	Poland	Near threatened	2002	IUCN	Glowacinski et al. 2002
F. pratensis	Sweden	Least concern	2020	IUCN	SLU Artdatabanken. 2020
F. pratensis	Germany	declining	1998	non IUCN	Binot et al. 1998
F. pratensis	Germany (Bayern)	endangered	2003	non IUCN	Schnittler et al. 1994
F. pratensis	UK	Endangered: extinct on the mainland; occurs on Channel Islands	1991	modified IUCN	Falk 1991
F. pratensis	Germany (Nordrhein-Westfalen)	endangered	2011	non IUCN	https://www.lanuv.nrw.de/
F. pratensis	Switzerland	vulnerable	1994	non IUCN	Agosti & Cherix 1994
F. paralugubris	Germany	endangered	1998	non IUCN	Binot et al. 1998
F. polyctena	Poland	Near threatened	2002	IUCN	Glowacinski et al. 2002
F. polyctena	Sweden	Least	2020	IUCN	SLU Artdatabanken. 2020

		concern			
F. polyctena	Germany	declining	1998	non IUCN	Binot et al. 1998
F. polyctena	Germany (Bayern)	declining	2003	non IUCN	Schnittler et al. 1994
F. polyctena	Germany (Nordrhein - Westfalen)	endangered	2011	non IUCN	https://www.lanuv.nrw.de/
F. polyctena	Switzerland	near threatened	1994	non IUCN	Agosti & Cherix 1994
F. uralensis	Poland	endangered	2002	IUCN	Glowacinski et al. 2002
F. uralensis	Sweden	Least concern	2020	IUCN	SLU Artdatabanken. 2020
F. uralensis	Germany	critically endangered	1998	non IUCN	Binot et al. 1998
F. uralensis	Switzerland	extinct	1994	non IUCN	Agosti & Cherix 1994
F. truncorum	Poland	Near threatened	2002	IUCN	Glowacinski et al. 2002
F. truncorum	Sweden	Least concern	2020	IUCN	SLU Artdatabanken. 2020
F. truncorum	Germany	endangered	1998	non IUCN	Binot et al. 1998
F. truncorum	Germany (Bayern)	critically endangered	2003	non IUCN	Schnittler et al. 1994
F. truncorum	Germany (Nordrhein - Westfalen)	critically endangered	2011	non IUCN	https://www.lanuv.nrw.de/
F. truncorum	Switzerland	endangered	1994	non IUCN	Agosti & Cherix 1994
F. aquilonia	Estonia	Least	2017	IUCN	https://elurikkus.ee/lists/public/speciesLists/

		concern			
F lugubris	Estonia	Least concern	2017	IUCN	https://elurikkus.ee/lists/public/speciesLists/
F. polyctena	Estonia	Least concern	2017	IUCN	https://elurikkus.ee/lists/public/speciesLists/
F. pratensis	Estonia	Least concern	2017	IUCN	https://elurikkus.ee/lists/public/speciesLists/
F. rufa	Estonia	Least concern	2017	IUCN	https://elurikkus.ee/lists/public/speciesLists/
F. lugubris	UK	Not a species of conservation concern	1991	modified IUCN	Falk 1991
F. lugubris	Ireland	In serious decline	2014	non IUCN	Breen 2014
F. aquilonia	Ireland	Not formally assessed: only one population remains	2014	non IUCN	Breen 2014
F. aquilonia	UK	Scarce (Nb)	1991	modified IUCN	Falk 1991
F. polyctena	Belgium (Flanders)	vulnerable	2003	non IUCN	Dekoninck et al. 2005
F. pratensis	Belgium (Flanders)	vulnerable	2003	non IUCN	Dekoninck et al. 2005
F. rufa	Belgium (Flanders)	vulnerable	2003	non IUCN	Dekoninck et al. 2005
F. rufa x polyctena	Belgium (Flanders)	indeterminate	2003	non IUCN	Dekoninck et al. 2005
F. aquilonia	Czechia	near threatened	2017		Hejda et al. 2017
F. aquilonia	Finland	Least concern	2019	IUCN	Paukkunen et al. 2019

F. polyctena	Finland	Least concern	2019	IUCN	Paukkunen et al. 2019
F. rufa	Finland	Least concern	2019	IUCN	Paukkunen et al. 2019
F. lugubris	Finland	Least concern	2019	IUCN	Paukkunen et al. 2019
F. pratensis	Finland	Least concern	2019	IUCN	Paukkunen et al. 2019
F. truncorum	Finland	Least concern	2019	IUCN	Paukkunen et al. 2019
F. uralensis	Finland	Least concern	2019	IUCN	Paukkunen et al. 2019
F. aquilonia	Norway	Least concern	2021	IUCN	https://www.artsdatabanken.no/rodlisteforarter/2021
F. polyctena	Norway	Least concern	2021	IUCN	https://www.artsdatabanken.no/rodlisteforarter/2021
F. rufa	Norway	Least concern	2021	IUCN	https://www.artsdatabanken.no/rodlisteforarter/2021
F. lugubris	Norway	Least concern	2021	IUCN	https://www.artsdatabanken.no/rodlisteforarter/2021
F. pratensis	Norway	Least concern	2021	IUCN	https://www.artsdatabanken.no/rodlisteforarter/2021
F. truncorum	Norway	Least concern	2021	IUCN	https://www.artsdatabanken.no/rodlisteforarter/2021
F. uralensis	Norway	Near threatened	2021	IUCN	https://www.artsdatabanken.no/rodlisteforarter/2021

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Table S2. Protection laws and regulations.

Country	**Reference**
Austria	NE NSchG, Nature Conservation Act 2000
Estonia	Regulations of the Minister of the Environment of the Republic of Estonia n. 51, RTL 2004, 69, 1134
Hungary	Decree 13/2001 v. 9, art. 2 and annex 5
Belgium	Royal Law Decree 22 September 1980; Annex IIIb, M.B. 11th September 1973 and subsequent updates Flanders: Forest decree and the Nature decree (annex II, III and IV) Brussels: Ordinance for Nature Conservation (annex II.2 and II.3) Wallonia: Nature conservation law (annex V) and Directive 2009/147 on the protection of birds governs the protection of a series of species in Walloon (annexes I to VII)
United Kingdom	Biodiversity Action Plan 2007, covers *Formica pratensis* but not *Formica rufa, F. lugubris* or *F. aquilonia* which have no general protected status in the UK. Note however that *Formicoxenus nitidulus* is a BAP species, and as it is dependent on *F. rufa* group species, this arguably extends some protection to them.
Scotland (in addition to UK regulations above)	*Formicoxenus nitidulus* is on the Scottish Biodiversity List but none of the *F. rufa* group wood ants are
Northern Ireland (in addition to UK regulations above)	*F. aquilonia* is a Northern Ireland Priority Species (Wildlife and Natural Environment Act (Northern Ireland) 2011)
Ireland	Red Lists covering the island of Ireland do not yet cover ants, so there is no protection beyond EU regulations (*Formica lugubris* listed as a “special concern” in the EU Habitats Directive)
Switzerland	Federal law for the protection of nature and landscape, LPN, RS 451, 1966; OPN, RO 1991-249, 16th January 1991, updated 1st June 2017

Germany	BNatSchG of 29th July 2009 -Federal Official Gazette I pag. 2542 / FNA 791-9 Bundesartenschutzverordnung (16. Februar 2005 (BGBl. I S. 258, ber. S. 896 / FNA 791-8-1) Annex 1 lists RWA (except for F. sanguinea) as especially protected species.
Czechia	Law about protection nature and landscape 114/1992 Coll give general regulation about protected species. Particular species are listed in declaration 395/1992 Col, here Formica spp. are mentioned as treaten
Poland	Regulation of the Minister of Environment dated December 16, 2016 on the protected species of animals. J. Laws 2016.
Finland	Law for the protection of nature 20.12.2996/1096 (last updates September 3 2021)

Tables S3. Protection laws of the Italian Regions.

Area	Region	Law Nr
Alpine area	Veneto	L.R. 53/1974
	Friuli Venezia-Giulia	L.R. 34/1981
	Liguria	L.R. 4/1999; L.R. 28/2009
	Valle d'Aosta	L.R. 16/1977
	Autonomous province of Trento	L.P. 11/2007
	Piedmont	L.R. 32/1982
	Lombardy	L.R. 33/1977; L.R. 10/2008
	Autonomous province of Bolzano	L.P. 27/1973; L.P. 6/2010
Apennines	Umbria	R.R. 1/1981 (abrogated in 2003); L.R. 28/2001
	Lazio	L.R. 4/1999; R.R. 7/2005
	Campania	R.R. 3/2017
	Tuscany	D.P.G.R. 48/R/2003, Tuscany Forest regulation
	Emilia Romagna	General Prescriptions and Forestry Police, approved by regional resolution 182/1995