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

Luca Di Nuzzo (1), Alberto Masoni (1), Filippo Frizzi (1), Elisabetta Bianchi (1), Maria Beatrice Castellani (1), Paride Balzani (1-2), Federica Morandi (1), Ylenia Sozzi (1), Chiara Vallese (1), Giacomo Santini (1,3), Renato Benesperi (1)

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 (Heineke & Pellmyr 2009, Cara 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 foliaceous 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 maintaining a perpetual 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 & Ellis 2016), and in the southern part of their range they are restricted to mountain areas, rarely occurring below 900 m 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 Formica 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 natural range (1200-1350 m a.s.l.) and the mean annual precipitation is 2325 mm and 1682.1 mm for Abetone (Bartolini et al. 2018) and Campigna (Gonnelli & Bottacci 2009), respectively.

In Campigna, the RWA population is subdivided into several independent sub-populations displaced in the area, one of which is located in Avermio Alto, occupies an area of about 8 ha, and was chosen for this study (Frizzi et al. 2020). In the area of Abetone, a single population exists and covers an area of about 10 ha. Despite previous identifications described RWA populations in both areas as F. lugubris based on morphological characteristics (Groppali & Crudelle 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⁻¹, CA: ~12 nests ha⁻¹). For each area, we randomly selected three F. paralugubris nests spaced at least 150 metres from each other. Six silver fir (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 & Crudelle 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 – Tab. S1 in Supplementary material), which have been previously used to compare lichen communities (Giordani et al. 2016, Hurtado et al. 2020).

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 & 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 plot 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 areas 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 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 in Supplementary material), 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 = 0.15) showed a clear separation of ant-visited trees compared to those from control sites. In both AB and CA sites, differences in species composition were tested. We applied the

| Site     | Trait | r²   | P    | Direction |
|----------|-------|------|------|-----------|
| Abetone  | GF_Cr| 0.3706| 0.001|           |
|          | GF_Fo| 0.0422| 0.514| 0         |
|          | GF_Fr| 0.0001| 0.998| 0         |
|          | RS_A | 0.2208| 0.021| +         |
|          | RS_S | 0.0100| 0.859| 0         |
|          | LA_n | 0.6009| 0.001| n         |
|          | LA_y | 0.4068| 0.001| +         |
|          | GF_Cr| 0.3658| 0.002|           |
| Campigna | 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| -         |
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npMANOVA revealed a significant effect of ant presence ($AB: F_{1,4} = 27.77, P = 0.001$; CA: $F_{1,4} = 11.15, P = 0.001$), but also significant variability among plots within each site ($AB: F_{1,4} = 5.50, P = 0.001$; CA: $F_{1,4} = 6.68, P = 0.001$).

CWM values varied according to ant presence (Tab. 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 ants. 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 ($D = 26.9$) compared to control areas ($R = 29.1, S = 43.8, D = 27.1$).

**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. tergicolous). 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 patterns compared to *F. paralugubris*, thus influencing cryptogam communities differently. *F. paralugubris* has been introduced in the studied forests, so we could 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 (Ben-esperi & 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 pH, roughness, water holding capacity, and tree species, and macroscale factors such as temperature (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 contributed equally. All authors contributed extensively to the ideas, writing and discussions. Comments from two anonymous reviewers greatly improved the manuscript. The work was funded by grants of the University of Florence to GS and RB.

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**References**

Aho T, Kuitunen M, Suhonen J, Jääntti A, Hakkar T (1999). Reproductive success of eurasion treecreepers, *Certhia familiaris*, lower in territories with wood ants. *Ecology* 80: 998-1007. - doi: 10.1890/0012-9658

Anderson MJ (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecology* 26: 32-46. - doi: 10.1111/j.1442-9993.2001.01070.x

André HM (1983). Associations between corticolous microarthropod communities and ephytic cover on bark. *Ecography* 8: 113-119. - doi: 10.1111/j.1600-0583.1985.tb01161.x

Asplund J, Wardle DA (2017). How lichens impact terrestrial community and ecosystem properties. *Biological Reviews* 92: 1720-1738. - doi: 10.1111/brv.12305

Bailey RH (1970). Animals and the dispersal of sorelidae from *Lecanora conicaeoides* NYL. Ex cromb. *The Lichenologist* 4: 256-256. - doi: 10.1071/IO24282970000254

Balzani P, Vizzini S, Frizzi F, Masoni A, Lessard JP, Bernasconi C, Franciscoe A, Ibarra-Issasi J, Brasso S, Cherix D, Santini G (2021). Plasticity in the trophic niche of an invasive ant explains establishment success and long-term coexistence. *Oikos* 130 (5): 691-696. - doi: 10.1111/oik.08217

Bartolini G, Grifoni D, Magno R, Torrigiani T, Gozzini B (2018). Changes in temporal distribution of precipitation in a Mediterranean area (Tuscany, Italy) 1955-2015. *International Journal of Climatology* 38: 1366-1374. - doi: 10.1002/joc.5351

Benesperi R, Tretiach M (2004). Differential land snail damage to selected species of the lichen genus *Peltigera*. *Biochemical Systematics and Ecology* 32: 127-138. - doi: 10.1016/j.bsec.2004.06.036

Buhl J, Hicks K, Miller ER, Persey S, Alinovi O, Sumpter DJT (2009). Shape and efficiency of wood ant foraging networks. *Behavioral Ecology and Sociobiology* 65: 451-460. - doi: 10.1007/s00265-008-0680-7

Caradonna PJ, Burkle LA, Schwarzb B, Resasco J, Knight TM, Benadi G, Blüthgen N, Dormann CF, Fang Q, Fründ J, Gauzen H, Kaiser-Bunbury CN, Winfree R, Vázquez DP (2021). Seeing through the static: the temporal dimension of plant-animal mutualistic interactions. *Ecology Letters* 24 (1): 149-161. - doi: 10.1111/ele.16323

Chapuisat M, Keller L (1999). Extended family structure in the ant *Formica paralugubris*: the role of the breeding system. *Behavioral Ecology and Sociobiology* 46: 405-412. - doi: 10.1007/s002650050566

Clarke KR, Warwick RM (2001). Change in marine communities: an approach to statistical analysis and interpretation. *Primer-E Ltd*, Plymouth, UK, pp. 262.

Domisch T, Risch A, Robinson EJH (2016). Wood ant foraging and mutualism with aphids. In: “Wood Ant Ecology and Conservation”. *Cambridge University Press*, Cambridge, UK, pp. 145-176. - doi: 10.1017/CBO9781107102614.008

Ellis S, Robinson EJH (2016). Internet food sharing within wood ant colonies: resource redistribution behavior in a complex system. *Behavioral Ecology* 27: 660-668. - doi: 10.1093/beha/ear025

Frizzi F, Masoni A, Quilghini G, Clampelli P, Santini G (2018). Chronicle of an impact foretold: the fate and effect of the introduced *Formica paralugubris* ant. Biological Invasions 20: 3575-3589. - doi: 10.1007/s10530-018-1757-x

Frizzi F, Masoni A, Migliorini M, Fanciulli PP, Cianferoni F, Balzani P, Giannotti S, Davini C, Frasconi Wndt C, Santini G (2020). A comparative study of the fauna associated with nest mounds of native and introduced populations of the red wood ant *Formica paraluguribis*. The European Journal of Soil Biology 101: 103241. - doi: 10.1016/j.ejsobi.2020.103241

Frouz J, Jílková V, Sorvari J (2016). Contribution of wood ants to nutrient cycling and ecosystem functioning. *Wood ants*: strategies, adaptations. *Kluwer Academic Publishers*, Dordrecht, The Netherlands, pp. 225. [online] URL: http://books.google.com/books?id=DB8sqJQXKToC

Grolli P, Cruden K (2005). The formiche del gruppo *Formica rufa* trapiantate nel Parco nazionale delle Foreste Casentinesi, Monte Falterona e Campigna [Red wood ants (*Formica rufa* group) introduced in the Foreste Casentinesi, Monte Falterona and Campigna National Park]. Quaderni di Studi e Notizie di Storia Naturale della Romagna, Cesena 20: 63-73. [in Italian]

Heikten K, Rohner M-S, Hoppert M (2007). Red wood ants (*Formica rufa* group) disperse brophyte and lichen fragments on a local scale. *Nova Hedwigia* 81: 147-163. - doi: 10.1017/S0024282906006369

Herrera CM, Pelimyr O (2009). Plant animal interactions: an evolutionary approach. *Blackwell Science Ltd.*, Oxford, UK, pp. 334. [online] URL: http://books.google.com/books?id=Q4A7.7rsc

Hurtado P, Prieto M, Martínez-Villalta J, Giordani P, Aragón G, López-Angulo J, Kožuškova A, Merinero S, Díaz-Peña EM, Rosas T, Benesperi R, Bianchi E, Grube M, Mayrhofer H, Nascimbene J, Wedin M, Westberg M, Martínez I (2020). Disentangling functional trait variation and covariation in epiphytic lichens along a continent-wide latitudinal gradient. *Proceedings of the Royal Society B: Biological Sciences* 287: 20192862. - doi: 10.1098/rspb.2019.2862

Kenney M (1999). Atlas of the land and freshwater mollusca of Britain and Ireland. *Brill, Leiden, The Netherlands*, pp. 272.

Lach L, Parr C, Abbott K (2010). Ant ecology. *Oxford University Press*, Oxford, UK, pp. 432. [online] URL: http://books.google.com/books?id=vDeRQ4UpAQN

Lavorel S, Grugulis K, McIntyre S, Williams NSG, Garden D, Dornough J, Berman S, Quétier F, Thébault A, Bonis A (2008). Assessing functional diversity in the fragment-ecosystem: a multi-fragmentary approach. *Functional Ecology* 22: 134-147. - doi: 10.1111/j.1365-2435.2007.01339.x

Lorentssen S, Mattsson J-E (1999). New Reports of soreida dispersed by ants, *Formica conicaeoides*. *The Lichenologist* 31: 204-207. - doi: 10.1017/S0024282999000262

Marini L, Nascimbene J, Nimis PL (2011). Large-scale patterns of epiphytic lichen species richness: photograph-dependent response to cli-
climate and forest structure. Science of the Total Environment 409: 4381-4386. - doi: 10.1016/j.scitotenv.2010.07.010
Masoni A, Frizzi F, Natali C, Bemasconi C, Ciofi C, Santini G (2019). Molecular identification of imported red wood ant populations in the Campagna Biogenetic Nature Reserve (Foreste Casentinesi National Park, Italy). Conservation Genetics Resources 11: 231-236. - doi: 10.1007/s12686-018-0982-7
Matteucci E, Benesperi R, Giordani P, Piervittori N, Isocrone D (2012). Epiphytic lichen communities in chestnut stands in Central-North Italy. Biotologia (Bratislava) 67: 61-70. - doi: 10.2478/v11759-011-0145-8
Nascimbene J, Lazzaro L, Benesperi R (2015). Patterns of β-diversity and similarity reveal biotic homogenization of epiphytic lichen communities associated with the spread of black locust forests. Fungal Ecology 14: 1-7. - doi: 10.1016/j.fusco.2014.10.006
Nash TH (2008). Lichen biology. Cambridge University Press, Cambridge, UK, pp. 81-105. [online] URL: http://books.google.com/books?id=Gp9OGAAAQBAJ
Robinson EJH, Stockan JA, Iason GR (2016). Wood ants and their interaction with other organisms. In: "Wood Ant Ecology and Conservation". Cambridge University Press, Cambridge, UK, pp. 177-206. - doi: 10.1017/CBO9781107261402.009
Stockan JA, Robinson EJH (2016). Wood ant ecology and conservation. Cambridge University Press, Cambridge, UK, pp. 304. [online] URL: http://books.google.com/books?id=otigDAAAQBAJ
Tab. S1 - Functional traits of lichen species.
Link: DiNuzzo_3897@suppl001.pdf