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ABSTRACT

Understanding the distribution of parasites is crucial for biodiversity conservation. Here, we studied the distribution of the ectoparasitic fungus *Laboulbenia formicarum* in native and invasive *Lasius* ants in a 2000 km^2 area. We screened over 16,000 ant workers in 478 colonies of five different species. We found that *Lab. formicarum* was rare in native *Lasius* species but infected 58% of the colonies of the invasive species *Las. neglectus*. At landscape scale, *Lab. formicarum* presence could not be explained by geographic and genetic distances between *Las. neglectus* colonies but was associated with hotter and dryer climatic conditions and its prevalence in colonies increased with urbanization. Within infected colonies, fungal prevalence varied from 0 to 100 percent within meters and was negatively correlated with impervious ground cover. In a changing world, our findings emphasize the importance of land-use and climatic factors in shaping the distribution and prevalence of fungal parasites.

1. Introduction

Parasites and pathogens are major, albeit understudied, components of ecosystems which often impose tremendous costs to human societies (Carlson et al., 2017; Frainer et al., 2018; Gómez and Nichols, 2013; Rocha et al., 2016; Torgerson, 2013). Understanding the spatial distribution of parasites is essential to the conservation of species and ecosystems (Frainer et al., 2018; Rocha et al., 2016; Weldon et al., 2004), to the control of invasive species and agricultural pests (Hall and Papierok, 1982; Meikle et al., 2015; Vega et al., 2009) and to human health (Thompson et al., 2010; Torgerson, 2013). Characterizing the ecological conditions favouring parasites is, however, challenging (Hall and Papierok, 1982; Johnson et al., 2019) as the spatial distribution of parasites depends on the availability of hosts (Ezenwa, 2004; Kołodziej-Sobocińska, 2019) and on the biotic and abiotic factors that control the parasites' range (Bradley and Altizer, 2007; Chakraborty et al., 2019).

Ectoparasites are ideal models to study the factors shaping the distribution of parasites. They live on the external body envelope of other organisms and are thus particularly exposed to environmental conditions in addition to being easily detected (Hopla et al., 1994; De Kesel, 1996; Kołodziej-Sobocińska, 2019). Laboulbeniales (Ascomycota; Laboulbeniomycetes; Laboulbeniales) are one of the largest groups of ectoparasitic fungi, with about 2325 species described in 145 genera (Haelewaters et al, 2020, 2021; Kirk, 2019; Reboleira et al., 2018; Rossi and Santamaría, 2012). They are

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obligate ectoparasites of arthropods and live attached to the cuticle of a wide variety of insects and of a few other taxa, including mites, harvestmen and myriapods (Pfliegler et al., 2016; Santamaria et al., 2017, 2020; Seeman and Nahrung, 2000). Laboulbeniales form thalli that can cover the entire body of their hosts and may penetrate through the cuticle (Tragust et al., 2016). Transmission usually occurs via spores upon direct contact between conspecifics (e.g. during mating; De Kesel, 1995; Knell and Webberley, 2004). Laboulbeniales are commonly found in ant species (Hymenoptera; Formicidae; Santamaria and Espadaler, 2015). To date, six Laboulbeniales species are known to parasitize 43 ant species from ten genera (Santamaria and Espadaler, 2015). However, little is known of the factors determining the spatial distribution and prevalence of Laboulbeniales in ants (Haelewaters et al., 2015b; Szentiványi et al., 2019). Laboulbeniales are often assumed to have adapted to the ecological niche of their hosts (De Kesel, 1996), to thrive best in densely packed host populations (De Kesel, 1993) or to have an affinity for moist habitats (Santamaria and Espadaler, 2015; Markó et al., 2016; Kołodziej-Sobocińska, 2019). Large-scale climatic variations affect the probability of infection of the ant *Myrmica scabrinodis* by the laboulbenian fungus *Rickia wasmanni* (Szentiványi et al., 2019), but it is not known whether landscape- and local-scale environmental conditions, such as elevation and land cover type, affect the distribution or infection success of the Laboulbeniales that parasitize ants.

To understand what determines Laboulbeniales' spatial distribution at landscape- and local-scale, we studied the ectoparasitic fungus *Laboulbenia formicarum,* that parasitizes *Lasius* ants, including one of the most widespread invasive ant species in Europe, *Lasius neglectus* (originating from Asia Minor; Herraiz and Espadaler, 2007; Ugelvig et al., 2008; Blatrix et al., 2018). Over 300 introduced populations of this species have been detected so far in Europe (Gippet et al., 2017; Espadaler and Bernal, 2020), of which four are known to be infected by *Laboulbenia formicarum* (Herraiz and Espadaler, 2007; Espadaler et al., 2011). The native range of *Lab. formicarum* is still unknown and has been the subject of contrasting hypotheses. First, the fungus could have been introduced in Europe recently. A possible origin is North America, as suggested by the spatial and temporal distribution of records for the species in both continents (Espadaler and Santamaria, 2012). Another origin could be any part of *Las. neglectus* native range if both organisms were co-introduced in Europe, with *Lab. formicarum* being lost in most *Las. neglectus* colonies during the invasion process – reduced parazitation in introduced populations is indeed observed in many taxa (Torchin et al., 2003). Alternatively, *Lab. formicarum* may be native to the European ant fauna and might have found a new suitable host species in invasive *Las. neglectus* ants.

To first assess whether *Lab. formicarum* is common in native *Lasius* species, or only occurs in *Las. neglectus*, we screened 412 colonies from four native *Lasius* species and 66 colonies of the invasive species *Lasius neglectus* (Van Loon et al., 1990) sampled across the landscape of the middle Rhône valley in France (~2000 km^2; Figs. 1–3).

We then focused on the invasive ant *Lasius neglectus*. Because dispersal is crucial in shaping species spatial distribution (Clobert et al., 2012), we tested the importance of horizontal and vertical transmission in explaining *Lab. formicarum* presence across the *Las. neglectus* colonies occurring in our study landscape. In *Las. neglectus,* colonies can extend over several hectares and are composed of multiple nests connected by trails (Espadaler et al., 2007; Ugelvig et al., 2008). Horizontal transmission occurs if *Las. neglectus* colonies transmit the fungus to each other via direct contact of their workers or reproductive individuals (males and females) or indirectly via vectors (e.g., commensals, other parasites). Geographically close colonies should thus be more likely to infect each other if horizontal transmission is an important driver in this host-parasite system. *Lasius neglectus* is dispersed throughout landscapes (and continents) via the transport of potted plants, soil or construction material (Ugelvig et al., 2008). *Lab. formicarum* spread could therefore occur vertically, when a portion of an infected colony is transported to a new location through human activities. Under this scenario, genetically close colonies should be more likely to be infected with *Lab. formicarum* than genetically distant colonies.

We then tested whether the presence and prevalence of *Lab. formicarum* were associated with environmental factors linked to climate (mean annual temperature and precipitation), land cover (vegetation cover, agriculture, urbanization) and topography (elevation and solar radiation). Finally, in 16 infected *Lasius neglectus* colonies, we tested whether within-colony spatial variation in *Lab. formicarum* prevalence was affected by local land cover types (open vegetation, forest, croplands, unsealed ways and impervious surfaces).

2. Material and methods

2.1. Study system

2.1.1. Study area

The study area is a 2000 km^2 zone located in South-East France, in the city of Lyon and its surrounding suburban and rural areas. Lyon is the second largest French metropolitan area after Paris. The area is characterized by a temperate climate with Mediterranean influences. This area is heavily invaded by the ant *Lasius neglectus* (Gippet et al., 2017, 2018).

2.1.2. Datasets

We used two different datasets to study the spatial distribution of *Lab. formicarum*. The first dataset is a sampling of native and invasive *Lasius* ants throughout the study landscape (1248 locations; Methods section 2.2; Table 1, Figs. 2 and 3). The second dataset focuses on 16 colonies of *Las. neglectus* infected by *Lab. formicarum*. In each of these colonies, several nests or trails were sampled in order to assess local (i.e. intra-colonial) spatial variation in fungus prevalence (Methods section 2.3; Figs. 3 and 5).

2.2. Landscape scale sampling and analyses

2.2.1. Sampling of native and invasive Lasius ants

In the study landscape, a total of 1248 locations were sampled during spring and summer 2011, 2012 and 2013. Sampling locations consisted of haphazardly selected patches with vegetation, generally close to or along roadsides on public land. Sampling locations were separated by at least 200 m in dense urban areas and by at least 500 m in suburban, residential and rural areas. Sampling was done by directly searching ant nests and trails on the ground, trees and shrubs. Samples were collected by hand using custom entomological aspirators. Each time a trail or nest of *Lasius* ants was discovered, ants were sampled. We considered that each sample corresponded to a unique ant colony, except for *Las. neglectus* because in this species, all nests and trails occurring locally are interconnected and belong to the same colony. Thus, if different samples of *Las. neglectus* were collected in the same sampling location, they were pooled together for analyses. All samples were stored in 96% ethanol at −20 °C. Ants were then identified to species level using morphological criteria (Seifert, 2007). Additional samples of *Las. neglectus* and native *Lasius* colonies were obtained from the local-scale ant sampling (see methods in section 2.3) and collated to this dataset.

Fig. 1. A *Lasius neglectus* worker infected by *Laboulbenia formicarum* (magnified leg on the right, arrows point thalli) with males taking off from the nest (Image: T. Colin).

2.2.2. Laboulbenia formicarum prevalence in native and invasive Lasius ants

Only samples containing at least 10 individuals were screened for the presence of *Lab. formicarum*. A total of 16,779 workers from 478 different colonies were screened for *Lab. formicarum* presence (Table 1), including 230 colonies of *Las. niger*, 118 of *Las. alienus*, 39 of *Las. paralienus*, 25 of *Las. emarginatus* and 66 of *Las. neglectus* (see Table 1 for details on the number of workers screened by colony). Workers were carefully examined under a stereomicroscope at 50× magnification and were considered infected if at least one *Lab. formicarum* thallus was observed on a single ant's cuticle (Fig. 1).

2.2.3. Geographic and genetic distance between Lasius neglectus colonies

A matrix of geographic distances among all 66 *Las. neglectus* colonies was calculated using the 'dist' function from the *stats* package in R v.3.6.2 (R core team, 2019). To assess the genetic distance between colonies, a total of 793 workers from 33 *Las. neglectus* colonies (mean ± standard deviation: 24 ± 3.6 workers per colony; range: 14–36) were genotyped at 12 microsatellite markers (see supplementary Material and Methods and Table S1 for details). We calculated the genetic distances between *Las. neglectus* colonies as *Fst/*(1-Fst*)* using the 'pairwise.fst' function from the *hierfstat* package in R (Goudet and Jombart, 2015).

2.2.4. Landscape scale environmental factors

Seven climatic, land cover and topographical variables were compiled to test their association with *Lab. formicarum* presence and prevalence in *Las. neglectus* colonies: (i) mean annual temperature (in °C; ~1 km resolution), (ii) mean annual precipitation (in mm; ~1 km resolution), (iii) elevation (25 m resolution), (iv) amount of solar radiation (in $kWh.m^{-2}$) estimated from the elevation map (i.e. 25 m resolution) for June to August 2013 with ArcGIS 10.1 (default parameters in Spatial Analyst Tools), v) the proportion of vegetated land cover (2.5 m resolution), vi) the proportion of impervious land cover (2.5 m resolution) and vii) the proportion of cultivated land cover (vector data) (see Table 2 for more information on variables and their sources). For each variable (except climatic variables), average values were calculated in a 100 m zone around the centre of the sampling locations invaded by *Las. neglectus*. We computed the Euclidean environmental distance between locations invaded by *Las. neglectus* using the 'dist' function from the *stats* package in R.

2.2.5. Statistical analyses

We used the 'dist' function of the *stats* package in R to construct a binary infection status distance matrix between 66 *Las. neglectus* colonies. Pairs of colonies that were both infected by *Lab. formicarum* or both non-infected were assigned a distance of '0', and pairs of colonies with one infected and one non-infected colony were assigned a distance of '1' (following Gilbertson et al., 2016). A Mantel test with 10,000 permutation was then performed using the 'mantel.rtest' function (R package *ade4*; Dray and Dufour, 2007) to test whether *Las. neglectus* colonies with the same infection status were geographically closer to each other than expected from a random spatial distribution. A second Mantel test with 10,000 permutations between the infection and genetic distance matrices ($N = 33$ colonies) was performed to test whether *Las. neglectus* colonies with the same infection status were genetically more similar. A third Mantel test between the infection and environmental distance matrices ($N = 66$ colonies) was performed to determine whether *Las. neglectus* colonies with the same infection status occurred in more similar environmental conditions than random. To test if the infection of *Las. neglectus* colonies *Lab. formicarum* was associated with specific environmental conditions, we used a generalized linear model (GLM) with binomial link function (R package *stats*; $N = 66$ colonies). Because the five environmental variables compiled were not independent from each other (especially land cover variables that are mutually exclusive), we summarized the five environmental variables into artificial uncorrelated variables using a Principal Component Analysis ('dudi.pca' function in R package *ade4*). We then used the axes of the PCA as explanative variable in the binomial GLM.

Finally, considering infected colonies only, we tested if prevalence, expressed as the proportion of infected workers in the colony, was associated with environmental conditions using a GLM with quasibinomial link function and weighted by the log number of workers screened (R package *stats*; $N = 38$ colonies). For this

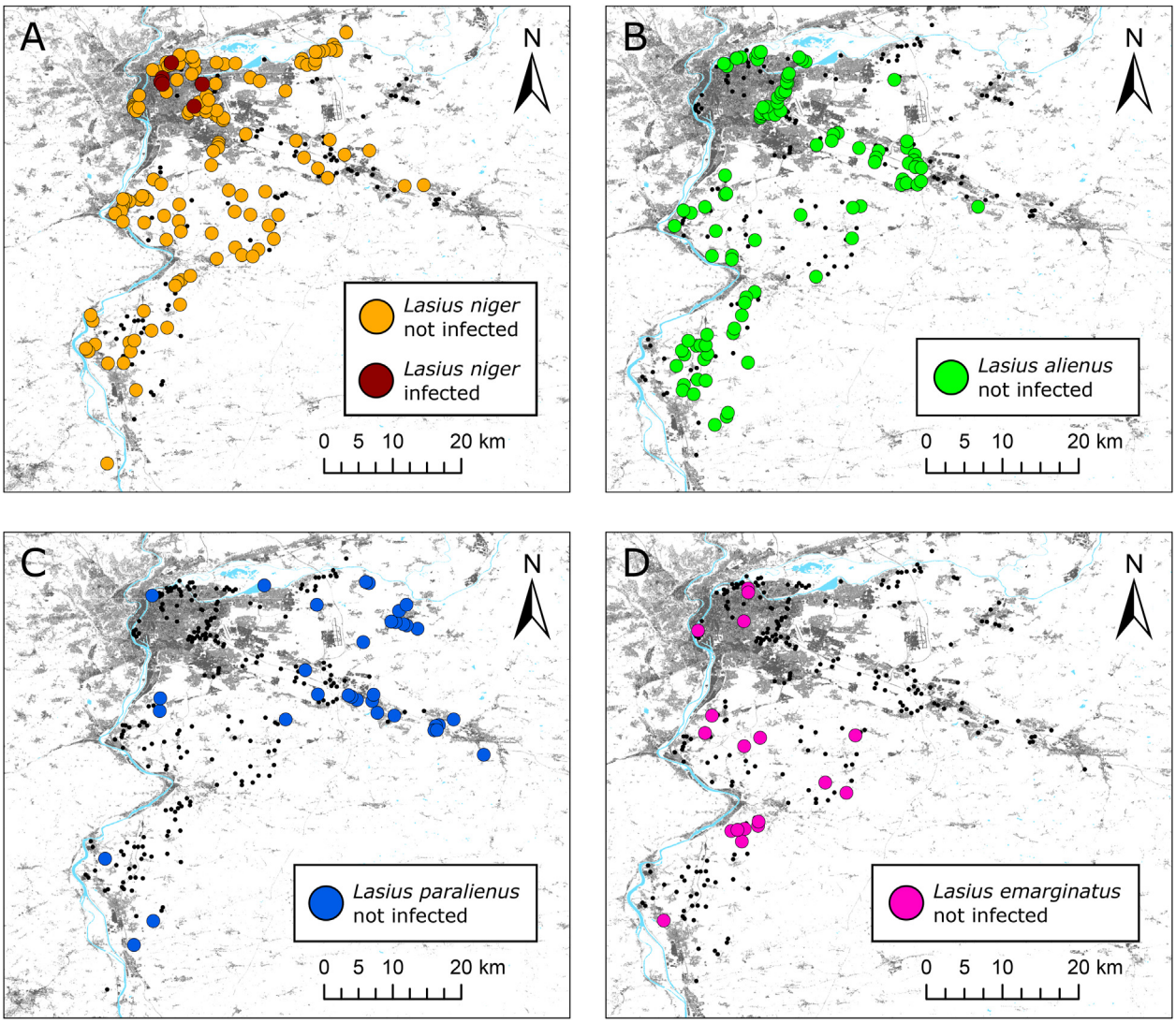

Fig. 2. Location of native *Lasius* colonies screened for *Lab. formicarum* presence. The fungus was only found in *L. niger*, in 11 colonies occurring at six different sampling locations (A-D). Small black dots represent sampling locations where the species was not found (or where not enough workers were sampled to perform screening).

GLM, we also summarized our five environmental variables using a PCA and used the PCA axes as explanatory variables.

The coefficients of determination (Nagelkerke's pseudo-R^2) of the models were estimated using the function 'r2_nagelkerke' from the *performance* package in R (Ludecke et al., 2019).

2.3. Local scale sampling and analyses

2.3.1. Measurement and sampling of infected Lasius neglectus colonies

To study if and how *Lab. formicarum* prevalence varied locally, within the extent of infected *Las. neglectus* colonies, we measured the surface area occupied by 16 colonies (out of the 38 infected colonies detected in the landscape; see Fig. 3) and sampled workers from several nests and trails within each colony (see section 2.3.2 for details). Colonies measurements were performed during spring and summer 2012 and 2013 by teams of two to five persons, and ants were detected by searching for trails and nest entrances visually. Workers were sampled every 20–40 m depending on land access, and each sample was georeferenced precisely. Colony boundaries were defined when no more *Las. neglectus* were found in a 50 m radius from the last location where *Las. neglectus* were detected. *Las. neglectus* occurrences were mapped with ArcGIS 10.1 (ESRI, Environmental Systems Research Institute, Redlands, 2012).

2.3.2. Laboulbenia formicarum prevalence within infected Lasius neglectus colonies

Depending on the extent of *Las. neglectus* colonies, 5 to 50 nests (or trails) were sampled (mean ± s.d. = 13 ± 10 samples by colony; total number of samples = 219). Samples contained between 6 and 106 workers (mean ± s.d. = 20 ± 11 workers by sample). A total of 4286 workers were screened. For each sample, workers were carefully examined under a stereomicroscope at 50× magnification and were considered infected if at least one *Lab. formicarum* thallus was observed on an ant's cuticle (Fig. 1). These samples were also used in the landscape-scale analyses (pooled by colony).

2.3.3. Local-scale environmental factors

To assess variations in land cover within the extent of colonies, satellite images were obtained for the June 1, 2012 from Google Earth Pro v7.3.2.5776, saved individually, and 5 m radius circles around the sampling points (i.e. nest or trail) were drawn. The

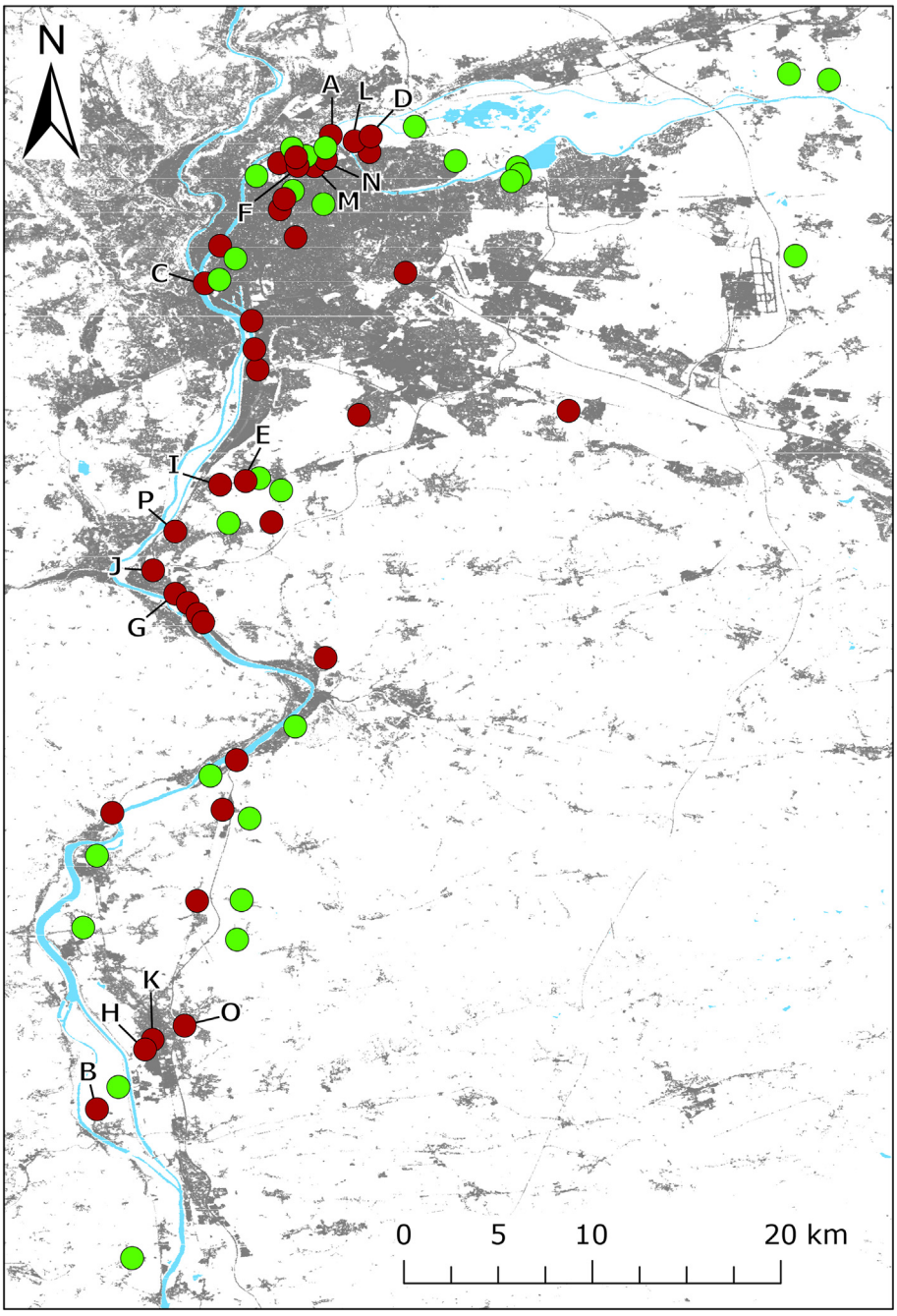

Fig. 3. Distribution of infected (red) and non-infected (green) colonies of the invasive ant *Lasius neglectus* in the study area. Map background shows waterways (blue) and urbanized areas (grey). Letters correspond to the 16 infected colonies presented in Fig. 4.

Table 1
Number of samples screened for the presence of formed thalli of *Laboulbenia formicarum* in colonies of the invasive ant *Lasius neglectus* and four native *Lasius* species occurring in the same study area. *Lab. formicarum* was found in the invasive species *Las. neglectus* and in the widespread native ant *Las. niger*.

Lasius species	Las. neglectus	Las. niger	Las. alienus	Las. paralienus	Las. emarginatus
Number of workers screened	9374	4306	2138	545	426
Number of colonies screened	66	230 (134 + 96)*	118 (94 + 24)*	39 (38 + 1)*	25 (18 + 7)*
Workers per colony (Mean ± s.d.)	142 ± 226	19 ± 6	18 ± 6	14 ± 4	17 ± 9
Number of colonies infected	38	11 (2 + 9)*	0	0	0
Percentage of colony infected (%)	57.6	4.8	0	0	0

* Number of colonies screened from each dataset (i.e., landscape-scale random sampling and *Las. neglectus* colonies measurements, respectively).

Table 2
Environmental variables used for landscape- and local-scale analyses.

Spatial scale	Environmental variable	Min - Max	Mean ± s.d.	Source
Landscape	Mean annual temperature (°C)	11.45–12.38	12.03 ± 0.22	www.worldclim.org
	Mean annual precipitations (mm)	805–914	829 ± 21	
	Elevation (m)	136–272	180 ± 29	www.geoportail.gouv.fr
	Solar radiation (kWh/m^2)	974 - 1156	1079 ± 24	
	Proportion vegetation	0–0.85	0.09 ± 0.18	European Settlement Map (2012) land.copernicus.eu
	Proportion urban area	0–0.86	0.38 ± 0.25	
	Proportion croplands	0.02–0.86	0.44 ± 0.22	Registre Parcellaire Graphique www.data.gouv.fr
Local	Proportion of tree cover	0–1	0.47 ± 0.37	Satellite images (2012)
	Proportion of open vegetation	0–1	0.24 ± 0.28	Google Earth Pro
	Proportion of unsealed ways	0–78	0.05 ± 0.14	
	Proportion of impervious surfaces	0–1	0.24 ± 0.33	

proportions of the circles corresponding to four different land cover types (tree cover, open vegetation, impervious surface and unsealed ways) were measured using ImageJ v1.52 (Schneider et al., 2012) (see Table 2 for more information on variables).

2.3.4. Statistical analyses

The effect of local land cover on the prevalence of *Lab. formicarum* was tested using a general linear mixed model (GLMM) with a binomial link function and colony identity as random effect (R package *lme4*; Bates et al., 2015). Land cover variables were summarized using a PCA, with the PCA axes used as explanatory variables. We determined the best-fitting model using a backward model selection procedure based on sequential one-term deletions using Chi-square tests ('drop1' function in R package *stats*; only additive models were considered) and a significance threshold of 0.05. The coefficient of determination (Nakagawa's pseudo-R^2) of the model was estimated using the function 'r2_nakagawa' from the performance package in R.

3. Results

3.1. Landscape-scale analyses

3.1.1. Presence of Lab. formicarum in Lasius neglectus and native Lasius species

The ectoparasitic fungus *Lab. formicarum* was detected in 58% (38 of 66) of *Las. neglectus* colonies (Figs. 3) and 5% (11 of 230) of *Las. niger* colonies screened (Table 1). The fungus was not detected in any of the other three *Lasius* species (Fig. 2). On a total of 230 colonies of *Las. niger* screened, 134 were sampled during the landscape-scale survey (i.e., among the 1248 randomly selected sampling locations) and 96 were sampled during the measurement of *Lasius neglectus* colonies (i.e., these colonies adjacent to infected *Las. neglectus* colonies). *Lab. formicarum* prevalence was significantly different between these two sets of colonies: 1.5% (2 colonies infected on 134 screened) for the first one and 9.4% (9 colonies infected on 96 screened) for the second one (Chi-square test: $\chi^2 = 3.87$, $P = 0.049$).

3.1.2. Landscape-scale variations in Laboulbenia formicarum presence and prevalence

3.1.2.1. Laboulbenia formicarum presence. There was no clear correlation between the infection status of *Las. neglectus* colonies and geographic distance (Mantel test: observed correlation = 0.04, P = 0.058) or genetic distance (Mantel test: observed correlation = 0.05, P = 0.12) between colonies. There was, however, a significant correlation between infection status and environmental distance (Mantel test: observed correlation = 0.08, P = 0.006), which indicates that ant colonies in similar environments were more likely to have the same infection status. A first PCA was performed with the seven landscape-scale environmental variables and all 66 *Las. neglectus* colonies. The first PCA axis explained 38.1% of the total variability and was associated with high mean annual temperature, low mean annual precipitation, low elevation and the absence of agricultural areas. The second PCA axis explained 26.7% of the total variability and was associated with high vegetation and low impervious cover (Fig. 4A). The probability of being infected by *Lab. formicarum* was positively correlated to the first axis of the PCA (Estimate = 0.38 ± 0.17, z = 2.2, P = 0.027; $R^2 = 0.11$; Fig. 4A), suggesting that ant colonies were more likely to be infected in areas characterized by high mean temperature, low mean precipitation, low elevation and low agricultural surfaces.

3.1.2.2. Laboulbenia formicarum prevalence. A second PCA was performed with the same seven landscape-scale environmental variables, but with the 38 infected *Las. neglectus* colonies only. The first PCA axis explained 34.2% of the total variability and was associated with high mean annual temperature, low mean annual precipitation, low elevation and a small proportion of agricultural areas. The proportion of infected workers was negatively associated with the first PCA axis, although not significantly (Estimate = −0.36 ± 0.19, z = −1.9, P = 0.06). The second PCA axis explained 27.9% of the total variability and was associated with high impervious and low vegetation covers (Fig. 4B). The proportion of infected workers was positively associated with the second PCA axis (Estimate = 0.58 ± 0.17, z = 3.4, P = 0.002; $R^2 = 0.55$; Fig. 4B), indicating that the prevalence of the fungus was positively associated with urbanization.

3.2. Local-scale analyses

A PCA was performed with the five local environmental variables and all 219 *Las. neglectus* nests (or trails) sampled across the 16 infected colonies measured. The first PCA axis explained 43.5% of the total variability and opposed high tree cover to open areas (i.e. impervious and open vegetation). The second PCA axis explained 31.8% of the total variability and opposed open vegetation and impervious surfaces (Fig. S1). In infected colonies, the proportion of infected workers in nests could vary from 0 to 100% within a few meters (Fig. 5) and was negatively associated with the second axis of the PCA (Estimate = −0.26 ± 0.04, z = −7.1, P < 0.0001) suggesting that prevalence was negatively associated with ground imperviousness (Fig. S1). However, the proportion of variance explained by this variable was very low (marginal $R^2 = 0.012$) as most of the explained variation was linked to colony identity (i.e. the random factor of the mixed model; conditional $R^2 = 0.55$).

4. Discussion

We screened over 16,500 individual *Lasius* ants from 478 colonies to detect the ectoparasitic fungus *Lab. formicarum* and understand how local and landscape-scale environmental conditions

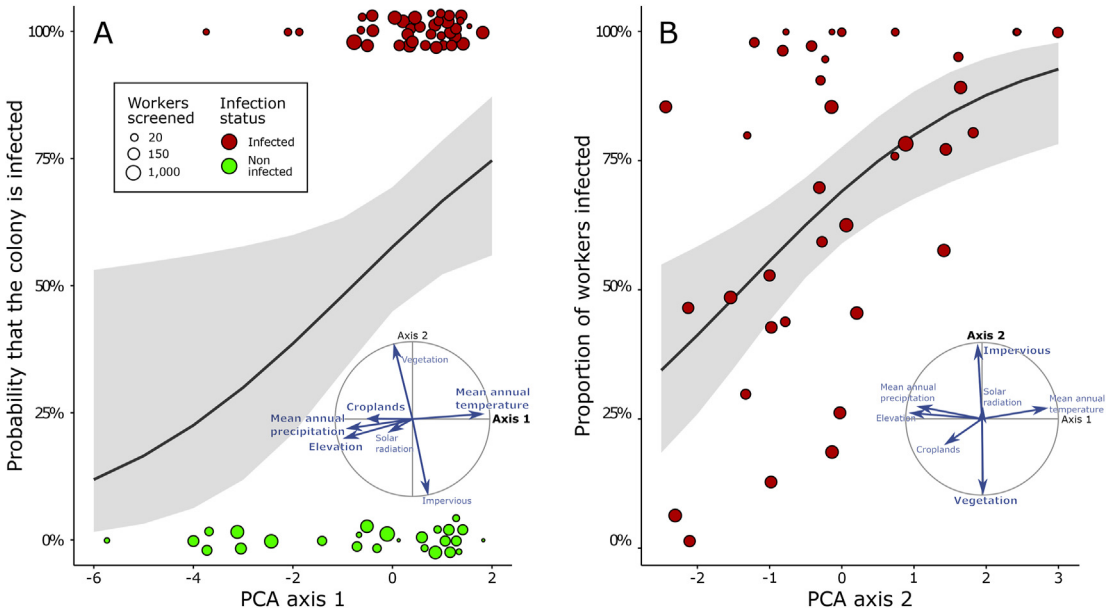

Fig. 4. (A) Relationship (mean ± 95% CI) between environmental conditions (PCA axis 1) and the probability of *Las. neglectus* colonies to be infected by *Lab. formicarum* ($N = 66$ colonies, observed values are 0 or 100% but slightly shifted for visual purpose). As illustrated by the bottom-right circle of correlations, the first PCA axis represents a gradient of temperature and precipitation. (B) Relationship (mean ± 95% CI) between environmental conditions (PCA axis 2) and *Lab. formicarum* prevalence in infected *Las. neglectus* colonies ($N = 38$ colonies). As illustrated by the bottom-right circle of correlations, the second PCA axis represents a gradient of urbanization as it is negatively correlated with the proportion of vegetated areas and positively correlated to the proportion of impervious surfaces (e.g., buildings, roads).

affect its distribution. The fungus was present but uncommon in colonies of *Lasius niger*, absent in four other native *Lasius* species, and common in the nests of the invasive ant *Las. neglectus*. At the scale of the landscape, the presence of *Lab. formicarum* in *Las. neglectus* colonies was positively associated with low elevation, the absence of agriculture and dry and warm environments. Its prevalence in infected colonies was positively associated with urbanization. The prevalence of the fungus also varied spatially at the scale of the colony and was negatively linked to impervious surfaces.

We did not detect *Lab. formicarum* in colonies of *Lasius emarginatus*, *Las. alienus* and *Las. paralienus* and the fungus was present in only 5% of the sampled colonies of *Lasius niger* (11 colonies infected among 230 screened). However, nine out of these eleven infected colonies were near infected *Las. neglectus* colonies. The prevalence of *Lab. formicarum* in *Las. niger* is six times higher when the species occurs near infected *Las. neglectus* (9.4% versus 1.5% when randomly sampled in the landscape). Laboratory experiments have shown that infected *Las. neglectus* can transmit *Lab. formicarum* to *Las. niger* (Tragust et al., 2015). Our findings suggest that cross-species transmission occurs between these two species in natural settings and that *Las. neglectus* might constitute a reservoir for *Lab. formicarum* to spill over the native species *Las. niger*.

Fifty-eight percent of *Las. neglectus* colonies were infected by the fungus (38 out of 66 colonies, Table 1). This was higher than expected from the literature, as *Lab. formicarum* had only been reported in four colonies of *Las. neglectus* in Europe, despite extensive sampling and monitoring (Herraiz and Espadaler, 2007; Espadaler et al., 2011; Espadaler and Bernal, 2020). The most extensive study to date screened nearly 5000 workers from 21 Hungarian *Las. neglectus* colonies without detecting *Lab. formicarum* (Tartally and Báthori, 2015). These results are consistent with the hypothesis of a recent introduction of *Lab. formicarum* in Western Europe (Espadaler and Santamaria, 2003). However, we cannot exclude that *Lab. formicarum* may be a native and widespread, albeit not abundant parasite of European ants. Among the randomly sampled *Las. niger* colonies, 1.5% were infected by *Lab. Formicarum*, which is in line with infection rates found in two native ant-parasitic Laboulbeniales in Europe (Báthori et al., 2014, 2015). *Lasius niger* is an extremely abundant ant species (Gippet et al., 2017) and a suitable host to *Lab. formicarum*. *Laboulbenia formicarum* could thus be a native parasite that regularly jumps from native ant species to invasive *Las. neglectus* colonies. This scenario was described in the Laboulbeniales fungus *Hesperomyces virescens*, a parasite that occurs at low prevalence in native ladybirds but that is common in the invasive ladybird *Harmonia axydris* (Ceryngier and Twardowska, 2013). Similarly, *Las. neglectus* may be a natural host for *Lab. formicarum*, both of them possibly co-introduced in some areas across Europe. Establishing the genetic profiles of North American and European populations of *Lab. formicarum* might help understand the origin and colonization history of the fungus (Haelewaters et al., 2015a).

We found no clear evidence that the geographic proximity between *Las. neglectus* colonies was associated with infection status. We expected, under a horizontal transmission scenario, that geographically closer colonies would have more similar infection status. For example, sexual transmission could occur if spores or thalli are dispersed by reproductive ant individuals, although young reproductive female and male ants do not appear to bear Laboulbeniales thalli (Haelewaters et al., 2015b). Sexual transmission is also unlikely in *Las. neglectus*, because this species rarely or never performs nuptial flights: females seem to mate with males from the same colony (Espadaler et al., 2007; although we witnessed males taking off from an infected colony, see Fig. 1). Cross-infection between spatially close *Las. neglectus* supercolonies cannot be ruled out in the very rare cases where separate colonies are not kilometres apart (Fig. 3); only one such instance is known to us, where two genetically distinct colonies are separated by a broad boulevard. Similarly, it has also been suggested that horizontal transmission may occur via ant-associated 'myrmecophilous' invertebrates (Santamaria and Espadaler, 2015), but again, *Las. neglectus* colonies are too distant to make such events likely.

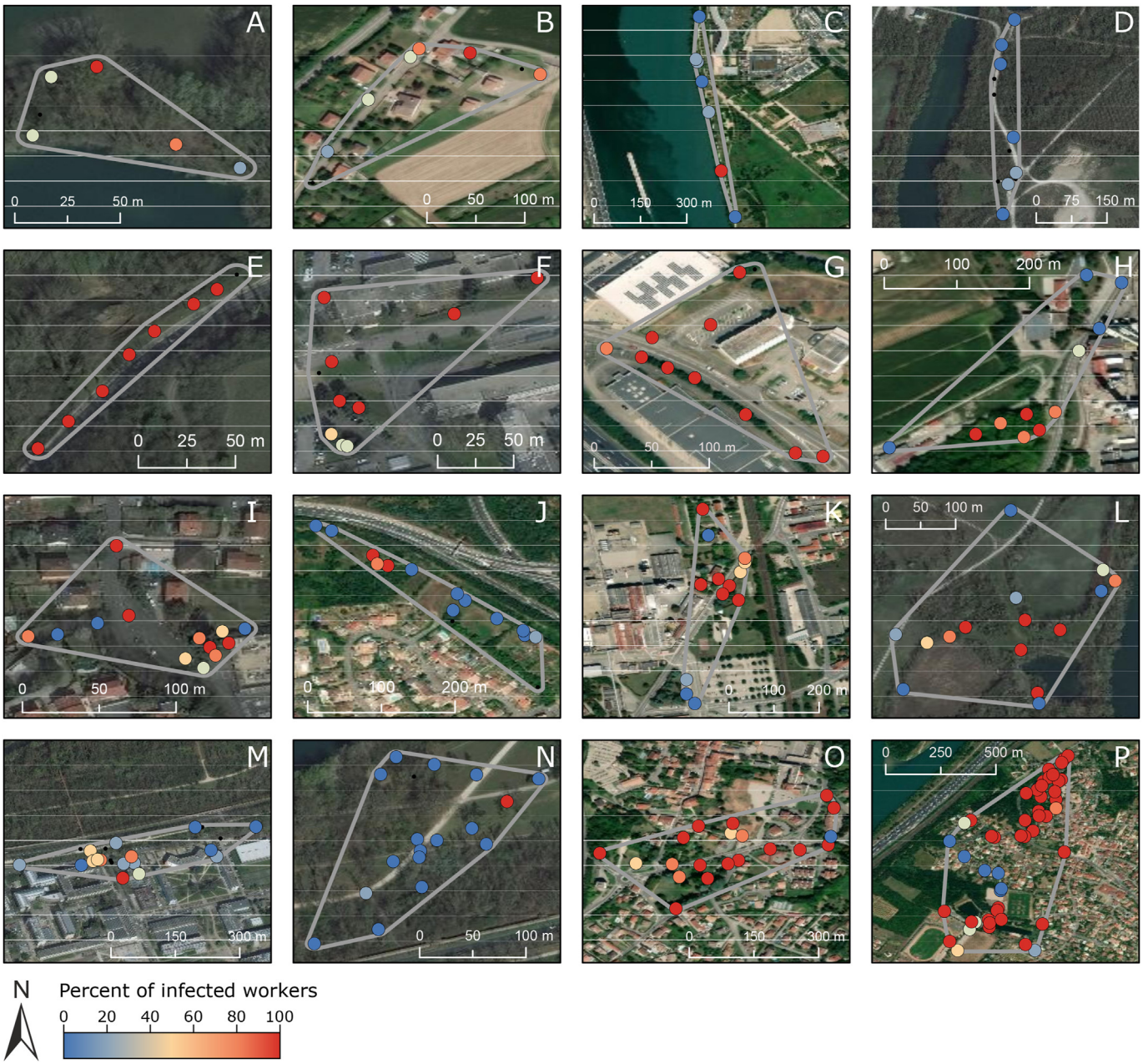

Fig. 5. Intra-colonial survey of *Lab. formicarum* prevalence. Each panel represents an infected *Las. neglectus* colony (see Fig. 2 for their respective position in the landscape). Each coloured dot represents a sample for which workers were screened for *Lab. formicarum* presence. Dots' colour indicates the percentage of infected workers. Grey line indicates the colony boundary. Background map is a satellite image from Esri, Digital globe.

We expected vertical transmission to explain the distribution of *Lab. formicarum*, but there was no evidence that genetic proximity between *Las. neglectus* colonies was associated with infection status. This surprising result suggests the vertical transmission of *Lab. formicarum* is not systematic. Vertical transmission may be uncommon if human-mediated dispersal is detrimental to *Lab. formicarum* (Gippet et al., 2019). Humans may also propagate uninfected portions of infected colonies because the intra-colonial prevalence of *Lab. formicarum* is extremely variable (Fig. 5). Finally, *Lab. formicarum* may disappear over time if the environmental conditions at the place of introduction are not favourable (*Las. neglectus* colonies kept in laboratory conditions lose the fungus in a few months; S. Tragust, *unpublished data*). Altogether, these results question the importance of both horizontal and vertical transmission and suggest that environmental limitation is a stronger determinant of *Lab. formicarum* distribution.

Our landscape-scale analysis showed *Lab. formicarum* presence was associated with warmer and dryer climatic conditions in low elevation areas. It has been hypothesized that humidity should favour *Rickia wasmanni*, a fungus that parasitizes *Myrmica* ants, because these ants live in moist environments (Santamaria and Espadaler, 2015). However, Haelewaters et al. (2015b) found no such trends across three distinct habitats, Markó et al. (2016) found no difference in fungal prevalence within *Myrmica* colonies from dry and humid sites and Szentiványi et al. (2019) found that *Rickia wasmanni* was more common in colder and dryer areas (Szentiványi et al., 2019). In addition, the most recently described ant-associated Laboulbeniales, *Rickia lenoirii*, was described from

ants in the genus *Messor*, which live in dry or arid habitats (Santamaria and Espadaler, 2015). Together with the literature, our results suggest ant-associated Laboulbeniales prefer warm and dry climates.

Fungal prevalence was also negatively associated with agriculture (Fig. 4A, first PCA axis). High concentrations of fungicides are commonly found in the soil and water surrounding crops (Zubrod et al., 2019). Laboulbeniales, including species associated with ants, are sensitive to fungicides (Gemeno et al., 2004; Pfliegler et al., 2016; but see Pech and Heneberg, 2015), and the contamination of agricultural areas by fungicides leaching into the environment may explain why *Las. neglectus* colonies located near crops were less infected by *Lab. formicarum*.

When focusing only on infected *Las. neglectus* colonies ($N = 38$) we found that urbanization was positively associated with fungal prevalence (Fig. 4B). A similar association between urbanization and Laboulbeniales prevalence was reported in *Hesperomyces virescens* parasitizing native ladybirds in the UK (Welch et al., 2001). It was suggested that urbanization increased the overlapping time of successive ladybug generations, increasing the probability that new fungus-free cohorts would mate with older infected individuals (Welch et al., 2001; Knell and Webberley, 2004). This mechanism could not explain our observations in ants because new workers are produced all year long (often with pulses of production in spring and fall and a diapause in winter; Hölldobler and Wilson, 1990). Environmental changes associated with urbanization, like increased heat or pollution (Grimm et al., 2008), may benefit the fungus, either directly by changing local environmental conditions or indirectly by altering *Las. neglectus* immunity or behaviour (Youngsteadt et al., 2015).

Finally, we found that the prevalence of *Lab. formicarum* was highly variable within colonies (from 0 to 100% within meters). It was negatively associated with impervious surfaces such as roads and buildings (Fig. S1). This correlation was weak, and differed from landscape-scale analysis, suggesting a scale-dependent relationship between Laboulbeniales prevalence and environmental conditions. The prevalence of ant-associated Laboulbeniales may also vary with time. At the individual level, the number of thalli of the Laboulbeniales *Rickia wasmanni* increases with the age of its hosts (i.e. *Myrmica scabrinodis* ants; Báthori et al., 2018). The high variability in numbers of Laboulbeniales thalli we observed within ant colonies may result from a heterogeneous spatial distribution of age cohorts within the nest. Such spatial age structures may originate in large ant colonies because ants gather and move their brood to optimize development (generally hot and dry places for pupae; Hölldobler and Wilson, 1990). The prevalence of *Lab. formicarum* also increases with time in *Las. neglectus* colonies (Tragust et al., 2015). Temporal fluctuations in the ectoparasite prevalence may complexify the relationship between Laboulbeniales and their hosts. Repeated sampling may be needed to further our understanding of ant-Laboulbeniales interactions (Haelewaters et al., 2015b).

Overall, our results show that environmental conditions and land use play an important role in shaping the distribution of ant-associated Laboulbeniales. Improving our understanding of this role might help predict current and future distribution of fungal parasites in a changing world. This knowledge will be crucial to protect endangered or important flora and fauna from threatening fungal parasites, and to control pests and invasive species.

Authors contribution

JMWG, TC, ST and BK designed the study. JMWG, TC, and BK morphologically identified the ants. AD performed DNA extraction and genotyping and BK processed microsatellite raw data. FW, MH, TC and JMWG screened the ants for *Lab. formicarum*. JMWG, JG and BK measured *Las. neglectus* colonies in the field. JMWG and TC processed the data. JMWG performed statistical analyses. JMWG, TC, ST, NM and BK wrote the first draft of the manuscript and all co-authors participated in improving the subsequent versions.

Availability of data and materials

Datasets supporting the conclusions of this article are included in Supplementary Material and Methods, Table S1, Fig. S1 and supplementary datasets (available at: https://github.com/JGippet/Datasets—Gippet-et-al.-2021—Fungal-Ecology).

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2021.101045.

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