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The introduced oak *Quercus rubra* and acorn-associated arthropods in Europe: An opportunity for both carpophagous insects and their ant predators

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Abstract

1. We studied the interactions involving oak acorns and associated arthropods focusing on the role of the introduced *Quercus rubra* in Italy.
2. We conducted our study in areas where native oaks coexist with *Q. rubra*. We carried out a survey on the arthropods inhabiting fallen acorns and a colonisation experiment. Furthermore, we conducted laboratory experiments documenting the interactions among the most abundant arthropod species on *Q. rubra*.
3. Acorns were colonised by ant species and carpophagous insects showing different preferences concerning acorns' position within the leaf litter and oak species. Acorns of *Q. rubra* were colonised frequently by ants (mostly *Temnothorax nylanderi*) and *Cydia fagiglandana*. *Cydia splendana* preferred native acorns, while *Curculio* beetles were ubiquitous. Our data suggest that ants occupy exclusively acorns previously attacked by *Cydia*, killing or displacing their caterpillars if still developing inside the acorns. Vertebrates may prefer to feed on *Q. rubra* acorns over native species.
4. *Quercus rubra* has a meaningful influence on native arthropods associated with oak acorns. The ecological consequences of this interaction are still not fully clear, yet our data suggest an increase in acorn-consuming insects, partly affecting the acorns of neighbouring native oaks, but also an increase of predatory ants.

KEYWORDS
acorn ants, alien species, ant-ecology, ants' behaviour, carpophagous insects, multitrophic interactions

INTRODUCTION

Oaks (Fagaceae: *Quercus* L., 1753) are among the most landscape-defining, dominant tree species in European and Mediterranean forests. One of the most iconic features of oaks is their abundant production of large seeds, protected within nuts known as acorns. They represent a significant trophic resource for a diverse range of vertebrates and insects that includes beetles, birds, moths, rodents, and ungulates (e.g. Bieberich, 2016; Bonal et al., 2007; Cornelis et al., 1999; Focardi et al., 2000; Jackson, 1980; Jimenez-Pino et al., 2011; Myczko et al., 2014, 2017 ; Ouden et al., 2005; Steele et al., 1996; Tixier et al., 1997). Vertebrates also play a crucial role in the dispersal of the acorns (e.g., Bieberich, 2016; Myczko et al., 2014). In addition to invertebrates that consume acorns as food, other

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animals may seek shelter within cavities inside the acorns. Ants are notorious for this role as they may entirely rebuild the acorns internal architecture to use them as nests (Herbers & Johnson, 2007; Karlik et al., 2016; Mitrus, 2019; Myczko et al., 2018; Seifert, 2017, 2018; Varoudis et al., 2018), as it happens with galls (Giannetti et al., 2019, 2021). A few species from the large, mostly Holarctic genus *Temnothorax* nest within acorns particularly frequently, so that all *Temnothorax* spp. are sometimes referred to as "acorn ants" despite many having a completely different ecology (Foitzik et al., 2004; Herbers & Johnson, 2007; Myczko et al., 2018; Prebus, 2017; Seifert, 2017, 2018). In fact, contrary to several other myrmicine ants that form very populous colonies and large or even permanent foraging trails, *Temnothorax* ants live in small societies and forage solitarily or in small groups, occasionally forming inconspicuous trails of just a few workers (Hölldobler & Wilson, 1990; Grasso et al., 1998, 1999; Steiner et al., 2011; Seifert, 2018). Despite such inconspicuousness, their impact in agroforestry systems may be relevant, although the ecological consequences of acorn colonisation by ants are rarely well-documented (Prebus, 2017; Seifert, 2018).

Quercus rubra L., also known as the northern red oak, is an eastern North American oak species that was introduced in Europe at the end of the 17th century, first as an ornamental species and then for reforestation and timber production (Magni, 2004; Merceron et al., 2017). During the 19th century, these plants were used in parks and gardens all over Europe, except for Scandinavia (Goeze, 1916; Nicolescu et al., 2020). Today, *Q. rubra* is widespread across the continent, covering about 350,000 ha and it is considered invasive in at least some European countries, while at the same time being still appreciated for silvicultural purposes (Henin & Vandekerkhove, 2016; Lavnyy & Savchyn, 2016; Major et al., 2013; Nicolescu et al., 2020; Van Loo et al., 2016; Woziwoda et al., 2014). Moreover, it appears one of the European tree species likely to benefit from future climate change scenarios, as it is predicted to possibly further expand its populations at the expense of native vegetation (Dyderski et al., 2018). Some studies have shown an impact of *Q. rubra* on native vegetation structure and soil properties (Chmura, 2013; Marozas et al., 2009; Stanek et al., 2020). On the other hand, the interactions between *Q. rubra* and animals interested in acorns were documented in Europe quite recently from Germany and Poland (Bieberich, 2016; Myczko et al., 2014, 2017, 2018). In particular, the European Jay *Garrulus glandarius* (Linnaeus, 1758) (Passeriformes, Corvidae) shows a strong preference for acorns of the native oak species over those of *Q. rubra*, whose dispersal may be restricted to shorter ranges as it is instead mostly mediated by rodents (Bieberich, 2016; Myczko et al., 2014; Wróbel & Zwolak, 2019). However, rodents too may tend to favour native oaks rather than *Q. rubra* (Bogdziewicz et al., 2019, 2020). Compared to those of native oaks, *Q. rubra* acorns were found to be less attractive to larvae of the tortrix moth *Cydia fagiglandana* (Zeller, 1841) (Lepidoptera, Tortricidae), while equally attractive to *Cydia amplana* (Hübner, 1799) and *C. splendana* (Hübner, 1799), and from almost completely unattractive to *Curculio* spp. weevils (Coleoptera, Curculionidae) to attractive but unfeasible to their development (Bogdziewicz, Bonal, et al., 2018; Bogdziewicz, Marino, et al., 2018; Myczko et al., 2017). Bogdziewicz, Bonal, et al. (2018) found that in the pre-dispersal stage, *Q. rubra* acorns in Europe were very unlikely to be predated by carpophagous insects compared to those of native European oaks, and compared to *Q. rubra* acorns in their native North American range. Interestingly, *Q. rubra* acorns reach maturity in 2 years while those of native oaks do so in the same season of flowering. As a result, the asynchronous acorn production in a forest hosting both *Q. rubra* and native oaks can be a stabilising resource for *Cydia* spp. and *Curculio* spp. populations, susceptible to the significant yearly variation of acorn availability (Fukumoto & Kajimura, 2011; Myczko et al., 2017). At the same time, larval development for these species is longer on *Q. rubra* acorns, which could mean a higher risk of predation by acorn-consuming vertebrates (Myczko et al., 2017). As far as ants are concerned, *Q. rubra* appears to significantly favour *Temnothorax crassispinus* (Karavaiev, 1926) in Eastern Europe, as these ants have a marked preference for *Q. rubra* acorns as nest cavities (Myczko et al., 2018).

Here we report the results of a first field survey and experimental evaluation on the arthropod fauna associated with fallen acorns of *Q. rubra* and of the native oaks *Quercus petraea* (Matt.) Liebl. and *Quercus cerris* L. in Italy. In particular, we aimed to obtain a qualitative and quantitative assessment of the carpophagous and predatory arthropods colonising fallen acorns in the oak forest litter and to document their variation in relation to the presence of *Q. rubra* in Southern Europe for the first time. Our investigation was carried through a field survey in which naturally present acorns were collected and examined, and an experiment in which acorns were artificially placed and examined a year later. In all cases, we aimed to identify and quantify the main acorn-colonising arthropods and to verify their preferences for acorns of different native oaks compared to those of *Q. rubra*, as well as the influence of acorn depth within the leaf litter. Under laboratory conditions, we also evaluated how acorn moisture could influence their colonisation by ants. Furthermore, we conducted laboratory experiments to evaluate how predatory arthropods of this system, represented by acorn-colonising ants, may interact with acorn-consuming insects. To this aim, we tested the existence of: (i) direct interaction consisting in predation or displacement between the two groups; (ii) indirect interactions based on the hypothesis that acorn colonisation by ants could be prevalently secondary, depending on previous opening and consumption of the acorn by carpophagous insect species.

MATERIALS AND METHODS

Study area

We selected two areas in oak forests near the village of Fornoli (Tuscany, Italy) which were used for both the field survey and the acorn colonisation experiment as well as the source of the arthropods later used in the laboratory experiments. The selected areas were the following:

- Area A populated by native *Q. cerris* oaks and introduced *Q. rubra* (44.2585, 9.9705, 135 m a.s.l.), comprising 20 plots (Figure 1a).
- Area B populated by the native oak trees *Q. cerris* and *Q. petraea* (44.2565, 9.9696, 165 m a.s.l.), comprising 20 plots (Figure 1b).

Each plot was a circular area on the ground (Ø = 2 m) between two oak trees of different species which were distant at least 2 m one from the other (measured by laser distance meter Leica Disto D2), containing acorns of both trees. Within each plot, no other trees or bushes occurred.

Arthropods identification

All specimens collected in the study area (either during the field survey or acorn colonisation experiment, but also those used in following laboratory experiments) were examined under a ZEISS Stemi 508 stereoscopic microscope (5–200× magnification range) with the support of an Axiocam Erc 5 s and ZEISS ZEN core software used to take morphometric measurements. Ants were identified according to the keys provided by Seifert (2018). Tortrix moth caterpillars (*Cydia* spp.) were identified according to Brown and Komai (2008), Villagrán et al. (2000), and Myczko et al. (2017). Moreover, beetles were identified according to the description provided by Conedera et al. (2005).

Field survey

We first carried out a field survey to identify and quantify the acorn-colonising arthropod species in our study area, and to understand their preference (if any) for acorns of certain oak species or for acorns positioned at a certain depth within the leaf litter. In September 2019, acorns were collected from plots located in both areas A and B. A total of 14–16 acorns were collected per plot, equally divided between the two oak species. As a result, 300 *Q. cerris* acorns and 300 *Q. petraea* acorns were collected from area B, while 300 *Q. cerris* acorns and 300 *Q. rubra* acorns were collected from area A. According to their position within the leaf litter, these acorns were then divided into three categories: (i) acorns found on top of the leaf litter (P1), (ii) acorns found in the middle of the leaf litter (covered by at least 5 cm of litter above, and at a distance of at least 5 cm from the soil layer) (P2), (iii) acorns found at the bottom of the leaf litter layer, directly in contact with the soil (P3). Finally, in the laboratory, they were checked for the eventual presence of holes, and arthropods inside.

Acorn colonisation (field experiment)

We carried out a field experiment on acorn colonisation with similar goals to those of the field survey, but this time limiting to 1 year the time available to any arthropod to find and colonise the acorns whose content we examined. In addition, during this experiment, acorns already fallen from trees were placed on the leaf litter, which meant they were unlikely to attract arthropod that start colonise younger acorns still on the trees.

A total of 960 acorns (480 of *Q. cerris*, 240 of *Q. rubra*, and 240 of *Q. petraea*) were selected from a larger set retrieved from the leaf litter of areas A and B during September 2019 (and examined in the field survey): only acorns without any trace of structural damage or mould were chosen. On each acorn, we then created a small hole (2 × 1 mm) on the lateral side (between the cupule and the style remains, see Figure 2) and applied a serial number for subsequent recognition.

In October 2019, we placed acorns within the plots of areas A (*Q. cerris*, n = 240; *Q. petraea*, n = 240) and B (*Q. cerris*, n = 240; *Q. rubra*, n = 240). Each plot received 12 acorns, equally divided between the two species, and placed at a distance of 10 cm one from another. These acorns were placed at different positions within the leaf litter, being equally divided between the three P1, P2, and P3

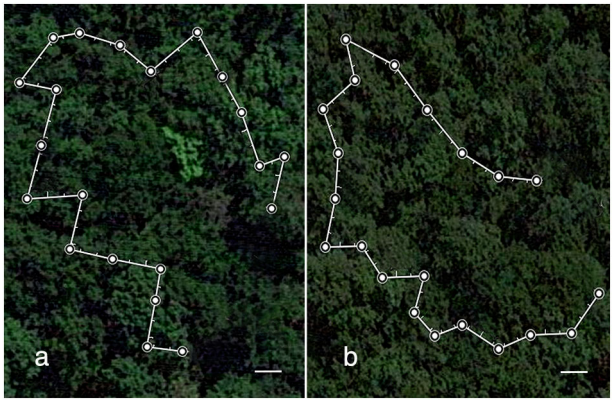

FIGURE 1 Area A, populated by native *Q. cerris* and introduced *Q. rubra* oaks (a); Area B, populated by the native oaks *Q. cerris* and *Q. petraea* (b). Dots indicate oaks, while circular plots were delimited in the areas crossed by the segments between two dots. Scale bars: 2 m

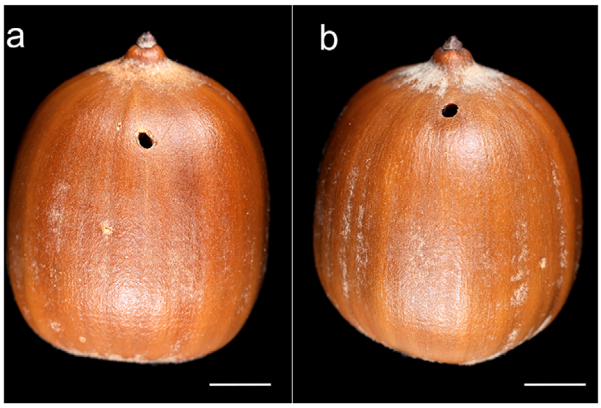

FIGURE 2 Acorns of *Q. rubra*: In (a), an artificial hole, in (b), and hole created by *C. fagiglandana* (b). Scale bars: 5 mm

categories illustrated in the field survey section above (n = 320 for each category). One year later (October, 2020) the plots were checked again, the experimental acorns were collected and checked for eventual presence of arthropods inside.

Setup for laboratory experiments

Five laboratory experiments were conducted with the same setup and only small variations, and were aimed to study (i) the effects of moisture over acorn colonisation by ants; (ii) the interactions between ants and carpophagous insects. Before describing each of these experiment in detail, first we provide here a description of their common characteristics.

All experiments were conducted during November 2020. The experimental apparatus consisted of two cylindrical arenas (Ø: 56.4 mm; H: 78.2 mm) connected by a horizontal tube (Ø: 14.9 mm; L: 65.5 mm). An ant colony was always inserted in one of the two arenas alongside 5 g of wood debris as shelter (unless in control groups without ants), while the other (acorn arena) hosted one or two acorns depending on the experiment. In each experiment, the horizontal tube was kept blocked with hydrophilic cotton for 2 h after ants were introduced in their arena, in order to facilitate their establishment in the area. All experiments started with the hydrophilic cotton being removed, granting ants access to the acorn arena.

In all laboratory experiments we used *Temnothorax nylanderi* (Foerster, 1850) as the model ant species due to its high abundance as acorn-colonising ant in the study area, and *Q. rubra* acorns. Experimental colonies and acorns were selected among those collected during the field survey and field experiment and maintained in the laboratory at 24°C. We selected only ant colonies containing a queen, brood and at least 40 workers and acorns without physical damage or mould and homogenous in size.

Effects of acorn moisture on ant colonisation (laboratory experiment)

We used 20 ant colonies and a set of 40 acorns, on which small holes (2 × 1 mm) were created on the lateral side (see the acorn colonisation experiment). These acorns were divided into two groups (20 acorns for each group), and each group was exposed to different humidity conditions in the laboratory, obtaining acorns with relative humidity (RH) = 18.5%–22.9% in one group (dry group) and RH = 29.1%–33.6% in the other (moist group) (Data S1). These two RH ranges were decided based on preliminary measurements conducted on the acorns retrieved during the field survey. In each trial, two acorns, one from the dry group and one from the moist group, were placed in the acorn arena, at a 5 cm distance from one another and both equally distant from the attachment of the connecting tube. The trial lasted 24 h, after which and the acorns were examined to evaluate ant colonisation. An acorn was considered colonised if it contained at least 60% of the ant worker population and the queen.

Effects of tortrix moths on acorn colonisation by ants and predation on the caterpillars (laboratory experiments)

We conducted four different experiments to evaluate the interactions between the tortrix moths (at the caterpillar stage) and the ants in different contexts. Experiments **A** and **B** were carried out to evaluate ant ability to enter acorns and the influence of tortrix moth caterpillars on this ability; experiment **C** was aimed to evaluate ant ability to enter acorns not previously modified by caterpillars; experiment **D** was aimed to evaluate the predatory ability of ants on caterpillars of different sizes.

Experiment A. We used 20 ant colonies (meeting the same requirements as in the previous experiment) and 60 acorns. We selected comparable sized acorns with similar conditions of humidity (mean RH = 20%, min-max: 19.2%–23.6%) (Appendix S1). Moreover, all selected presented a hole created by *C. fagiglandana* tortrix moth which was at least partly sealed with silk, indicating the presence of the caterpillar inside. They were divided into two groups (n = 30 each) according to two conditions observed during field surveys: in one group, the hole was completely sealed (CS) with silk (forming a uniformly tightly meshed wall), while sealing was incomplete (IS) in the acorns of the other group (forming an imperfect wall with holes of about 0.12–0.33 mm radius) (Figure 3). Each experimental apparatus contained both a CS and an IS acorn: ants were given access to 20 acorn arenas, while the remaining 10 were used for control experiments in which no ants were present. We checked whether ants entered or not into the acorns: to do so, since the beginning of the trials, we performed 10-min scan samplings every 30 min during 6 h and a final one 24 h after the start. The experiments ended after 24 h, and then all acorns were examined to check the status of the caterpillars, which were considered dead if they did not move after being touched with an entomological forceps.

Experiment B. The experimental procedure was the same as in experiment **A**, but in this case, we tested 32 acorns (16 IS and 16 without any silk coverage) (Figure 3; Data S1) and 16 ant colonies.

Experiment C. Single intact acorns (without any hole previously excavated by caterpillars) (n = 30) were offered to the ant colonies (n = 30) (Data S1). After 5 days, we checked whether ants had entered into the acorns or not.

Experiment D. We used 30 ant colonies and 60 acorns (Data S1) with a hole caused by *C. fagiglandana*, and 60 caterpillars equally

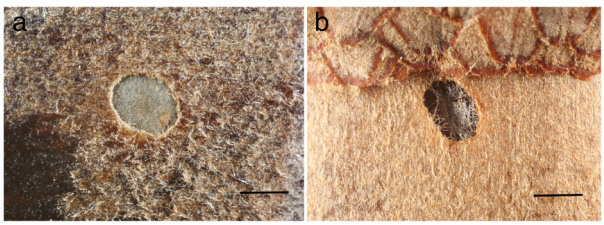

FIGURE 3 Holes created by *C. fagiglandana* on *Q. rubra* acorns. Hole completely closed by silk threads – CS (a) and hole only partially closed – IS (b). Scale bars: 1 mm

divided into three groups according to their total body length (size range 1: 3.1–4.6 mm; size range 2: 7.6–11.3 mm; size range 3: 15.3–20.0 mm). The acorns were cut into two halves and a caterpillar was placed inside each, then the two halves were re-attached back together using plasticine. Ants were given access to half of the acorns, while the remaining half constituted the control group. The caterpillars of each of the size ranges were equally divided between the two groups of acorns. Since the trials started, 10-min scan samplings were performed after 1, 2, 3, 4, and 24 h. During each scan sampling, we assessed whether the caterpillar was being attacked, and whether it was still alive or not. Caterpillars were considered dead if they did not move after being touched with entomological forceps.

Statistical analyses

Statistical analyses were performed by using the software IBM SPSS 27 and R 4.0.3 (R Core Team, 2021). Chi-squared tests, followed by the analysis of standardised residuals whenever useful, were used to compare the frequency of acorn colonisation under different conditions and by different arthropod groups. Kaplan–Meier tests followed by pairwise log-rank comparisons were conducted to analyse differences between the survival ability of tortrix moth caterpillars of different size ranges.

RESULTS

Field survey

In total, 702 of the acorns collected in September 2019 were colonised by arthropods (58%), while the remaining 498 (42%) were not. Colonised acorns were occupied by the ant species *Crematogaster sordidula* (Nylander, 1849) (n = 6), *Myrmecina graminicola* (Latreille, 1802) (n = 6), *Temnothorax nylanderi* (n = 80), *T. parvulus* (Schenck, 1852) (n = 29) and *T. unifasciatus* (Latreille, 1798) (n = 9), by the curculionid beetles *Curculio* spp. (n = 287) and by the tortrix moths *C. fagiglandana* (n = 85) and *C. splendana* (n = 206) (Data S2).

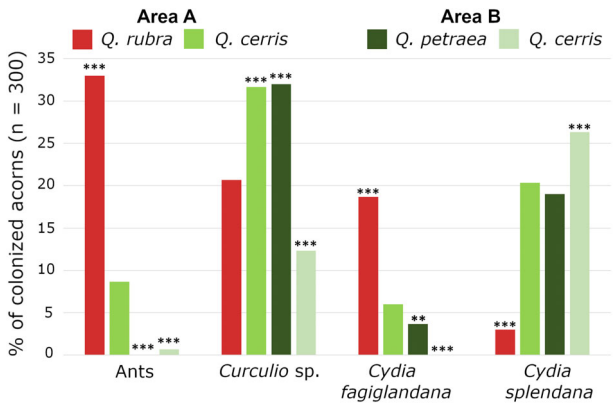

FIGURE 4 Colonisation of acorns in relation to their oak group (field survey data). Asterisks above the bars indicate significant differences from the expected according to the analysis of standardised residuals (* if $p < 0.05$; ** if $p < 0.01$; *** if $p < 0.001$)

Statistical analyses highlighted a significant difference in the frequency of acorn colonisation by all arthropods according to the four different oak groups (ants: $\chi^2_{(3)} = 227.15$, $p < 0.001$; *Curculio* sp.: $\chi^2_{(3)} = 44.18$, $p < 0.001$; *C. fagiglandana*: $\chi^2_{(3)} = 89.88$, $p < 0.001$; *C. splendana*: $\chi^2_{(3)} = 62.89$, $p < 0.001$) (Figure 4).

Moreover, each arthropod group showed a different pattern of colonisation according to the analysis of standardised residuals (Figure 4, Data S3). Ant presence strongly concentrated on *Q. rubra* acorns (observed frequency higher than the expected, 33%), while they avoided *Q. cerris* from area B and *Q. petraea* (observed lower than the expected, 0%–9%). Colonisation frequency by *Curculio* beetles had highest scores in *Q. petraea* and *Q. cerris* from area A (higher than the expected, 32%), while being lower on *Q. rubra* (21%) and lowest on *Q. cerris* from area B (lower than the expected, 12%). On the other hand, the pattern of *C. fagiglandana* was more similar to that of ants, as its colonisation frequency was highest on *Q. rubra* (higher than the expected, 19%), lower on *Q. cerris* from area A (6%) and lowest on *Q. petraea* and *Q. cerris* from area B (lower than the expected, 0%–4%). Contrariwise, *C. splendana* showed a very low colonisation rate on *Q. rubra* (lower than the expected, 3%) while it attained higher frequencies on *Q. cerris* from area A and *Q. petraea* (19%–20%), and its highest frequency on *Q. cerris* from area B (higher than the expected, 26%).

For each examined arthropod group there was a significant difference in the frequency of acorn colonisation according to acorn position within the leaf litter (ants: $\chi^2_{(2)} = 38.75$, $p < 0.001$; *Curculio* sp.: $\chi^2_{(2)} = 250.91$, $p < 0.001$; *C. fagiglandana*: $\chi^2_{(2)} = 58.14$, $p < 0.001$; *C. splendana*: $\chi^2_{(2)} = 405.12$, $p < 0.001$). Moreover, each arthropod group showed a different pattern of colonisation according to the analysis of standardised residuals (Figure 4, Data S3). Ants showed an observed colonisation frequency higher than the expected on P1 acorns (17%), but lower for P2 and P3 (5%–7%). On the other hand, *Curculio* beetles were significantly less frequent than expected on P1 and P2 acorns (5%–14%), while they showed a very high frequency (higher than the expected) on P3 acorns (48%). *Cydia fagiglandana* was more frequent than the expected on P1 acorns (12%) while less frequent on P2 acorns (8%) and absent from P3 acorns (less frequent than the expected). Finally, *C. splendana* showed a very low presence on P1 and P3 acorns (lower than the expected, 0%–6%) while a very high colonisation frequency of P2 acorns (higher than the expected, 50%) (Figure 5).

The holes of acorns colonised by *C. fagiglandana* were characterised by a complete silk coverage (CS) in 59.45% of the acorns and incomplete silk coverage (IS) in 29.72%, while no silk coverage at all was present in the remaining 10.83%. No such coverage was detected in acorns colonised by *Curculio* sp. or *C. splendana*, while 55.95% of those colonised by *T. nylanderi* and *T. parvulus* showed a marginal trace of silk on the hole (Figure 6d).

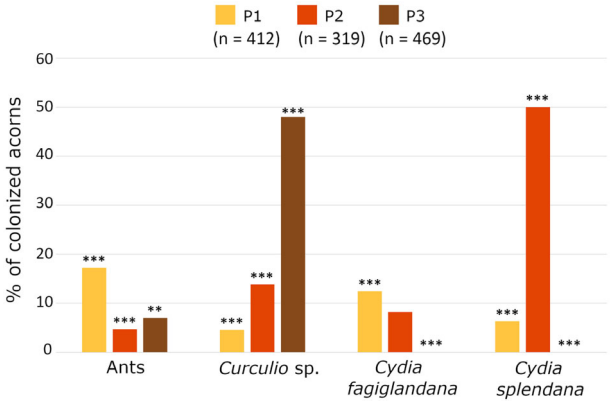

FIGURE 5 Colonisation of acorn groups in relation to their position within the leaf litter (above the leaf litter - P1, in the middle of the leaf litter - P2, below the leaf litter - P3) (field survey data). Asterisks above the bars indicate significant differences from the expected according to the analysis of standardised residuals (* if $p < 0.05$; ** if $p < 0.01$; *** if $p < 0.001$)

Acorn colonisation field experiment

Overall, only 49.5% of the experimental acorns placed in October 2019 were successfully retrieved and collected in October 2020 (Data S2 and S3). The frequency of lost acorns varied significantly according to the oak species of the acorns ($\chi^2_{(3)} = 93.95$, $p < 0.001$): it was 70% for *Q. rubra* (higher than the expected), 57% for *Q. cerris* from area A (higher than the expected), 48% for *Q. cerris* from area B and 27% for *Q. petraea* (lower than the expected). It was also significantly different according to the acorns' position within the leaf litter ($\chi^2_{(2)} = 7.25$, $p < 0.027$): lost acorns were 47% of the ones placed in P1, 56% of P2 (higher than the expected) and 46% of P3 (Data S3).

The acorns hosted the ant species *C. sordidula* (n = 3), *Lasius emarginatus* (Olivier, 1792) (n = 3), *T. nylanderi* (n = 38) and *T. unifasciatus* (n = 3), *Curculio* sp. beetles (n = 11), and *C. fagiglandana* (n = 1). *Curculio* beetles were split between *Q. cerris* from area A (n = 5) and *Q. petraea* (n = 6), always in P3 acorns, while the sole *C. fagiglandana* collected came from a *Q. cerris* acorn of area B (P3). Due to the paucity of data regarding the other groups (which was expected for acorn-consuming insects), statistical analyses were only carried out on ants. Acorns colonisation frequency by ants was found to be significantly different according to different oak groups ($\chi^2_{(3)} = 181.15$, $p < 0.001$) and also according to the acorns' position within the leaf litter ($\chi^2_{(2)} = 12.31$, $p < 0.002$) (see Figure 7; Data S3). Ants were particularly frequent on *Q. rubra* acorns (higher than the expected, 58%), lower on those of *Q. cerris* from area A (12%), while they scored their lowest rates on *Q. petraea* and *Q. cerris* from area B (lower than the expected, 0%–2%). On the other hand, they were more frequent than expected on P1 acorns (higher than the expected, 21%), while less frequent on P2 acorns (8%) and P3 acorns (lower than the expected, 7%).

Effects of acorn moisture on ant colonisation (laboratory experiment)

The results showed significant differences in the choice between the two categories of humidity, *T. nylanderi* choosing to colonise acorns from the drier group more often (30% vs. 20%, $\chi^2_{(1)} = 6.40$; $p = 0.026$).

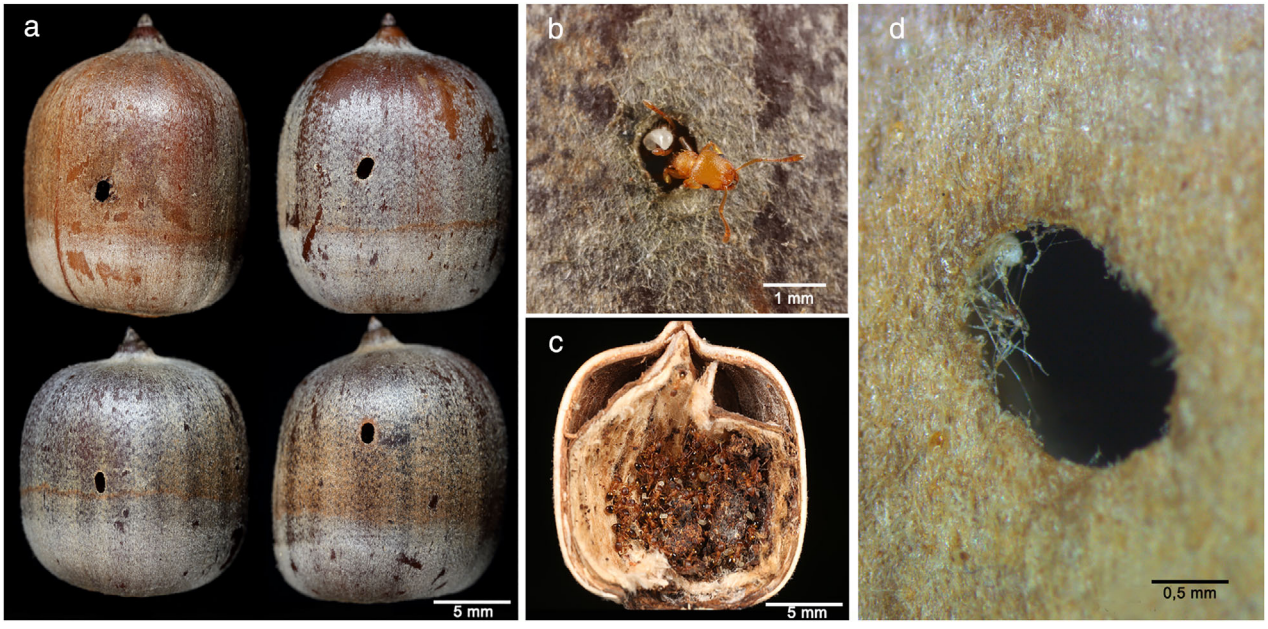

FIGURE 6 (a) *Quercus rubra* acorns colonised by ants (*Temnothorax nylanderi*); (b) workers of *T. nylanderi* on the hole of a *Q. rubra* acorn; (c) colony of *T. nylanderi* inside a *Q. rubra* acorn; (d) hole of a *Q. rubra* acorn colonised by *T. nylanderi*, *Cydia* silk threads are still visible

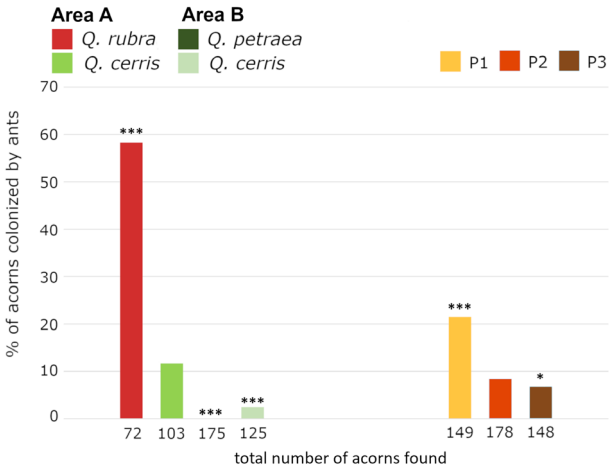

FIGURE 7 Ant colonisation of acorns retrieved from the colonisation field experiment in relation to the oak groups (left) and their position on the ground (right). Asterisks above the bars indicate significant differences from the expected according to the analysis of standardised residuals (* if $p < 0.05$; ** if $p < 0.01$; *** if $p < 0.001$)

Effect of tortrix moths on acorn colonisation by ants (laboratory experiments)

Experiment A. Ant colonisation preference significantly differed according to the conditions of the acorns' hole when choosing between CS and IS acorns: 15 of the IS acorns were colonised as opposed to only 5 of the CS acorns ($\chi^2_{(1)} = 10.0$; $p = 0.002$). Ants were able to enter the acorns mostly between 2 and 3 h after the experiment started and always colonised only one of the two acorns, without moving into the other during the course of the trials. Acorn colonisation by ants was always coupled with attacks against the caterpillars, which in 4 cases left the acorns during the 24 h scan sampling, and in 16 cases died before the end of the experiment (Figure 8).

Experiment B. Ants showed no significant preference between acorns without silk coverage and IS acorns ($\chi^2_1 = 1.000$, $p = 0.639$): 9 of the acorns without silk coverage were colonised as opposed to 7 of the IS acorns.

Experiment C. Acorns without pre-existing holes were never colonised by ants.

Experiment D. Ants ultimately killed all the caterpillars independently from their size, while all caterpillars from the control group were still alive by the end of the experiment. Aggression was carried out by a combination of biting and stinging and was cooperative (involving at least two ants) for the larger caterpillars (size range 2 and 3) (Figure 9). We observed 30% of the size range 1 caterpillars and 20% of the size range 2 caterpillars (but none of the size range 3 caterpillars) leaving their acorns once attacked by the ants before being eventually killed. The caterpillars' size had a significant influence over their survival ability (Kaplan–Meier test: $\chi^2_{(2)} = 49.75$; $p < 0.001$): size range 1 caterpillars were all killed within 3 h, size range 2 within 4 h and size range 3 were all found dead at the 24 h scan sampling (Figure 10). There was a statistically significant difference in survival distributions between all size ranges according to pairwise log-rank comparisons, as larger caterpillars survived longer: size range 1 versus size range 2, $\chi^2_{(1)} = 17.128$, $p < 0.001$; size range 1 versus size range 3, $\chi^2_{(1)} = 35.106$, $p < 0.001$; size range 2 versus size range 3, $\chi^2_{(1)} = 13.744$, $p < 0.001$ (Figure 10).

DISCUSSION

Recently emphasised arthropod decline and its severe potential outcomes on ecosystems functioning show the need for a deeper commitment into effective monitoring of arthropods and the consequences they face for habitat alterations. Their involvement in multitrophic scenarios, and the ecosystem services/disservices they can offer in both natural and agroforestry systems deserve more attention (e.g., Burgio et al., 2015; Campanaro et al., 2011; Leather, 2017; Pizzolotto et al., 2018; Schowalter et al., 2018; Yang & Gratton, 2014). In the context of Mediterranean oak forests, our study suggests that insect assemblages associated with acorns of *Q. rubra* and other coexisting oak species are very similar across Europe. Furthermore, our data demonstrate that all groups of insects commonly associated with fallen acorns of native oaks can colonise acorns of *Q. rubra*. In the Italian forests, we obtained results similar to previous

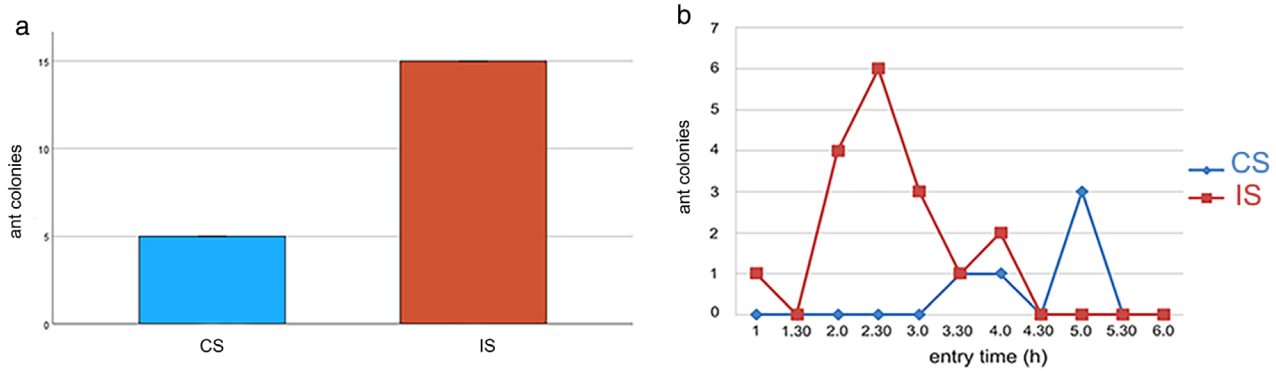

FIGURE 8 (a) Acorn preferences of *T. nylanderi* in relation to the condition of the acorn's hole created by *C. fagiglandana*: Incomplete silk coverage (IS) and complete silk coverage (CS). b) Number of ant colonies entering CS and IS acorns during the first 6 h of the experimental trials

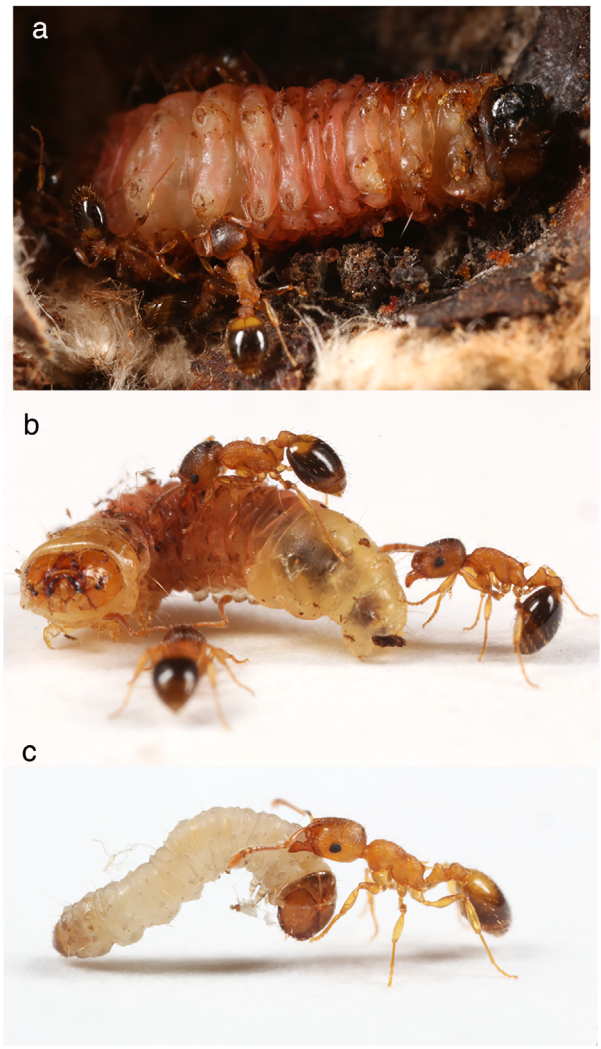

FIGURE 9 *T. nylanderi* workers attack caterpillars of *C. fagiglandana*: (a) size range 3 caterpillar killed by the ants within the acorn, (b) size range 2 caterpillar attacked by three ants once escaped away from the host acorn, (c) size range 1 caterpillar killed and transported back to the acorn by a single ant worker

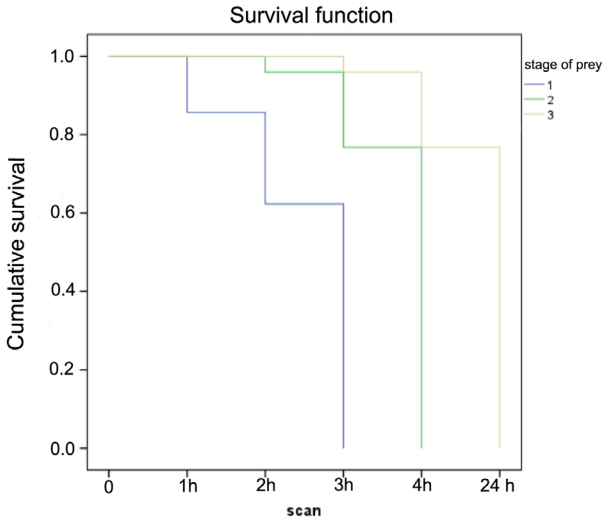

FIGURE 10 Survival time rate of *C. fagiglandana* caterpillars attacked by ants. Scan samplings were performed after 1, 2, 3, 4, and 24 h

surveys carried out in Poland, recording *Cydia fagiglandana*, *C. splendana*, *Curculio* beetles, and *Temnothorax* ants (Myczko et al., 2017). We were unable to identify *Curculio* larvae at the species level, a problem which also Myczko et al. (2017) encountered. However, the four potential candidate species of the Polish fauna suggested by Myczko et al. (2017) are also widespread in Italy (these are *C. elephas* (Gyllenhal, 1836), *C. glandium* Marsham, 1802, *C. pellitus* (Boheman, 1843), and *C. venosus* (Gravenhorst, 1807), see Colonnelli, 2003). On the other hand, Myczko et al. (2018) reported on the abundance of *T. crassispinus* (Karavaiev, 1926), mostly associated with *Q. rubra* acorns, but recorded no other ant species. In our case, the ant fauna associated with acorns was similarly dominated by *Temnothorax* but resulted to be more diverse, comprising 6 species. The most abundant species was *T. nylanderi*, which is the Western sibling species of *T. crassispinus*, while the second most abundant was *T. parvulus*, also a member of the same species-group (Csősz et al., 2015).

However, according to our data, the relationship of *Q. rubra* acorns with these arthropods was characterised by some interesting differences compared to previous studies. Similar to what was found in Poland (Myczko et al., 2018), ants preferred to colonise the large *Q. rubra* acorns rather than those of native oak species. However, contrary to the situation documented there (Myczko et al., 2017), *C. fagiglandana* did not avoid *Q. rubra* acorns and instead preferred them over those of native oaks. On the other hand, *C. splendana*, which in Poland showed no preference between native and *Q. rubra* acorns, avoided *Q. rubra* acorns in our Italian site. Finally, *Curculio* beetles, uninterested in *Q. rubra* acorns in Poland in the study conducted by Myczko et al. (2017), even facing a low developmental success with them according to Bogdziewicz, Bonal, et al. (2018), but important predators according to Bogdziewicz, Marino, et al. (2018), were frequent colonisers in our area of study. These interesting differences may be the result of different local conditions between the respective study sites in Italy and Poland. The most impactful differences may lay in the macroclimate of the two regions, determining oak development cycles and acorn production. Another factor may be the presence of different native oak species: for instance, *Q. robur* L., 1753, reported by Myczko et al. (2017), was absent from our study area. Secondarily, it is also possible to speculate that different populations of insect species feeding on acorns may have developed different adaptive responses when facing the local establishment of *Q. rubra*. Complex competitive interactions between these species may also play a significant role in shaping local abundances and their year-to-year fluctuation (for example *C. elephas* activity on senescent acorns may limit *C. fagiglandana*, see Jimenez-Pino et al., 2011), and may have a significant influence on their population dynamics at the local scale which are still not fully understood. Considering the significant variation from one country to another, a satisfactory assessment of such phenomena at the European level is certainly far from being achieved.

Ants are key factors in many ecosystems and their abundance, community composition, and interactions with both biotic and abiotic factors may offer useful information for a better understanding of ecological dynamics and multitrophic network in which they can be involved (e.g., Arnan et al., 2018; Castracani et al., 2015; Gibb et al., 2017; Lach et al., 2010; Lawes et al., 2017; Segat et al., 2017). They frequently interact with most of the biotic components of their environment, and often mainly act as predators (Hölldobler & Wilson, 1990; Lach et al., 2010; Cerdá & Dejean, 2011; Grasso et al., 2015; Nepi et al., 2018): most ants represent a model of generalist predators having a positive effect on the reduction of phytophagous insects (e.g., Campolo et al., 2015; Castracani et al., 2017; Cerdá & Dejean, 2011; Giannetti et al., 2019; Hölldobler & Wilson, 2009; Schifani et al., 2020). Our data testify the predatory ability of *T. nylanderi* in respect to *C. fagiglandana* (similarly to *Cydia* species of agricultural importance, see Schifani et al., 2020), and the ecological importance of this interaction is worth further investigation. Ants seem to colonise only those acorns which had previously been occupied by tortrix moths, and if the moths' caterpillars are encountered inside them, they are suitable preys at least for *T. nylanderi*. Since caterpillars of *C. fagiglandana* first enter acorns that are still attached to the branches and then complete their life-cycle on the ground, the fact that *T. nylanderi* prefers drier acorns and those that are found on the most superficial layer of the leaf litter may increase the frequency of encounters between these species. Further investigation should document what are the conditions that make *C. fagiglandana* caterpillars either build a "complete" silk wall on the hole of their acorns (with a relatively effective anti-ant performance) or an "incomplete" silk wall which was more easily penetrated by *T. nylanderi*: this peculiar dichotomy was hitherto undescribed. Finally, it is worth noting that the predatory behaviour of *T. nylanderi* may contribute to the control of *C. fagiglandana* in other contexts since both are widespread across very different types of forested environments; *C. fagiglandana* is considered among the most important carpophagous insects of evergreen oaks such as the holm oak *Q. ilex* L., 1753 and the cork oak *Q. suber* L., 1753, and can also target other Fagaceae of great ecological and agroforestry interest such as the chestnut *Castanea sativa* Mill., 1768 or the European beech *Fagus sylvatica* L., 1753, while *T. nylanderi* is common across many types of European forests (Jimenez-Pino et al., 2011; Myczko et al., 2017; Seifert, 2018; Speranza, 1998).

Acorns' position within the leaf litter influenced the frequency of colonisation by most arthropod groups. We detected a clear preference by ants for acorns positioned in the top layer of the leaf litter (as mentioned above). On the contrary, *Curculio* beetles were more abundant on the bottom layer, and *C. splendana* in the intermediate one. While such pattern for plurennial ant colonies suggests they actively relocate to newer acorns in the upper leaf litter layers, it is unclear whether *Curculio* beetles and *C. splendana* moths, whose life cycle is obviously shorter, also move often from acorn to acorns. In the hypothesis that individuals are mostly found in the same acorns they started to colonise on the trees, other factors may have determined this unequal abundance. The differences we observed may partly result from competitive or predatory interactions between arthropods which are difficult to disentangle (e.g., see Jimenez-Pino et al., 2011 regarding *Curculio*; Stukalyuk & Radchenko, 2011 regarding *T. parvulus*). Microclimatic conditions and the risk of acorn predation by vertebrates are certainly no less important. In ants, the physical characteristics of the nest are key to ensure protection from predators, unfavourable temperatures and desiccation, and to favour optimal brood development (Blüthgen & Feldhaar, 2010; Mitrus, 2015, 2019; Pratt & Pierce, 2001; Solida et al., 2011). Winter temperatures have a major influence on survival rates for *Temnothorax* ants living in acorn nests, and acorns close to the surface may guarantee more favourable warmer conditions during the period of activity, while drier conditions may be important to avoid bacterial and fungal contamination which pose a great danger to the colonies of social insects (Herbers & Johnson, 2007; Hood & Tschinkel, 1990; Karlik et al., 2016). The same differences in humidity and temperature are likely impactful for moths and beetles too. On the other hand, vertebrates that feed on acorns such as birds or mammals may find acorns placed on the top of the leaf litter more easily. Most can discriminate between intact acorns with higher nutritional value and acorns already damaged or partially emptied by the action of insects, which may reduce the threat they pose to nesting ants (Dixon et al., 1997; Focardi et al., 2000; Muñoz & Bonal, 2008; Pyare et al., 1993; Steele et al., 1996). Concerning our experiment, these animals may have played a role in the disappearance of acorns placed during the colonisation experiment, but missing acorns were not significantly associated with a particular position within the leaf litter. However, *Q. rubra* acorns in general disappeared in greater numbers from the sites in which we experimentally placed them, which may mean that they are more frequently consumed by vertebrates compared to native acorns.

In conclusion, the influence that *Q. rubra* acorns have on the acorns-associated arthropods likely affects the fitness of coexisting native oaks which for now remains undocumented. In our study area, *Q. cerris* acorns were more frequently colonised by ants and by *C. fagiglandana* if they occurred in the presence of *Q. rubra*. While the establishment of *Q. rubra* may be beneficial to the abundance of acorns-consuming carpophagous insects (Myczko et al., 2017), it also seems to offer significant nesting opportunities *Temnothorax* ants which are generalist predators. The ecological consequences of these interactions deserve further investigation.

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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Data S1 Data of acorns used in laboratory experiments.

Data S2 Field survey and field experiment data

Data S3 Chi-squared results and analysis of standardised residuals

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