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Niche construction by two ectomycorrhizal truffle species (*Tuber aestivum* and *T. melanosporum*)

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ABSTRACT

Niche construction by biota has been frequently reported for animals and plants, but fungi have received less attention. For mycorrhizal fungi, mutualistic niche construction has been proposed where partners construct niches for each other. However, few eco-evolutionary mechanisms leading to mutualistic symbiotic feedback have been described. Here we report niche construction by two ectomycorrhiza-forming species of truffles, *Tuber aestivum* and *T. melanosporum*. The soils of 263 spots of these *Tuber*, which create bare patches (brûlés), have been monitored (9–10 years) in natural forests to analyze whether they meet the criteria to satisfy the mutualistic niche construction process. Soil habitat modification by these *Tuber* can be seen in their brûlés, where the vegetation of arbuscular mycorrhizal plants and soil organisms are largely suppressed. *Tuber melanosporum* brûlés were smaller more productive, and with lower vegetation cover than *T. aestivum*. Interactions among soil carbonates, pH, and brûlé size are associated with increased carpophore productivity, indicating positive feedback between habitat modification and fungal fitness. Both species modified the habitat differentially. *Tuber aestivum* brûlés had higher pH and lower total carbonate than soils outside. *Tuber melanosporum* brûlés had lower total carbonate and higher exchangeable Ca^{2+} and active carbonate than soils outside. In conclusion, brûlés show a mutualistic niche construction process because modifies their soil environment and increases the fitness of both *Tuber* spp. Enhanced carbonate weathering by truffles resulted in lower soil quality with a negative impact on plants, indicating that mutualistic niche construction does not equally benefit both partners.

1. Introduction

Ecological theory has traditionally focused on how organisms adapt to their environment. However, environmental modification by organisms and their legacy over time are processes that act as sources of modified selection and modified phenotypes through a process called niche construction (Day et al., 2003; Odling-Smee et al., 2003, 2013; Pausas and Bond, 2022). Matthews et al. (2014) proposed three criteria to identify a niche construction process (Criteria 1 and 2) and to determine when it affects evolution (Criterion 3).

- Criterion 1: *An organism must significantly modify environmental conditions.*
- Criterion 2: *Organism-mediated environmental modifications must influence selection pressures on a recipient organism.*
- Criterion 3: *There must be an evolutionary response in at least one recipient population caused by environmental modification.*

The term ecosystem engineering has frequently been used as an equivalent term, although that term focuses less on the evolutionary implications of these processes (Pausas and Bond, 2022). Niche construction is a broad concept and includes examples from animals (dam

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building by beavers; landscape modification by fungus-growing termites), plants (dune-building by marram grass and their associated arbuscular mycorrhizal fungi), and microorganisms (through plant-soil feedbacks). Plants and their associated microbes bring changes in biological, chemical, and physical soil properties, and such environmental modifications, which at first sight might seem trivial, produce crucial selection pressures that increase fitness for some species and decrease fitness for others. Mutualistic symbioses, such as those formed between mycorrhizal fungi and plants, are prime examples of niche construction. However, few mutualistic niches have been described in detail, and many eco-evolutionary mechanisms leading to symbiotic feedback still need to be discovered (Peay, 2016). Habitats, considered suitable for certain plants, are sometimes not colonized without a suitable mycorrhizal fungal partner (Nuñez et al., 2009; Peay 2016). The differential modification of plant-soil feedback by different guilds of mycorrhizal fungi indicates that the mutualistic niche for one species might reduce the fitness of another species (Kadowaki et al., 2018).

A clear example of niche construction by ectomycorrhizal fungi is formed by truffles of the genus *Tuber* Micheli ex Wiggers. *Tuber* spp. are hypogeous ascomycetes growing in a mutualistic symbiosis with the roots of host plants such as *Quercus* L., *Corylus* L., *Populus* L., *Fagus* L., and *Cistus* L. Upon ripening, *Tuber* ascocarps emit distinctive species-specific volatile organic compounds that allow them to be located by mammals such as wild boar or pigs, trained dogs, or insects. After location, truffles are picked up by those animals, and through that mechanism, their spores are dispersed (Ceruti et al., 2003). Plants colonized by several *Tuber* species show an almost bare zone around the trunk of their host plants, called brûlé, where the growth of other non-host plants and soil organisms are inhibited (Fig. 1). There are several hypotheses about how those bare patches, which look like burnt sites, originate. The allelopathy hypothesis relates to the production of phytotoxic substances that affect seed germination, seedling establishment, and/or the growth of adult plants other than the host tree. An alternative parasitism hypothesis noted that these plant species, which are commonly colonized by arbuscular mycorrhizal fungi, are colonized by the truffle mycelium and potentially acquire carbon from these non-hosts without exchanging them for nutrients (Plattner and Hall, 1995; Gryndler et al., 2014; Schneider-Maunoury et al., 2018, 2020). The brûlé structure is mainly associated with three species of *Tuber*: The Mediterranean *T. melanosporum* Vittad. (Black truffle), the cosmopolitan *T. aestivum* Vittad. (Summer truffle), and the Chinese *T. indicum* Cooke and Massee (Streiblová et al., 2012). On the other hand, in natural forests, most studies on *T. aestivum* and *T. melanosporum* do not provide statistical data on brûlé development, carpophore production, and other quantitative data associated with truffle ecology.

Fig. 1. Brûlé formed by *T. aestivum* associated with *Q. rotundifolia* in a natural forest (Central Spain). Photo by T.W. Kuyper.

The brûlés created by *T. melanosporum* and *T. aestivum* provide attractive biological models in calcareous soils to analyze edaphic modification associated with these *Tuber* spp. (García-Montero et al., 2006, 2007, 2009a, 2009b, 2012; Menta et al., 2014). These studies demonstrated an impact of brûlé development on soil calcium carbonate and pH balances, and these soil chemical modifications, as a form of niche construction, could have feedback effects on the performance of these *Tuber* species and their host and non-host plants (García-Montero et al., 2009a, 2012). Bragato (2014) proposed that the brûlé could be a case of perturbational niche construction. His study in one brûlé created by an unidentified *Tuber* species demonstrated a significantly lower contribution of large macro-aggregates inside the brûlé compared to the adjacent area that was covered by grasses. Changes in soil aggregation were likely driven by the disappearance of grasses in the brûlé, which modified the impact of freeze-thaw cycles on soil aggregation and soil aeration that could have improved the conditions for sporocarp formation by the truffle due to lower resistance for sporocarp enlargement. Bragato (2014) put these observations explicitly in the niche construction framework of Odling-Smee et al. (2003, 2013).

The possibility that the edaphic chemical changes that occur during the development of brûlés are also an instance of niche construction has, to the best of our knowledge, not been studied before. The general objective of the present study was to evaluate, in the framework of niche construction theory, soil chemical changes caused by two truffle species, *T. melanosporum,* and *T. aestivum*, based on long-term observations of many wild truffle populations in Mediterranean forests located in Central Spain around the Natural Park of "Alto Tajo". In our study, we focus on criteria 1 and 2 by Matthews et al. (2014) as sufficient to demonstrate niche construction.

2. Material and methods

2.1. Study area

Brûlés of *T. melanosporum* and *T. aestivum* were monitored in 9 municipalities of central Spain (Sigüenza and Peralejos de las Truchas, province of Guadalajara; Medinaceli, Arcos de Jalón and Chaorna, province of Soria; Masegosa, El Tobar, Cueva del Hierro and Beteta, province of Cuenca). The study area is within a 40 km radius around the town of Huertahernando (province of Guadalajara; geographic coordinates: 40° 49′ 25″ N, 2° 17′ 12″ W). The exact geographical coordinates of the *Tuber* brûlés are not given here as they were confidentially provided to the first author by professional truffle hunters who participated in this study and who wanted to preserve the exact locations that they exploited. The study area is a mountainous region with elevations ranging from 1000 to 1450 m.a.s.l. in a supra-Mediterranean bio-climatic belt. Its lithology is predominantly Jurassic and Cretaceous limestone and dolomite. Soils are frequently lithic and rendzic leptosols with considerable surface stoniness. The mean annual temperature is 10.8 °C, and the mean monthly minimum and maximum temperatures are 3.3 °C and 18.5 °C, respectively. The mean annual precipitation is 650 mm.

The vegetation of the study area falls within the Mediterranean Ibero-Levantina Province Castellano Maestrazgo-Manchega Subprovince geobotanical classification (Rivas-Martínez, 1987; Peinado et al., 2017). In the study area, two main and one intermediate forest types constitute the most important habitats where both truffle species abound. Both *Tuber* species form ectomycorrhizal associations with *Quercus faginea* Lam. and *Q. rotundifolia* Lam. (syn. *Q. ilex* L. subsp. *ballota* (Desf.) Samp.). The vegetation types are classified as (forest type 1) *Cephalanthero rubrae – Quercetum fagineae* and (forest type 2) *Junipero thuriferae – Quercetum rotundifoliae*. Both *Quercus* species co-occur in the intermediate forest type (forest type 3). Compared to type 1, intermediate type 3 has a more xeric lithology with rocky soils exposed to the sun compared to type 1. Forest type 2 is the most xeric type.

2.2. Brûlé characterisation

Tuber melanosporum has been harvested in these forests since the end of the 1960s, and *T. aestivum* since the early 2000s. With the help of seven professional truffle hunters who harvested those brûlés regularly, we monitored (i) 119 natural *T. aestivum* brûlés for 9 years (2007–2015) and (ii) 144 natural *T. melanosporum* brûlés for 10 years (1994–2000 and 2013–2015). Some of the brûlé data used in this study have been updated in the field during 2013–2015 from data initially published in García-Montero et al. (2007, 2014). Truffles were collected with the help of trained dogs and identified, according to Riousset et al. (2001).

In each brûlé, we determined host tree identity, size (m^2) of the brûlé, the percentage of the brûlé surface occupied by plants other than the host of *Tuber* spp., and sporocarp (truffle) production. The maximum annual production of sporocarps was calculated as the largest quantity of fresh sporocarps picked during a harvest season over a minimum period of 9 years. Maximum annual production or productivity (instead of average yield) was chosen to standardize and compare brûlés in the most favorable year for each site. At the beginning of the monitoring of each brûlé, we measured its longest and shortest axis and estimated brûlé area using an ellipse model. In brûlés with an irregular shape, we used a combination of several small ellipses. In 2015, at the end of the monitoring period, we measured the percentage of the brûlé surface occupied by plants other than the ectomycorrhizal hosts of *Tuber* spp. of the 34 brûlés of *T. melanosporum* in forest type 2, we could not measure the percentage of the brûlé surface occupied by plants in 32 *Tuber* brûlés, because access to this area in Beteta was unexpectedly restricted. For the same reason, we could not collect soil samples in Beteta. Our data set on *T. melanosporum* in forest type 2 is therefore restricted to 2 brûlés in the locality Cueva del Hierro.

2.3. Soils

Soil samples were taken in 90 brûlés, 45 of each species. Soil sampling inside brûlés had to be restricted because of the high economic value of these brûlés for truffle hunters and the disturbance caused by soil sampling. In all, 134 soil samples were taken: 90 inside *vs* 44 outside brûlés. We included the data of pH, total organic carbon (TOC), and total and active carbonate of 40 *T. melanosporum* brûlé soils (20 inside *vs* 20 outside) from García-Montero et al. (2009b) in the dataset. For economic reasons, not all parameters were analysed in the 134 soil samples. The sample size used in the statistical analysis of each soil parameter is shown in Supplementary Table 1. This set was used for testing significant differences between soils in brûlés of *T. aestivum*, soils in brûlés of *T. melanosporum*, and soils outside brûlés. These data also allowed testing for soil modification by each truffle species. For that, we compared 20 samples of brûlés of *T. aestivum* with 20 samples outside brûlés (at a distance greater than 2 m outside the visible brûlé boundary) and 20 samples of brûlés of *T. melanosporum* with 20 samples outside brûlés. Some comparisons (inside/outside) of *T. melanosporum* brûlés have been previously published in García-Montero et al. (2009b). Here we use an expanded set of brûlé characteristics (surface size, sporocarp productivity, plant cover in the brûlés, and soil characteristics), and extend the statistical analysis.

The uppermost 30 cm of each soil profile was sampled because both *Tuber* species fruit in this range (Verlhac et al., 1990). Soils were sampled using the same spade (0.20 m blade) and a tape measure. The samples included a mixture of 1 kg of various soil horizons. Soil horizons could not be recognized because of the constant mixing of horizons due to the continuous digging by the harvesters, their dogs, and wild animals. Sampling was done according to FAO (1990). The following soil properties were assessed: pH in water, total calcium carbonate (equivalent carbonate), total organic carbon (TOC), and exchangeable cations following the methods of ISRIC (1995). Active carbonate (carbonate extractable with ammonium oxalate) was determined according to AFNOR (1982). Active carbonate is the finely divided fraction of carbonates measuring <50 μm; it is susceptible to rapid mobilization and is chemically very reactive. The amount and fraction of active carbonate are often used as a proxy for predicting iron chlorosis in the vineyard and orchard agroecosystems (Tagliavini and Rombolà, 2001).

2.4. Statistics

We first tested, through a two-way analysis of variance, whether the three forest types differed in size, plant cover, and sporocarp productivity for both truffle species, based on the sample of 263 brûlés. We repeated the analysis for 90 brûlés of which we had soil chemical data, to test for differences between forest types and truffle species in soil properties. Finally, we tested through a paired *t*-test for differences in soil properties inside and outside the brûlés, based on 20 paired samples for each species. In some of the analyses, the following extreme outlier values were discarded after testing (Grubbs test): the value of 150 m^2 of the size of a *T. melanosporum* brûlé; the values of 125.55 $cmol^+ kg^{-1}$ of exchangeable Ca^{2+}, and 125.6 and.

116.9 mg g^{-1} of TOC inside the brûlés of *T. melanosporum*; and the value of 80 mg g^{-1} of TOC inside the brûlé of *T. aestivum*. Soil data were additionally analysed through Principal Component Analysis (PCA).

Data were checked for normality (Shapiro-Wilk and Kolmogorov-Smirnov tests) and homogeneity of variances (Levene test). Transformations (log or square root) were made in case of departure from ANOVA assumptions, as in the values of brûlé size, soil pH, % total carbonate, % active carbonate, exchangeable Ca^{2+}, Mg^{2+}, and K^+. In addition, non-parametric tests (Kruskal-Wallis test; Spearman test) were used when data did not comply with ANOVA assumptions, as in the values of the ratio soil % active/total carbonate and TOC. We also used analysis of covariance to compare *Tuber* productivity among forest types while controlling for brûlé size. Following Ramsey and Schaffer (1997), after log-transformation, we interpreted the results as multiplicative effects on the group median. The family-wise error rate was controlled with the Tukey-Kramer procedure. Most analyses were done in R v. 3.0.2 (R Core Team, 2013), but some complementary analysis was done with Statgraphics Centurion 18v Software (2019(1982–2018).

3. Results

3.1. Characteristics of brûlés

Two-way analysis of variance showed that truffle species, forest type, and the interaction truffle species × forest type were significant sources of variation (*Tuber*-spp: $F_{1,261} = 5.13$, $p = 0.024$; forest type: $F_{2,260} = 13.33$, $p < 0.001$; *Tuber*-spp × forest type: $F_{2,260} = 21.63$, $p < 0.001$). Brûlés of *T. aestivum* (119 samples) and *T. melanosporum* (144 samples) showed significantly different characteristics in the three forest types (Fig. 2, Supplementary Table 2). There was a significant difference in *T. melanosporum* sporocarp productivity between forest types, with type 2 (the driest forest) being significantly less productive than the two other forest types.

The productivity of *T. aestivum* did not differ between forest types. *Tuber melanosporum* was much more productive than *T. aestivum* in forest types 1 and 3, but not in type 2 (Fig. 2A).

Two-way ANOVA for brûlé size showed that truffle species, forest type, and the interaction truffle species × forest type were significant sources of variation (*Tuber*-spp: $F_{1,261} = 113.50$, $p < 0.001$; forest type: $F_{2,260} = 8.59$, $p < 0.001$; *Tuber*-spp × forest type: $F_{2,260} = 10.36$, $p < 0.001$). Brûlés of *T. aestivum* were, on average, much larger than those of *T. melanosporum* (Fig. 2B). Brûlé size of *T. melanosporum* was smallest in forest type 2, and this coincided with the lowest productivity. Brûlé size of *T. melanosporum* was largest in the forest type 3, whereas it was smallest for *T. aestivum* in that forest type; therefore, species-specific differences in brûlé size were significant in both the most mesic (type 1) and xeric (type 2) forest type, but not in the intermediate type 3.

Two-way ANOVA showed that truffle species was a significant source

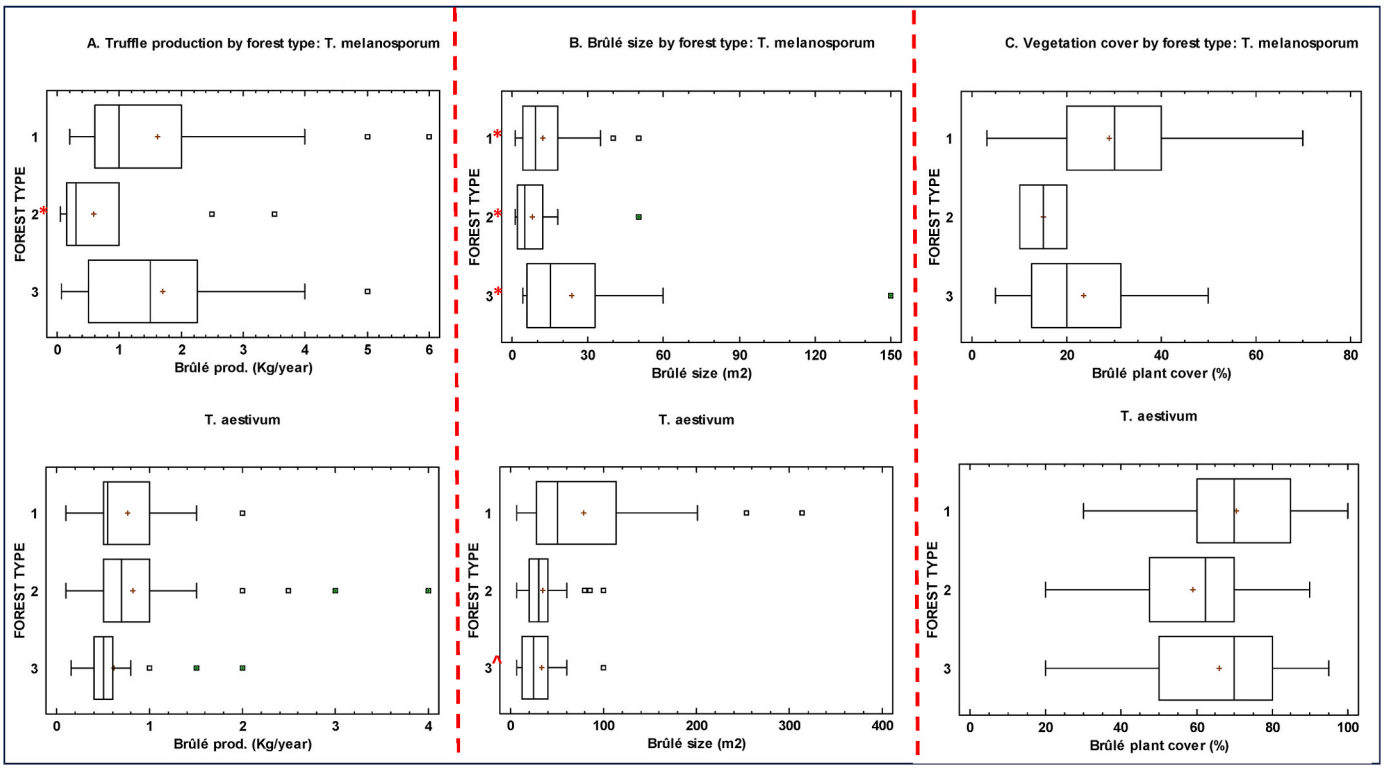

Fig. 2. Brûlé characteristics of *T. melanosporum* and *T. aestivum* in the different forest types. Sporocarp productivity (A), brûlé size (B), and vegetation cover (C). Forest type 1: formation of *Quercus faginea*, forest type 3, mixed forest with *Q. faginea* and *Q. ilex*, forest type 2: forest with *Q. ilex* [ˆp < 0.05; $^{+}$p < 0.01; *p < 0.001].

of variation in brûlé vegetation cover, whereas forest type and the interaction truffle species × forest type were not (*Tuber*-spp: $F_{1,229} = 226.04$, $p < 0.001$; forest type: $F_{1,228} = 3.15$, $p = 0.078$; spp × forest type: $F_{1,229} = 0.02$, $p = 0.890$). *Tuber melanosporum* reduced vegetation cover in the brûlé much more than *T. aestivum* (Fig. 2C). Outside brûlés, vegetation cover was close to 100%.

Brûlé size was significantly correlated with sporocarp productivity of both *T. melanosporum* (log [prod] = −1.1732 + 0.4912 log [size], $r^2 = 0.24$, p < 0.001) and *T. aestivum* species (log [prod] = −1.3937 + 0.2691 log [size], $r^2 = 0.10$, p < 0.001). This regression indicates that with a doubling of the brûlé area, the yield increases by 41% in *T. aestivum* (2ˆ0.4912 = 1.41 times) and by 21% in *T. melanosporum* (2ˆ.2691 = 1.21 times). While in the same brûlé area, the productivity of *T. melanosporum* is much higher than that of *T. aestivum* (Fig. 3). Because of the significant interaction between truffle species × forest type for both productivity and

brûlé size, we repeated the analyses for that correlation for forest sites separately. For *T. aestivum*, the relationship between brûlé size and its productivity was significant but did not depend on forest type (forest type: $F_{2,116} = 1.95$, $p = 0.17$; log [size]: $F_{1,117} = 13.23$, $p < 0.001$).

For *T. melanosporum*, the relationship between brûlé size and its productivity was significant and depended on forest type (forest-type: $F_{2,141} = 14.85$, $p < 0.001$; log [size]: $F_{1,142} = 28.66$, $p < 0.001$; log [size] × forest type: $F_{2,141} = 3.79$, p = 0.02).

We analysed the relationship between (non-host) plant cover in brûlés and sporocarp productivity. In *T. aestivum*, the positive relationship between plant cover in brûlés and sporocarp productivity was significant and did not depend on forest type or the interaction between

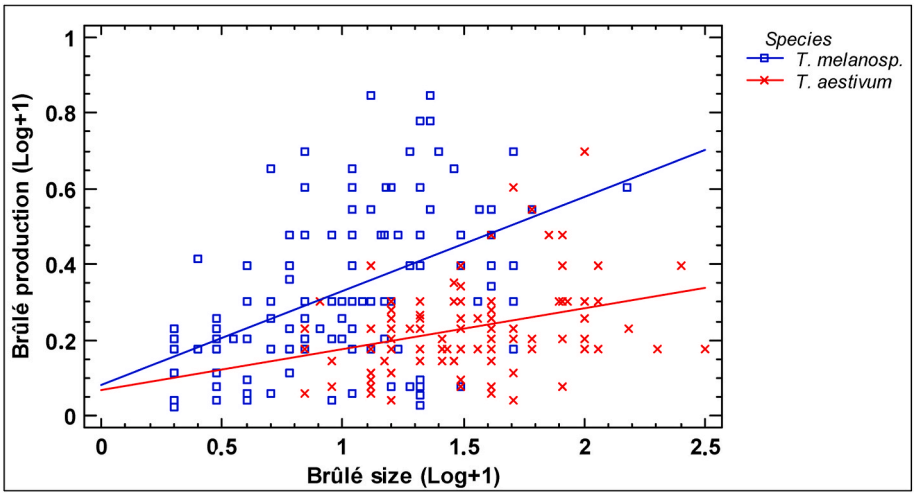

Fig. 3. The relationship between brûlé size and maximum productivity for *T. aestivum* (red) and *T. melanosporum* (blue).

those two variables (forest type: $F_{2,116} = 1.22$, $p = 0.30$; plant cover: $F_{1,117} = 5.50$, $p = 0.02$; plant cover × forest type: $F_{2,116} = 0.49$, $p = 0.61$). However, for *T. melanosporum,* the relationship between brûlé plant cover and the productivity was only significant if the effect of the forest type was included (forest type: $F_{1,142} = 6.19$, $p = 0.014$; plant cover: $F_{1,142} = 0.26$, $p = 0.61$; plant cover × forest type: $F_{1,142} = 6.72$, $p = 0.01$). The significant interaction between plant cover and forest type was because, in forest type 1, productivity increased when plant cover decreased, whereas, in forest type 3, productivity increased when vegetation covers increased.

3.2. Soils inside and outside brûles

In our analysis of soil modification by both truffle species we compared three categories, viz., brûlés of *T. aestivum*, brûlés of *T. melanosporum*, and soils outside brûlés (Fig. 4; Table 1). There was a significant difference in pH among the three groups (square root transformed data of pH, $F_{2,131} = 7.86$, $p < 0.001$). There was no effect of forest type ($p = 0.188$). Soils from *T. melanosporum* brûlés were, on average, 0.10 and 0.25 units of pH higher than soils of *T. aestivum* brûlés and soils outside brûlés, respectively (Fig. 4A). Both differences were significant. Soils from *T. aestivum* brûlés showed, on average, 0.14 units of pH higher than soils outside the brûlés, a difference that was also significant. Whereas forest type did not impact soil pH, the pH modification in *T. aestivum* brûlé showed significant interactions with brûlé size, with larger brûlés resulting in a stronger pH increase compared to neighboring soil ($p = 0.030$). The effect of plant cover on brûlé pH was marginally significant ($p = 0.080$). There was no relationship between sporocarp productivity and pH.

Total carbonate content was significantly higher outside brûlés and inside brûlés of *T. melanosporum* than inside brûlés of *T. aestivum*, indicating modification of the carbonate pool by the latter truffle species (Fig. 4B). Two-way ANOVA showed that soil group and forest type were significant sources of variation for total carbonate content, whereas the

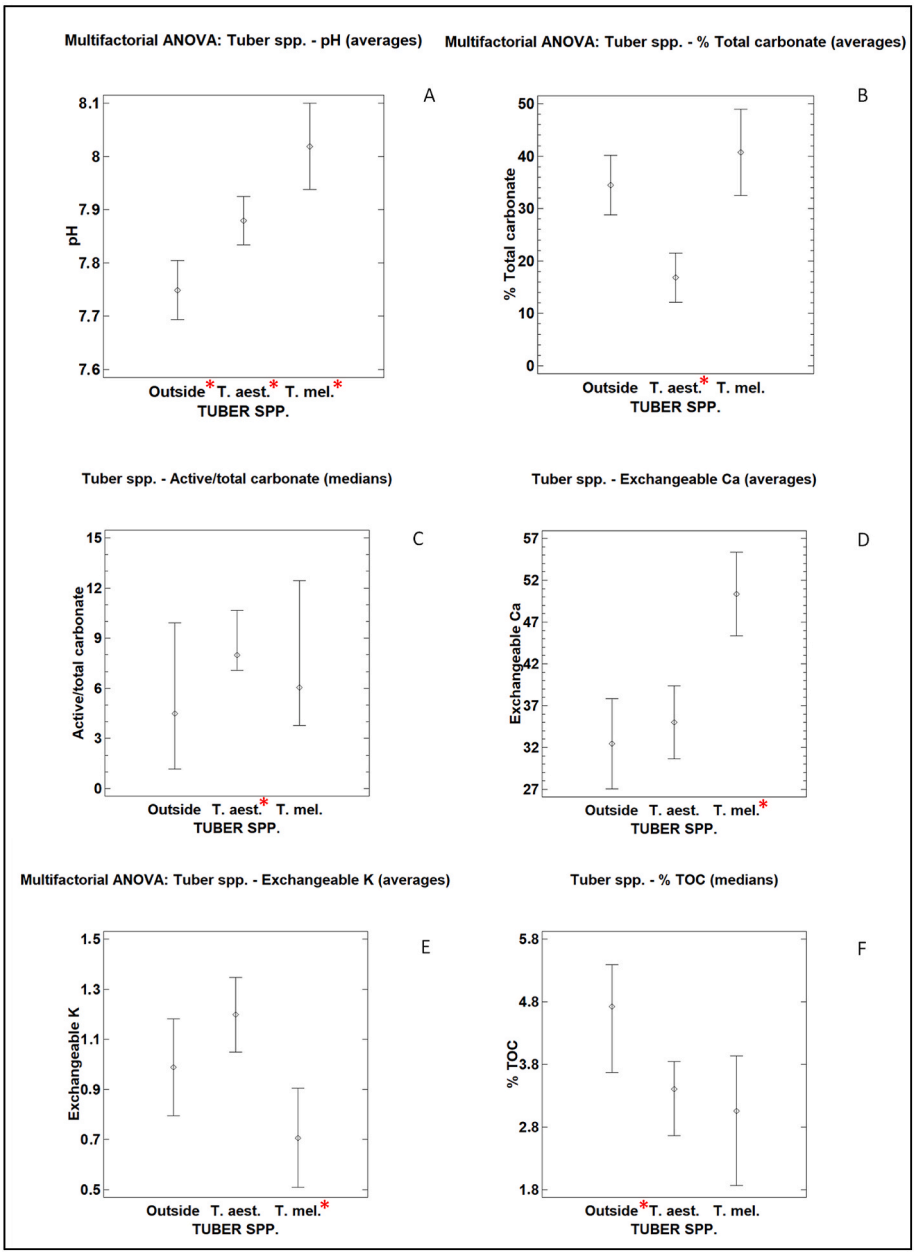

Fig. 4. Comparisons of average or median values of soil properties among the three groups of soils of *T. aestivum*, *T. melanosporum,* and soils outside the brûlés; pH (A), total carbonate (B), active/total carbonate ratio (C), exchangeable Ca^{2+} (D), exchangeable K^{+} (E), and total organic carbon (F) [*$p < 0.05$].

Table 1
Soil properties of 45 brûlés of *T. aestivum*, 45 brûlés of *T. melanosporum*[1] and 44 Samples outside the brûlés. [*[1]Data partly published in García-Montero et al. (2007, 2014) added with data collected 2013–2015. [2]Brûlé size in m[2]. [3]Maximum annual production of* Tuber *sporocarps in each brûlé was calculated as the greatest quantity of fresh carpophores, in Kg, picked during a harvest season.[4](g/100 g). [5]Exchangeable cations expressed in cmol $^{+}$ kg^{-1}*].

[1]134 Soil samples	Soils outside of the brûlés			Soils inside *T. aestivum* brûlés			Soils inside *T. melanosporum* brûlés		
	N	Average (SD)	Max/Min	N	Average (SD)	Max/Min	N	Average (SD)	Max/Min
[2]Brûlé size	–	–	–	45	45.69 (36.55)	153/6.00	45	1.73 (1.47)	6.00/0.15
[3]Brûlé maximum production	–	–	–	45	0.65 (0.46)	2.00/0.10	45	12.90 (10.77)	60.00/2.00
Brûlé plant cover: surface without plants (%)	–	–	–	45	32.40 (19.35)	80.00/5.00	45	72.84 (13.16)	95.00/30.00
pH	44	7.77 (0.24)	8.17/7.00	45	7.88 (0.17)	8.16/7.48	45	7.96 (0.24)	8.35/7.10
[4]Total carbonate	44	32.85 (25.08)	94.51/0.21	45	16.83 (12.58)	66.26/1.11	45	36.83 (28.01)	85.76/1.46
[4]Active carbonate	44	1.71 (2.06)	9.99/0.00	45	1.42 (1.01)	3.86/0.00	45	2.26 (2.13)	8.42/0.00
[4]Active/total carbonate	44	9.62 (18.44)	95.61/0.00	45	12.40 (11.78)	46.31/0.00	45	19.48 (26.42)	88.96/0.00
[4]TOC	44	4.64 (2.36)	11.12/0.06	45	3.38 (1.23)	8.10/1.37	28	3.75 (3.10)	12.56/0.10
[5]Ca^{2+}	30	32.43 (17.40)	72.43/9.62	45	35.01 (20.67)	125.50/6.16	35	50.36 (24.17)	122.55/8.64
[5]Mg^{2+}	30	5.56 (4.03)	16.10/0.42	45	6.56 (5.55)	19.62/0.21	35	5.43 (3.82)	17.48/0.41
[5]K^{+}	30	0.86 (0.45)	1.99/0.07	45	1.20 (1.07)	7.48/0.28	35	0.49 (0.28)	1.47/0.19

interaction soil group × forest type was not (log-transformed data of soil carbonate content; soils: $F_{2,131} = 6.59$, $p = 0.019$; forest type: $F_{2,131} = 4.48$, $p = 0.013$; interaction soil group × forest type: $p = 0.724$).

Brûlé characteristics of *T. aestivum* (size, productivity, vegetation cover) did not show significant relationships with total carbonate. In *T. melanosporum* brûlés, there was a significant relationship between productivity and total carbonate, which more productive sites exhibiting lower carbonate content (sqrt [% total carbonate] = 7.33033–1.53143 * sqrt [brûlé productivity, $r^2 = 0.11$; $p = 0.026$), indicating some modification of the total carbonate pool by that species.

There were significant no differences in active carbonate content among the three groups of soils due to large variations in active carbonate content between the three forest types, with very low contents in the most xeric forest type. Active carbonate content was highest in brûlés of *T. melanosporum* and lowest in brûlés of *T. aestivum*. The ratio active/total carbonate content was highest in brûlés of *T. melanosporum* and lowest outside brûlés, and the difference between these two soil groups was statistically significant (Fig. 4C). As this ratio is influenced by both effects of truffle species on total carbonate (a reduction by *T. aestivum*) and active carbonate (an increase by *T. melanosporum*), both truffle species showed a higher ratio compared to areas outside brûlés. In the most xeric forest type (type 2), the ratio active/total carbonate content was very low, likely reflecting moisture limitations on carbonate dynamics. Brûlé characteristics of *T. aestivum* did not have an impact on both parameters, whereas in brûlés of *T. melanosporum* there were positive relationships between brûlé size and productivity on the one hand and active carbonate and/or the ratio of active/total carbonate on the other hand. These relations were for brûlé size (log [% active carbonate] = - 0.0202982 + 0.444812 × log [brûlé size+1]; $r^2 = 0.29$; $p < 0.001$); and for the ratio of active/total carbonate (log [% active/total carbonate] = 0.278966 + 0.64649 * log [brûlé size+1]; $r^2 = 0.10$; $p = 0.031$). For brûlé productivity, the relation was: log [act/tot carbonate] = 0.558964 + 0.331396 × sqrt [prod]; $r^2 = 0.10$; $p = 0.031$).

There was no significant difference in exchangeable Mg^{2+} between the three soil types. Exchangeable Ca^{2+} was significantly higher in *T. melanosporum* than in *T. aestivum* brûlés or soils outside brûlés (Fig. D; $F_{2,107} = 7.54$, $p < 0.001$), an effect consistent with the stronger modification of calcium carbonate, as assessed through the ratio active/total carbonate. For both Mg^{2+} and Ca^{2+}, both truffle species did not exhibit significant relationships with brûlé characteristics.

There were significant differences between the three soil groups in soil exchangeable K^{+} (Fig. 4E; $F_{2,107} = 8.83$, $p < 0.001$). *Tuber aestivum* brûlés showed significantly higher K^{+} than *T. melanosporum* brûlés. For both truffle species, there were no relationships between brûlé characteristics and K^{+}.

TOC content was significantly higher outside brûlés than inside brûlés of both *Tuber* species (Fig. 4F; Kruskal-Wallis test), indicating an impact on both soil inorganic and soil organic components. There were no relationships between TOC content and *T. aestivum* and *T. melanosporum* brûlé characteristics.

Soil properties were summarized using Principal Components Analysis. The PCA that included all soil samples that were completely surveyed (91 cases) is shown in Supplementary Fig. 1. The first axis, PC_1, accounted for 29% of the variance, PC_2 23%, and PC_3 16% (Bartlett's Test: $p = 0.0$, n = 91). The first axis separated soils with high contents of active carbonates, Ca^{2+}, and a high active/total carbonate ratio from soils with high contents of Mg^{2+} and represents a separation between brûlé soils and soils outside brûlés. PC_2 separates sites with high pH, total carbonate, and K^{+} and separates forest types, with the most xeric forest being recognized by higher pH and total carbonate content. PC_3, which explains 16% of the variance, separates sites with high TOC content and high total carbonate content from sites where both soil properties are low. This likely coincides with brûlés created by *T. melanosporum* and *T. aestivum*.

3.3. Differential niche construction by T. aestivum and T. melanosporum

Soil properties outside brûlés of *T. aestivum* and *T. melanosporum* did not exhibit significant differences, except for pH, which was much higher in soils outside the brûlés of *T. aestivum* than in soils outside the brûlés of *T. melanosporum* (Table 2).

Soils of brûlés of *T. aestivum* had significantly higher pH than soils

Table 2
Soil properties inside and outside brûlés of *T. aestivum* and *T. melanosporum1*. n = number of pairwise comparisons in a paired *t*-test. [*ns: not significant; *: 0.01<p < 0.05; **: 0.001<p < 0.01; ***: p < 0.001*]. [*[1](g/100 g); [2]Exchangeable cations expressed in cmol $^{+}$ kg^{-1}. [3]Part of the soil parameters of* T. melanosporum *brûlés have been published before in García-Montero et al. (2009b)*].

T. aestivum brûlés	n	Inside	Outside	p
pH	20	7.91	7.63	***
[1]Total carbonate	20	16.96	36.23	**
[1]Active carbonate	20	1.62	2.58	n.s.
[1]Active/total carbonate	20	11.6	12.6	n.s.
[1]TOC	20	3.15	4.12	*
[2]Ca^{2+}	20	33.9	34.8	n.s.
[2]Mg^{2+}	20	6.15	6.26	n.s.
[2]K^{+}	20	1.09	0.85	n.s.
[3]*T. melanosporum* brûlés	n	Inside	Outside	p
pH	20	7.89	7.91	n.s.
[1]Total carbonate	20	21.66	33.63	***
[1]Active carbonate	10	3.17	1.67	**
[1]Active/total carbonate	10	44.3	12.5	**
[1]TOC	10	2.89	4.94	n.s.
[2]Ca^{2+}	10	44.0	27.8	*
[2]Mg^{2+}	10	3.24	4.18	n.s.
[2]K^{+}	10	0.62	0.87	n.s.

outside brûlés and significantly lower contents of total carbonate and TOC. Soils of brûlés of *T. melanosporum* had significantly lower carbonate and significantly higher active carbonate, active/total carbonate ratio, and Ca^{2+} than soils outside brûlés (Table 2).

4. Discussion

This study demonstrated significant soil modification by two truffle species, *T. aestivum* and *T. melanosporum*. Modifications are already visible from the creation of small to large bare patches with little or no vegetation cover (Fig. 1), and these sites, known as brûlés, are known by truffle hunters as the locations where they collect their highly-priced bounty. Brûlés differ from surrounding soils in several soil chemical properties, including a higher content of active carbonates (and hence a higher active/total carbonate ratio), Ca^{2+}, and a lower content of TOC. Our data also showed significant differences in habitat modification by both truffle species, with differences in brûlé area, the extent to which vegetation of non-host plants is reduced, pH, and Ca^{2+}.

Are these habitat modifications an instance of niche construction by ectomycorrhizal fungi? Matthews et al. (2014) listed two criteria that enable the recognition of niche construction. The first criterion of significant modification of environmental conditions is clearly met by both species, as shown by Tables 1 and 2, and the PCA in Supplementary Fig. 1. In addition, the second criterion is also met by both species because the formation of brûlés increased the fitness of these truffles. Truffles are hypogeous fungi that form sporocarps below-ground and therefore need active dispersal, often by mammals that are attracted by truffle aroma. Truffle spores are not digested in the stomachs of these mammals, therefore survive the gut passage, and their dung then acts as truffle inoculum (Ori et al., 2018; Piattoni et al., 2014). If we consider sporocarp production a proxy for spore production and hence for fungal fitness (Pringle and Taylor, 2002), the positive relationship between brûlé area (a proxy for its age) and sporocarp productivity implies increasing fitness with larger areas of modified habitat. The result by Liu et al. (2016) and Oliach et al. (2022) showing an increase in the number of truffle sequences upon ageing of plantations also supports the claim that brûlé formation enhances fungal fitness by reducing competition with other ectomycorrhizal fungi and partly eliminating arbuscular mycorrhizal fungi and plants, unless these plant species are favorable companion species.

Regarding the second criterion of Matthews et al. (2014), which implies that environmental modifications must influence selection pressures on recipient species, the creation of brûlés selects herbaceous plant species that are better able to withstand the conditions created by the truffles. González-Armada et al. (2010) noted that inside brûlés there is a larger representation of annual plant species. Among the species listed as characteristic for brûlés they also mentioned plants that are non-mycorrhizal or only sparsely colonized by arbuscular mycorrhizal fungi. In our sites, *Digitalis obscura* L. was often present in brûlés, a species known for its ability to grow on sites with high exchangeable Ca^{2+}, low exchangeable Mg^{2+}, and low P availability (Roca-Pérez et al., 2005). Taschen et al. (2020) compared six perennial plant species for their sensitivity to truffle brûlés and potential positive impact on truffle production. From this limited survey, it seems that sensitivity and positive impact are negatively correlated, suggesting that truffles could 'select' those plant species that have a subsequent beneficial impact on truffle production. However, a study by Barou et al. (2023) suggested that *Thymus vulgaris* L. suppresses rather than enhances truffle production, especially when it is arbuscular mycorrhizal. Menta et al. (2014) and Pinto et al. (2017) described how *T. aestivum* brûlés caused changes in soil fauna, with some collembolans benefitting from the changes in soil chemical conditions, while other collembolan species declined.

Truffle brûlés also exert a selective impact on arbuscular mycorrhizal and ectomycorrhizal fungi. Mello et al. (2015) observed a reduced arbuscular mycorrhizal fungal species richness inside *T. melanosporum* brûlés, and Taschen et al. (2020) observed decreased abundance of arbuscular mycorrhizal fungi in these brûlés. Napoli et al. (2010) and Mello et al. (2011) also noted a reduced species richness of ectomycorrhizal fungi, especially of members of the Basidiomycota, inside brûlés, equally indicating the selection exerted by truffles when creating brûlés. This shift toward a larger representation of Ascomycota in brûlés than outside brûlés was also noted by Taschen et al. (2015). There is almost certainly a selection on the ectomycorrhizal host tree of truffles as well. The habitat modification caused by truffles increased the content of active carbonates and could increase the risk for iron chlorosis in the host trees (Hall et al., 2007). In this regard, the amount of active carbonate is often used as a proxy for predicting iron chlorosis in agroecosystems (Tagliavini and Rombolà, 2001). The brûlé could therefore transform the mutualistic relationship into unbalanced interactions, as proposed by García-Montero et al. (2009a, 2012). Soils with a high content of active carbonate in the brûlés lead chlorosis, which favors increased mycorrhization by *Tuber* spp., which in turn promotes the formation of new amounts of active carbonate according to a feedback model. The concept of the mutualistic niche, as a generalized case of mycorrhizal fungal niche construction (Peay, 2016), needs to be nuanced, as the niche construction by the ectomycorrhizal does not inevitably enhance the fitness of the tree. Our studies confirm that brûlé construction generates a low-quality productive environment with negative effects on soil quality (Innangi et al., 2020). Streiblová et al., (2012) concluded that *T. melanosporum* and *T. aestivum* brûlés had established an efficient environmental strategy to gain and control the space needed for their survival and reproduction, where they suppress the richness and biodiversity of indigenous organisms. It is interesting that Mamoun and Olivier (1993) observed increased growth of hazel but reduced mycorrhizal colonization by *T. melanosporum* after the addition of iron. Niche construction by truffles has been well-known by truffle cultivators. Dessolas et al. (2008) and Chevalier and Palenzona (2011) proposed that *Tuber* spp. "*prepare their nest to produce carpophores*". Our report on niche construction by truffles, therefore, expands the earlier suggestion by Bragato (2014) on the perturbational niche construction by truffles by changing aggregate sizes and soil porosity.

Matthews et al. (2014) and Laland et al. (2016) indicated that fulfilling these two criteria suffices for meeting the definition of niche construction as an ecological process. They added a third criterion to evaluate the evolutionary consequences of niche construction, viz. that there must be an evolutionary response in at least one of the species that has been caused by the environmental modification. We did not address that evolutionary question in our study. However, we consider it likely that there are evolutionary responses in those arbuscular mycorrhizal plant species that are both suitable companion species that enhance truffle productivity and escape from the detrimental effects that truffles exert on the surrounding vegetation and/or on the ectomycorrhizal host that must cope with increased risk of chlorosis. The contrary data reported by Taschen et al. (2020) and Barou et al. (2023) on the ecological impact of *Thymus* species would be consistent with local differentiation and evolution.

Whereas our data show that both *T. aestivum* and *T. melanosporum* construct their own niche, both species do so in somewhat different ways. This differential effect can be clearly seen from the fact that soils outside brûlés of both species are largely similar, whereas the brûlés are dissimilar. While these differences may reflect habitat choice by both truffle species in what would seem homogeneous sites, we would argue that these differences reflect problems in delimiting brûlés.

Both truffle species co-occur in calcareous regions of southern Europe and then compete depending on environmental conditions. Habitat characteristics of *T. melanosporum* and *T. aestivum* were provided by Jaillard et al. (2016) and Robin et al. (2016), respectively. However, these authors did not report on habitat modification by truffles. Wehrlen et al. (2009) indicated that *T. aestivum* is less climatically demanding and has a wider distribution area in Europe than *T. melanosporum*. *Tuber aestivum*, as opposed to *T. melanosporum*, can produce sporocarps in soils with more variable water levels and can

withstand flooding (Thomas, 2021). It is also able to better withstand dry conditions, as the brûlé size of that species was not lower in the most xeric forest type, whereas that of *T. melanosporum* was significantly reduced. A study by Piñuela et al. (2021) indicated that under irrigation *T. melanosporum* replaced *T. aestivum*, confirming higher drought tolerance of the latter species. Replacement of *T. melanosporum* by *T. aestivum* has been reported by Turgeman et al. (2012) and García-Montero et al. (2014). Both species seem to benefit from climate change (higher temperatures), and areas suitable for cultivation will likely increase in central Europe (Čejka et al., 2020).

In most cases, *T. aestivum* brûlés were larger and non-host plant cover was greater than *T. melanosporum* brûlés. Other differences between both species are visible in the PCA (Supplementary Fig. 1), Tables 1 and 2, and Supplementary Table 2. Soils of *T. melanosporum* brûlés had higher pH than those of *T. aestivum*, but the difference in pH in properties inside and outside brûlés was larger in the latter species. There was also a significant positive relationship between *T. aestivum* brûlés size and soil pH. These data fit with observations by Gryndler et al. (2017) that liming increased the mycelial biomass of *T. aestivum*, and observations by Hilszczańska et al. (2018) that calcium carbonate content was the best predictor for *T. aestivum* productivity. The active/total carbonate ratio was higher in brûlés of *T. melanosporum* and was significantly positively correlated with brûlé area, suggesting the ongoing formation of active carbonates from the total carbonate pool. Higher exchangeable Ca^{2+} in brûlés of *T. melanosporum* would fit with ongoing modification of the total carbonate pool. Nevertheless, in both species, the exact mechanisms of brûlés formation are still poorly known.

Brûlés of both species showed significantly lower contents of TOC, demonstrating that niche construction has consequences for both the organic and inorganic components of the soil. It had been hypothesized that truffle species possess some saprotrophic activity during sporocarp formation. However, a study by Zeller et al. (2008) demonstrated that truffles do not exhibit saprotrophy during sporocarp development. The mechanism for soil organic C loss may be due to a lower input of fresh litter (through the reduction in vegetation cover). However, the amounts of C lost are much larger than can be explained by differential inputs of fresh C. It is more likely that the creation of brûlés results in environments that are more conducive to decomposition. In that respect, it may be noteworthy that Innangi et al. (2020), who also observed lower TOC inside brûlés of *T. aestivum* than outside, reported no changes in peroxidase activity inside brûlés compared to outside brûlés. A hydrolytic enzyme, related to the breakdown of more labile C sources, was significantly lower in brûlés than outside. One hypothesis is that inside brûlés, where total carbonate content was reduced, organic C that was protected by those carbonates was released and became available for decomposition (Virto et al., 2018). This mechanism could equally explain the observations by Bragato (2014) on the decline of the fraction of macroaggregates in brûlés and the protection of organic carbon in these macro aggregates. Transformation of carbonates may be caused by organic-acid production by truffle species, but the high carbonate content of these soils would buffer against pH decrease. Poitou (1988) showed that, *in vitro*, *T. melanosporum* mycelia acidify the environment and dissolve calcium carbonate. Callot (1999) reported an experiment on the growth conditions of *T. melanosporum* mycelia where pH values of 5.5–6 were reached in all cultures. However, to the best of our knowledge, there are no reports of oxalate or citrate exudation by truffle mycelia.

Truffle species can persist for at least twenty years on the same tree (Oliach et al., 2022). Truffle hunters even provided anecdotal evidence that brûlés have persisted for over fifty years. However, habitat modification cannot go on indefinitely, and with the exhaustion of the carbonate pool, the productivity of brûles should decline. Queralt et al. (2017) showed that *T. melanosporum* extraradical mycelium biomass was more abundant in productive brûlés than in non-productive ones. With the decline of truffle biomass, habitat modification declines. It may be possible that the brûlé is the arena for an oxalate-carbonate cycle, where fungi dissolve carbonate through the exudation of oxalic acid, and bacteria subsequently oxidize this calcium oxalate into carbonate (Verrecchia et al., 2006), but the mechanism has not often been demonstrated (Cromack et al., 1979).

In conclusion, brûlé is an example of niche construction by ectomycorrhizal fungi in a limited spatial sense, where habitat modification is visible, and measurable in terms of soil chemistry, soil physics (Bragato, 2014), and soil biology -*Mello* et al. *(2011) reported significant differences in fungal species composition inside and outside brûlés; Mello* et al. *(2013) described differences in bacterial communities inside and outside brûlés*-. However, it is still necessary to deepen the brûlé niche construction processes by analyzing other soil parameters, e.g., N, which likely will be reduced after the disappearance of soil organic matter (Table 1), P, micronutrients like Fe and Zn (whose bioavailability will decrease due to increased active carbonate) could further demonstrate soil habitat modification. Therefore, additional research on brûlé niche construction is required, focusing, for example, on quantitative relations between truffle mycelial biomass and the extent of habitat modification or on the legacy effects in brûlés after the truffles have disappeared.

On the other hand, habitat modification by fungi could go beyond brûlé, and this phenomenon could be more widespread. Niche construction by ectomycorrhizal fungi, which had already been mentioned as a keyword by Peay (2016), is ubiquitous, as these fungi pave the way for tree establishment. This is visible in the case of pine and eucalyptus co-invasion and their associated ectomycorrhizal fungi, which sometimes show significant ecosystem effects. For example, Chapela et al. (2001) reported massive losses of soil organic carbon after pines with their associated ectomycorrhizal fungi invaded paramo grasslands in Ecuador, and Dickie et al. (2010) linked such co-invasions to invasion meltdown. Another prominent example of niche construction by ectomycorrhizal fungi is that formed by mat-forming fungi (Cromack et al., 1979). Trappe et al. (2012) listed some changes in soil chemical properties caused by these mat-forming fungi. Another example is the structure called shiro formed by the ectomycorrhizal fungus *Tricholoma matsutake* (S. Ito & S. Imai) Singer. Lian et al. (2006) describe changes in fungal communities after shiro formation. Therefore, a more general review of niche construction by ectomycorrhizal fungi would be necessary, where their edaphic habitat modifications can be discussed in more detail. A better understanding of mutualistic niche construction processes by mycorrhizal fungi could contribute to improving sustainable forestry, environmental, conservation, and agricultural engineering and management, such as proposed by García-Montero et al. (2017) through the “rhizoculture” approach.

Author contributions

LGGM, TWK, and VJM designed the study. LGGM, IVA, and CM performed the field and laboratory tasks. VJM, TWK, and LGGM performed and discussed the statistical analyses. LGGM, TWK, and VJM wrote the manuscript draft. All authors edited the manuscript and approved the final version.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data and code used to generate the results of this study are available at https://zenodo.org/deposit/8025273

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Appendix A. Supplementary data

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