Eco-archaeological excavation techniques reveal snapshots of subterranean truffle growth

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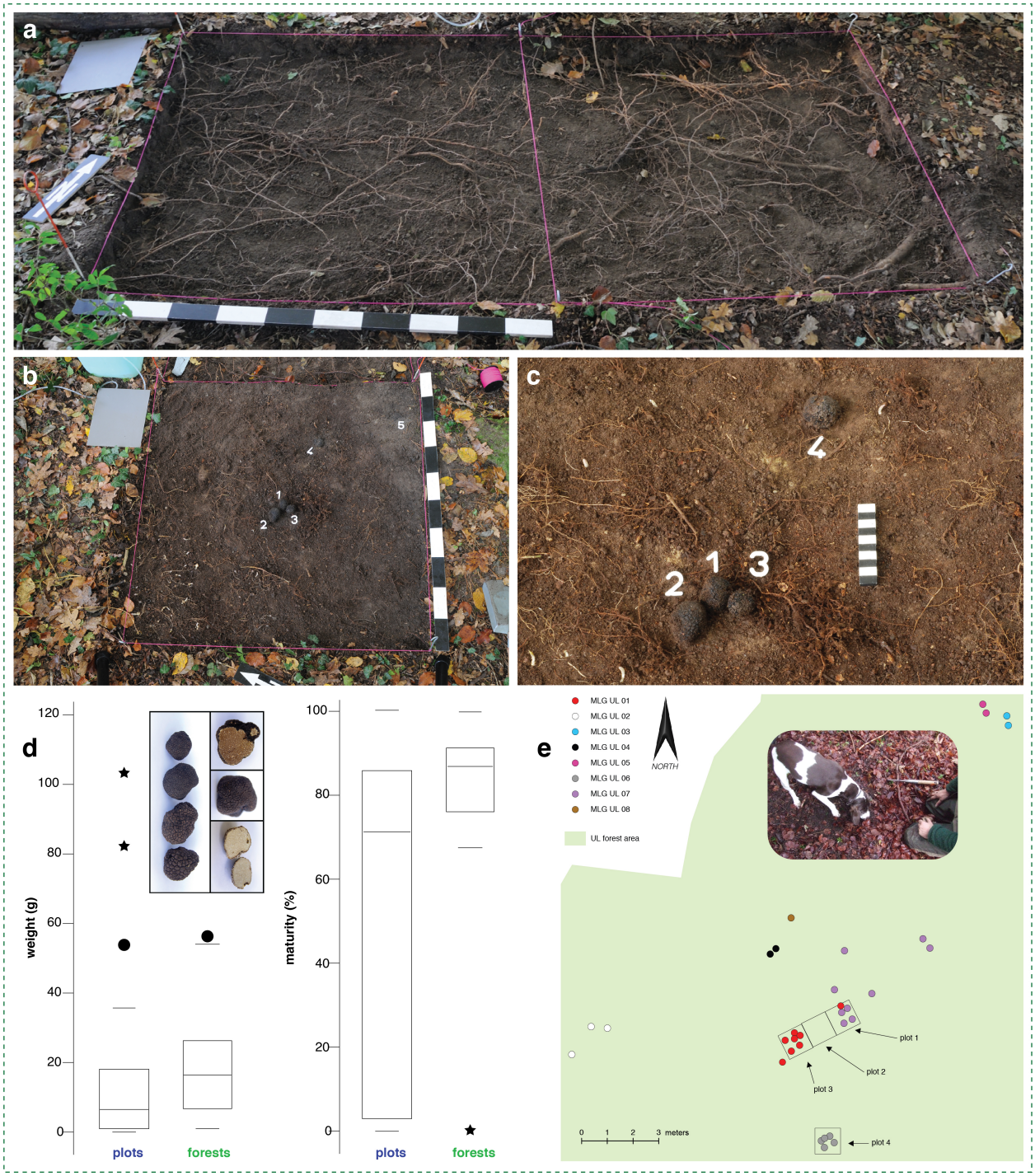

Graphical Abstract Our eco-archaeological excavations in different natural habitats in southern Germany provide snapshots of the subterranean formation, maturation, and genetic variation of *in situ* Burgundy truffles (*Tuber aestivum*) and should encourage cross-disciplinary studies to

further improve harvest practices and management strategies of one of the most coveted gourmet foods.

Abstract

Despite its status as a highly-prized and coveted fungi in gastronomy, many aspects of the subterranean life cycle of the Burgundy truffle (*Tuber aestivum*) are still unknown, because *in situ* observations of the formation and maturation of truffle fruitbodies remain difficult. Here, we adopted a suite of archaeological fine-scale excavating techniques to provide unique spatiotemporal snapshots of Burgundy truffle growth at three sites in southern Germany. We also recorded the relative position, fresh weight, maturity level and genotype composition of all excavated fruitbodies. Varying by a factor of thousand, the fresh weight of 73 truffle ranged from 0.1–103.2 g, with individual maturity levels likely representing different life cycle stages from completely unripe to fully ripe and even decaying. While only a slightly positive relationship between fruitbody weight and maturity level was found, our results suggest that genetically distinct specimens can exhibit different life cycle stages at the same period of time and under the same environmental conditions. We therefore argue that truffles are likely able to grow, mature and ripe simultaneously between early summer and late winter of the following year. Our case study should encourage further eco-archaeological truffle excavations under different biogeographic settings and at different seasons of the year to gain deeper insights into the fungi's subterranean ecology. The expected cross-disciplinary findings will help truffle hunters and farmers to improve their harvest practices and management strategies.

Keywords: archaeological excavation; fruitbody formation; fungal phenology; gourmet food; truffle ecology, *Tuber aestivum*

1. Introduction

Truffles are ectomycorrhizal fungi of the phylum Ascomycota and produce subterraneous fruitbodies (i.e. ascocarps or sporocarps). With an ever-growing number of newly described truffle diversity (*Tuber*, Tuberaceae) from around the world that is clearly exceeding 200 species (Bonito et al., 2013; Guevara et al., 2013; Reyna and Garcia-Barreda, 2014; Berch and Bonito, 2016; Suwannarach et al., 2017; Wan et al., 2017; Arthur et al., 2018; Eberhart et al., 2020), only about a dozen of them are of commercial importance (Boa, 2004; Bonito et al., 2010; Chevalier and Frochot, 2002; Hall et al., 2003). Recent advancements in molecular biology have contributed to a better understanding of evolutionary origins, population dynamics and symbiotic relations of different truffle species (Martin et al., 2010; Murat and Martin, 2016). However, many aspects of the subterranean life cycle of the genus, such as the formation, duration, maturation and deterioration of their fruitbodies, remain enigmatic and require innovative scientific endeavours (Büntgen and Egli, 2014; Büntgen et al., 2017). Interdisciplinary and international research projects are now indicating that Burgundy truffles (*Tuber aestivum*) are more widely distributed in ecologically suitable habitats across central Europe than previously recognised (Stobbe et al., 2012; Molinier et al., 2016c; Čejka et al., 2020; Puliga et al., 2021).

A marked long-term decline in the annual rates of winter harvests of the Périgord black truffle (*Tuber melanosporum*) across its Mediterranean natural and cultivated environments since the 1970s has been associated with warmer and drier summers in southern Europe (Büntgen et al., 2011, 2012, 2019). An ever-growing global demand for the Périgord and other *Tuber* species has contributed to the recent expansion of a vibrant 'truffle cultivation industry'. The ecologically sustainable truffle sector likely also appears economically lucrative under suitable environmental conditions in parts of California, Chile, southern Europe, South Africa, Australia

and New Zealand (Hall et al., 2003; Stobbe et al., 2013a; Reyna and Garcia-Barreda, 2014; Thomas and Büntgen, 2019).

The fruiting-bearing seasons of most truffle species are in winter. For instance, the Périgord black truffle is usually harvested between November and March, whereas the white Piedmont truffle (*Tuber magnatum*) is mainly harvested from late summer to winter (Callot, 1999; Tejedor-Calvo et al., 2021), and rarely after January. The Burgundy truffle, however, can produce fruitbodies during a much longer season from at least June until March of the following year (see Čejka et al., 2020 for a review of the ecological requirements of Burgundy and Périgord truffles). The Burgundy truffle is also less selective to soil properties, plant hosts and climate conditions as compared to other truffle species (Büntgen et al., 2012; Stobbe et al., 2013a). The optimal mean annual temperature for Burgundy truffles is ~10° C, and the ideal July and January temperatures are ~20° C and ~2° C, respectively (Čejka et al., 2020). The optimal annual precipitation total for Burgundy truffle growth is ~700 mm, with overall dryer conditions in summer. The species' optimal soil pH is ~7.5, and favourable host trees include *Carpinus betulus*, *Corylus avellana*, *Fagus sylvatica*, *Ostrya carpinifolia*, *Picea abies*, *Pinus nigra*, *Pinus sylvestris*, *Quercus cerris*, *Q. petraea*, *Q. pubescens*, *Q. robur*, and *Tilia cordata*. Given their wide ecological range (Schwärzel, 1967; Hall et al., 2008; Čejka et al., 2020), it has been argued that Burgundy truffles are one of the most abundant edible fungi in mixed broadleaf forests and cultural land on calcareous soils in central Europe (Stobbe et al., 2012).

In contrast to other high-value forest mushrooms, such as *Boletus* spp., *Cantharellus* spp. and *Laccaria* spp., which can develop their fruitbodies during a few days (Moore et al., 2008; Halbwachs et al., 2016), hypogenous truffle fruitbodies are expected to grow more slowly (Callot, 1999; Olivier et al., 2012; Zarivi et al., 2015). However, it is still unknown how much

time truffles need to develop and how many generations of fruitbodies can occur within one ‘growing’ season between the formation of primordia and the realisation of a fully mature and ripe gleba (Büntgen et al., 2017). The species’ subterranean life cycle dynamics, together with complex host interactions and the lack of non-destructive investigation techniques (Luoma et al., 1991), are the main reasons for the yet little understood biotic and abiotic drivers of truffle growth (Pacioni et al., 2014). Localising truffles *in situ* is only possible with the help of well-trained sniffing dogs or, very rarely, with domestic truffle-pigs (or occasionally also by searching and flowing truffle-specific flies, such as *Suillia tuberiperda*), which all have the olfactory sense capable of detecting the volatile aroma of fruitbodies from a distance of up to 50 meters (Olivier et al., 2012; Splivallo et al., 2011; Splivallo and Culleré, 2016). Consequently, our knowledge about the truffles’ hidden belowground mode of life is restricted to only those fruitbodies that were detected (Trappe and Claridge, 2010). In fact, we simply do not know how selective a harvest is and how well it represents the actual abundance of fruitbodies – the absence of evidence is no evidence of absence. Non-systematic and non-destructive harvests are therefore likely dominated by specific aroma concentrations, fruitbody dimensions and locations, as well as hunter/dog ability. The aroma profile of truffles seems to be influenced by individual genotypes (Molinier et al., 2015), and not by the geographical origin or maturity level (Niimi et al., 2021). Since the development of aromas most likely depends on the presence of specific bacteria (Splivallo and Culleré, 2016), no correlation between gleba colour and aroma intensity has been reported. Depending on the percentage of fully pigmented spores, and thus the level of maturity and ripening (Molinier et al., 2016b), the gleba of Burgundy truffles can vary from white-ochre to dark brown. Differences in the colour and texture of fruitbodies have been proofed useful visual criteria for species identification and distinction (Chatin, 1887), which may

otherwise be considered genetically identical, such as *Tuber aestivum* and *Tuber uncinatum* (Molinier et al., 2013).

Here, we introduce a novel eco-archaeological approach to study *in situ* aspects of subterranean life cycle dynamics and genetic structures of the Burgundy truffle in three different natural habitats in Baden-Württemberg, southern Germany. We apply a suite of fine-scale excavation techniques to reveal high-resolution, spatiotemporal snapshots of the composition, size and developmental stage of a wide-range of individual truffles. Our approach not only exposes fruitbodies in the investigated soil layers, but also relates each of them to its edaphic environment, and further describes the obtained genotype composition. Finally, we outline innovative pathways of cross-disciplinary research to help truffle hunters and farmers to improve their harvest practices and management strategies.

2. Material and methods

2.1 Truffle excavations

Three natural truffle habitats in southern Germany around 47°50' N and 8°50' E, where Burgundy truffles (hereinafter consequently and exclusively referred to as truffles) are most abundant between autumn and winter (Stobbe et al., 2012), were selected for our eco-archaeological excavation experiments (**Table 1**). Two plots were located in mixed beech forests on glacial moraine deposits (UL; Ueberlingen) and on scree material of Upper Jurassic limestone (RT; Rietheim). The third plot was located in a pure Norway spruce (*Picea abies*) stand on calcareous shallow rendzina (BG; Balgheim), which was previously a mixed beech forest that was stocked with Norway spruce some decades ago (Stobbe et al., 2013b; Moser et al., 2017). Truffles were found frequently at all of the experimental sites, and each site was monitored

144 regularly to ensure that no harvesting took place at any of the plots during at least three months
145 before the excavations were conducted. The excavations were performed at two points in time
146 during the approximate regional-specific peak season of fruitbody production between October
147 and December. All of the 1 × 1m excavation plots were selected by trained truffle dogs that
148 identified and marked the presence of fruitbodies, but were not allowed to dig any of them out. A
149 row of three neighbouring plots and one plot approximately three meters away were excavated in
150 UL (28–29th October 2011). A row of three contiguous plots was excavated in BG, and only one
151 plot was excavated in RT (both 11th December 2011).

152 We used standard archaeological equipment and techniques for the stratigraphically precise
153 two-dimensional excavation of all eight plots (e.g., trowels, small tools, and brushes). The
154 location of each truffle fruitbody was levelled, measured and mapped relative to its surrounding
155 soil profile. After a preliminary levelling of the intact original surface horizon, using a hose level
156 instrument with a precision of 0.5 cm, the leaf litter was removed and the top of the O horizon
157 was exposed, which was again levelled and photographed. The process was repeated for each of
158 the remaining soil horizons, as we carefully followed the boundaries of the soil stratification
159 (**Fig. 1**). The excavated soil was sieved at three-millimetre mesh size to detect and document
160 small specimens. However, wet-sieving as described by Pacioni et al., (2014) was not possible
161 during our field experiments, which likely affected the detection of the smallest primordia. Once
162 discovered *in situ*, each fruitbody was kept in its original position and mapped relative to the
163 proximate soil profile. Moreover, we visually examined any possible connections between the
164 truffle peridium and surrounding plant fine root systems (**Fig. 2**). After exposure and
165 photography, the exact position of each fruitbody was recorded, and each specimen was labelled
166 and packed in zip-lock polyethylene bags for further laboratory analyses. Plots were vertically

167 excavated in thin strata until far into the unaltered mineral subsoil, and the resulting sections
168 were documented.
169 Furthermore, we extracted 12–16 soil cores of 5 cm increment on a regular grid at 0–5 and
170 10–15 cm soil depth in each plot (except for the additional plot in UL). These samples were used
171 to analyse the presence of truffle ectomycorrhizas (ECM) on fine root tips. In one plot each in
172 UL and BG, soil was also sampled at a depth of 20–25 cm. In RT, we also collected soil
173 containing roots in close proximity of eleven truffles supplementary to the regular plot sampling.
174 All soil cores were stored in plastic bags and kept cool until further laboratory treatment. After
175 our truffle dogs indicated that no ripe truffles were present at deeper soil levels, all plots were
176 backfilled, and the terrain was restored to its original state. Finally, the truffle dogs harvested
177 'all' ripe fruitbodies in the surrounding forests up to 50 meters from the excavated plots, and all
178 truffles and their position were recorded electronically and converted into a spatially explicit
179 inventory. These data were processed with a surface mapping program (Golden Software,
180 Golden CO), and photos were rectified and geo-referenced by means of the PhoToPlan module
181 in AutoCAD. Each truffle was gently washed under tap water and morphologically described.
182 The fresh weight of each specimen was documented, and its stage of maturity determined
183 microscopically according to a modified protocol from Zeppa et al., (2004), which evaluates the
184 presence and pigmentation of spores (Molinier et al., 2016b). Last but not least, a piece of the
185 gleba was extracted from each fruitbody and stored at -20° C for the assessment of maternal
186 genotyping.
187
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2.2 Genotyping of ectomycorrhizal root tips and statistical investigations

From each 5 cm increment soil core, the roots were extracted and washed under low running water, gathered in a 1 mm sieve, and then placed in a petri dish. Between zero and 13 potential *Tuber aestivum* ECMs were morphologically detected under a binocular (Agerer, 2006; Molinier et al., 2016b). ECMs were individually placed in lysis buffer for subsequent genotyping. DNA was isolated from ECM tips and from the gleba of all truffles using the DNeasy® 96 Plant Kit and following the manufacturer's instructions (Qiagen, Hilden, Germany). To identify *Tuber aestivum* on ECM tips, we used primer pairs that target a *Tuber aestivum* specific single-copy gene (TuGM4108) (Todesco et al., 2019). We applied the same conditions as indicated by these authors with minor modifications, i.e. the JumpStart™ REDTaq® ReadyMix™ reaction Mix (Merck KGaA, Darmstadt), performed the reaction in 15µl and performed 40 cycles on a Veriti Thermal Cycler (ThermoFisher Scientific, Switzerland). The PCR products were stained with MiDORI Green Xtra (NIPPON Genetics Europe), visualized using a c300 digital imager (Azure Biosystems, Dublin, USA) and checked for presence/absence of the band. Positive samples were further genotyped at the individual level using eleven nuclear simple sequence repeat (nSSR) markers as well as the mating type following protocols of Molinier et al., (2016a). To identify multilocus genotypes (MLGs) and true clones (Psex analysis), we used GenAlEx 6.512b software in Excel 2016 (Peakall and Smouse, 2012) and MLGsim with 1000 simulations (Stenberg et al., 2003), respectively. Psex was calculated over all sites as well as for each site/population separately. The truffle MLGs in UL were subsequently mapped with ArcGIS 10.2 (ESRI, Redlands, California, USA).

Spearman's rank correlation coefficients were applied to assess meaningful relationships between the weight and maturity of fruitbodies. To evaluate differences of maturity or weight

213 within and between the excavated truffles and those detected by our dogs outside the excavation
214 plots, the Mann-Whitney U test was calculated. Due to different numbers of truffle ECMs in the
215 two soil horizons at 0–5 cm and 10–15 cm, the total number of *Tuber* ECMs was summed for
216 each quadrat and horizon, and analysis of variance was applied. All tests were performed after
217 checking for homoscedastic and heteroscedastic distributions of residuals.
218
219 **3. Results**
220 *3.1 Truffle characteristics and locations*
221 A total of 40 *Tuber aestivum* fruitbodies were discovered in six excavation plots, with 4–13
222 specimens per plot (**Table 2**). In two out of eight excavation plots (UL 2, BG 2), which were not
223 directly selected by our dogs, though were adjacent to the dog-selected plots, no truffles were
224 found. Fresh weight of the individual fruitbodies was between 0.1 g (UL1 and RT1) and 103.2 g
225 (UL1), and the average weight was 18 g. Maturity stages ranged from 0 % (no spores) to 100 %
226 (fully developed with abundant pigmented spores), with a mean of 55 %. Ten fully unripe
227 truffles (maturity 0 %) were very small (0.1–1.2 g), whereas the weight of the seven fully ripe
228 fruitbodies (maturity 100 %) ranged from 0.9–31.5 g. Truffles of different maturation stages
229 were found in the immediate proximity to each other. A total of 33 fruitbodies in the three forest
230 surroundings of the excavation plots were located by our dogs, harvested and recorded (**Table**
231 **2**); raising the question of how many truffles our trained dogs missed (see discussion below).
232 With an average fresh weight of 19 g, these 'reference' truffles varied from 0.9–56.0 g (both at
233 BG). Maturity values were between 0 and 100 % (not recorded at UL), with a mean of 78 %
234 (compared to 55 % of the excavation harvests), and a significantly different maturity distribution
235 (p-value = 0.01) (**Fig. 3**). Only one small 2.9 g fruitbody (out of 35 specimens) that was detected

236 by our dogs in the surrounding forests was fully unripe (0 % maturity), whereas ten of the 40
237 excavated plot truffles were fully unripe (0 % maturity). The weight of all 73 truffles ranged
238 from 0.1–103.2 g and their and maturity varied from 0–100 % (**Table 2**; **Fig. 4**). A significantly
239 positive Pearson's correlation coefficient was found between the maturity and weight of all 73
240 fruitbodies (rho = 0.57, *p*-value < 0.01, n = 58). Within each site, we also found significantly
241 positive Pearson's correlation coefficients for RT (rho =0.74, *p*-value <0.01, n= 23) and UL (rho
242 = 0.71, *p*-value = 0.01, n = 15), but not for BG (rho = 0.13, *p*-value = 0.59, n = 20).
243 All 40 excavated truffles were situated within the upper 5–10 cm organic layer of the O
244 horizon (which itself is important information for cultivators), and the underpart of each
245 fruitbody was directly above or slightly within the lower mineral soil. A few truffles were
246 growing marginally out of the upper soil surface through the litter layer, and the exposed fraction
247 of the peridium was characterised by substantially smaller warts. Growing around a tree root, one
248 fruitbody indicated a clear upward growth direction (**Fig. 3c**). Truffle locations were neither
249 correlated with distance nor with any kind of ground vegetation, nor with the thickness of the
250 litter and humus layer (**Fig. 1c**). No obvious pattern in the relative position of fruitbodies to fine
251 and coarse plant roots was found (**Fig. 2**). Moreover, we found no macroscopic evidence for
252 truffle mycelium at the fruitbodies, and mycelium connections to any mycorrhizas.
253
254 *3.2 Truffle genotypes*
255 We collected 724 ECM root tips from 224 soil cores that exhibited similar morphotypes as
256 truffles. After genotyping, 374 ECM root tips proofed to be *Tuber aestivum* (i.e. herein
257 abbreviated as truffle). Although we found truffle ECMs in all three soil layers, significantly
258 more truffle ECMs were detected between 10 cm and 15 cm, as compared to the uppermost soil

layer where the majority of truffle fruitbodies was located. Even at the depth of 20–25 cm we found more truffle ECMs than in the top soil layer of both plots (**Fig. 5**). We detected truffle ECMs in all six plots, including also the two middle plots in UL and BG, in which no truffle fruitbodies were present (**Table 3**; **Fig. 1c**).

The multilocus genotyping (MLG) of 11 nSSR loci and the mating type was successful for 88 % of the 73 analysed fruitbodies and for 67 % (91 % with ≥ 7 nSSR loci) of the 374 ECM samples. Particularly ECM samples from UL amplified less well and some loci were therefore missing, but when analysing the present ones, the overall pattern supported the results obtained from non-missing data. MLG analysis of the 40 truffles collected within the six excavation plots revealed 13 different individuals (MLGs) that formed truffle tissues (**Tables 2**, **3**). In UL, eight different MLGs were detected within and outside the excavation plots (**Fig. 6**). All truffles that were found within a plot showed the same MLG with the exception of one truffle found at a lower depth of 20–25 cm in plot 1. This truffle showed the same MLG as the ones found in the excavation plot 3 (MLG UL01; **Table 2**, **Fig. 6**). It also formed all the truffle ECMs found in plot 3 as well as in plot 2, where no fruitbody was found, but no ECM of this individual was detected in plot 1. There, all ECMs sampled at all depths were formed by the individual MLG UL07, which produced four truffles at the upper layer in plot 1 as well as in the surrounding forest. In BG, one dominant individual (MLG BG01) produced all truffle ECM root tips identified in the three plots and the truffles found in plot 1 and 3, as well as in seven of the nine truffles found in the surrounding area, the remaining two being produced by a second individual (**Tables 2**, **3**).

At this site, we additionally sampled soil containing mycorrhizal roots around eleven truffle fruitbodies. For only four we identified the presence of a total of 21 truffle ECM root tips. In

three cases all ECMs were formed by the same individual that produced the tissue of the respective truffle, and for one truffle that was found outside the excavation plot, we found another ECM formed by an individual not matching any of the multilocus genotypes detected in any other sample (**Table 3**).

4. Discussion

4.1 Truffle characteristics and locations

Our eco-archaeological experiment revealed the occurrence of different truffle fruitbody sizes and maturity levels in autumn (**Table 2**; **Fig. 4**). Varying life cycle stages, including extremely small and very large specimens (circa 0.1–100 g), as well as completely unripe (white) and totally overripe (rotten) fruitbodies were found in all excavation plots and their surrounding reference forest sites (**Figs. 3**, **6**). Genetically distinct individuals produced fruitbodies of varying sizes and maturation levels at the same point in time, indicating that a single specimen can exhibit different life cycle stages simultaneously. As opposed to the Piedmont white truffle, Burgundy truffles benefit from extended fruiting seasons between early summer and late winter (Callot, 1999; Le Tacon, 2016). Our findings agree with results of three independent truffle surveys in Hungary and Switzerland (Büntgen et al., 2017), which reported different maturation levels at different fruitbody sizes and at different periods of time. Comparison of our results with those from other *Tuber* species is problematic since most of them have much shorter growing seasons and likely only one life cycle. For example, *Tuber melanosporum* commonly, but not exclusively (Rebière, 1967), fruits in its Mediterranean habitats from the end of December until early March (Callot, 1999; Garcia-Barreda et al., 2021), and only small and unripe primordia between 0.5–1.0 mm have been described to form in summer and autumn (Pradel, 1914; Pacioni

et al., 2014). It has been reported that several months are needed from the induction and initial production of primordia to fully mature *Tuber melanosporum* fruitbodies (Pacioni et al., 2014; Le Tacon et al., 2013; Garcia-Barreda et al., 2021).

Truffles were found near the soil surface just within or below the litter horizon and the organic topsoil (O horizon). However, one fruitbody in UL was found at 20–25 cm soil depth (**Fig. 1c**). Our results confirm the reported experience of truffle hunters and farmers, including co-authors of this study and their colleagues from different parts of Europe, which suggests that Burgundy truffle fruitbodies are seldomly harvested below the O horizon. However, soil properties likely have an influence on fruiting depth, and our plots were generally characterised by very thin humus layers of only 2–10 cm that are typical for calcareous soils with high pH values and high mineralisation rates associated with dense, loamy and compacted mineral subsoil. ECM fungi usually dominate in the upper soil horizons (Dickie et al., 2002), and in hypogeous genera such as *Tuber* that have a limited hyphal differentiation (Agerer, 2006), fruitbodies are mainly found in the upper soil horizon where carbon resources are accessible via the mycorrhizal root tips. Though we did not quantify the number of fine roots in the different soil layers, we qualitatively observed a much higher abundance of fine roots in the upper two soil layers. The highest number of *Tuber* ECMs was found in the two lower layers, whereas most fruitbodies were found in the uppermost organic layer. A vertical stratification of the mycelia of fungal species (Lindahl et al., 2007; Solly et al., 2017), as well as the presence of ECM species on root tips is well known (Genney, 2006). A shift between the presence and abundance of mycelia and ECM root tips has been observed for some ECM species (Genney, 2006). Likewise, the presence of fruitbodies does not always correspond to the presence and abundance of soil mycelia or ECM root tips (Anderson, 2007).

In our study, the presence of truffle fruitbodies, together with their genotypes, generally agreed well with their abundance with one exception in site UL. At this site, we found one truffle located at 20–25 cm depth that showed the genotype of ECMs and truffles present in neighbouring plots, and no ECM root tips of this genotype was detected in its immediate vicinity. Whether these were missed because they were located further down in the soil profile, outside of the excavation plot, or the mycelia have grown from the neighbouring plot into this location, remains unknown. Vertical structuring of genotypes within soil profiles was previously described for the larch bolete (*Suillus grevillei*), which is an ECM fungus associating with *Larix* spp. (Zhou et al., 2001). Not much is known about the vertical distribution of truffle species, and we are aware of only one study that described the mycelial abundance of white truffles within soil profiles (Iotti et al., 2018). However, white truffles differ substantially from Burgundy and Périgord black truffles in their ecology and habitat preferences with fruitbodies being produced until deep down in the soil profile and with only very few ECM root tips formed (Riccioni et al., 2016). Unlike black truffles in general, white truffles usually do not exhibit a thick and melanised peridium that protects their fruitbodies from drying out. The relatively good drought resistant peridium of black truffle fruitbodies might be a reason why they can grow in the upper soil layers whereas their more fragile and non-melanised mycelial and mycorrhizal structures are preferentially located further down in the profile. Another reason for the preferred location of ECM root tips in deeper soil layers might be differences in the pH concentration between soil layers. Most pronounced was the vertical stratification of *Tuber* ECMs in the Norway spruce forest at site BG, where the upper soil layers were acidic due to stronger soil surface acidification by conifer litter as compared to the input of organic matter from broadleaved trees at all other excavation sites.

4.2 Truffle genotypes

Our genetic analyses of truffles and ECM root tips within the excavation plots of all three sites showed that single individuals formed several fruitbodies and dominated at the ECM root tips in the respective plots. Other, small-scale studies of spatiotemporal genetic patterns of Burgundy and Périgord truffles showed that only a few dominating individuals produced several fruitbodies that were present over several years, whereas most others were only found once (60–90 %; Murat et al., 2013; Molinier et al., 2016a; Taschen et al., 2016; De la Varga et al., 2017; Schneider-Manoury et al., 2019). When ECM root tips were considered, analyses indicated that genotypes forming ECMs and truffles spatially overlapped, and ECMs were mostly detected for those genotypes that formed several fruitbodies in several years, although some genotypes at ECMs were not detected in truffles *vice versa* (Murat et al., 2013; Taschen et al., 2016; De la Varga et al., 2017; Schneider-Manoury et al., 2019). Since our temporally-constrained eco-archaeological experiment only revealed a short snapshot of time, we cannot ascertain that the truffle individuals are dominating our plots at ECMs and that truffle producers persist over several years. Since the size of each of them was at least one square meter (based on the occurrence at ECM roots and truffles), it is likely that they were perennial when considering a mycelium growth rate of about 15 cm per year (Iotti et al., 2002; Gryndler et al., 2013). Larger individuals point towards a life strategy investing in vegetative mycelial growth, whereas small and ephemeral individuals indicate resource investment in sexual spore production for colonization and survival (Douhan et al., 2011). Burgundy and Périgord truffles seem to follow both strategies based on the limited number of studies performed on spatiotemporal genetic patterns (Murat et al., 2013; Molinier et al., 2016a; Taschen et al., 2016; De la Varga et al., 2017; Schneider-Manoury et al., 2019). For the Périgord truffle, it has been further reported that

374 individuals generally operate either maternally, which means that they form their fruitbody
375 tissues and are present at ECM root tips to acquire the needed carbon, or paternally by
376 contributing only genetic material for sexual reproduction (Selosse et al., 2017). These paternal
377 genotypes are often found once in spores extracted from single fruitbodies and are therefore
378 mostly short-lived individuals of unknown niche and structure.

379 Since we found no obvious horizontal relationship between truffles and tree roots (**Fig. 2**),
380 our findings contrast to the often-reported occurrence of black truffles near the fine roots of their
381 host trees (Callot, 1999). While this might be true for truffle orchards with undisturbed root
382 systems, it seems less so for natural forest sites where the root systems of *Tuber* mycorrhized and
383 *Tuber* non-mycorrhized trees are overlapping. The co-occurrence of plant and fungal species
384 could be another factor of heterogeneous root and mushroom webs in the upper soil horizons.
385 Since it is impossible to detect *in situ* hyphal connections from the truffles to soil mycelium and
386 mycorrhizas macroscopically, it remains unclear if small and large, or young and old truffles
387 differ in the way in which they are connected to the soil mycelium or host tree mycorrhiza. We
388 also still do not know whether truffles switch from mycorrhizal to saprotrophic status during
389 their life cycle. While such a fundamental change in supply strategy has been suggested from
390 sporadic field observations (Callot, 1999), it has been refuted by ^{13}C pulse labelling experiments
391 (Le Tacon et al., 2013). Genome sequencing of truffle species has shown a significant reduction
392 in genes involved in plant cell wall degradation as seen for other ECM fungi, which questions
393 their saprotrophic capability to acquire carbon from plant litter (Murat 2018). However,
394 *Tuberaceae* have a rich complement of genes involved in chitin degradation and other
395 components of fungal and bacterial tissues, allowing them to saprotrophically gain carbon from
396 these tissues (Miyauchi, 2020). Future eco-archaeological excavations should include field

microscopic investigations of the putative mycelium connections between truffles, soil and mycorrhizas.

At all reference forest sites, our dogs found truffles that were larger than those that were excavated (**Fig. 4**). The reference fruitbodies were not only bigger but also significantly more mature; only one fully unripe truffle was detected by our dogs. This finding partly diverges from data collected in a truffle orchard in central Hungary (Büntgen et al., 2017), where trained truffle dogs usually detect fruitbodies of a wide range of maturation stages. While our case study might be biased by the limited sampling dates in winter, which could lead to a lower proportion of overall young and/or unripe truffles, the divergence from the Hungarian findings are indicative of poorly understood differences in the aroma profile, as well as the hunting behaviour and detection capability of the dogs (Olivier et al., 2012).

5. Conclusions

Our eco-archaeological approach helps to illuminate the enigmatic belowground life cycle of truffles. Generally applicable to hypogeous fungi, fine-scale excavations can reveal insights into the relationship between the weight and size of fruitbodies. Our results suggest that truffle fruitbodies can grow, mature and ripe simultaneously. We also show that genetically distinct specimens can exhibit different life cycle stages at the same period and under the same environmental conditions.

Since a better understanding of the formation and maturation of truffle fruitbodies will help hunters and farmers to improve their harvest practices and management strategies, more eco-archaeological truffle excavations are needed for unravelling the species' life cycle that seems more complex than previously thought.

Informed consent

All named authors declare that they are agreed to publish this manuscript.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Author contributions

Ulf Büntgen (U.B.), Simon Egli (S.E.) and Martina Peter (M.P.) designed the study. Ulf Büntgen (U.B.), Willy Tegel (W.T.), Ulrich Stobbe (U.S.), Rengert Elburg (R.E.), Ludger Sproll (L.S.), Virginie Molinier (V.M.) and Simon Egli (S.E.) conducted fieldwork and analysed the data. Rengert Elburg (R.E.) and Will Tegel (W.T.) coordinated the archaeological work. Martina Peter (M.P.) and Virginie Molinier (V.M.) performed the genetic analysis and strengthened their interpretation. Ulf Büntgen (U.B.), Simon Egli (S.E.) and Martina Peter (M.P.) wrote the paper with input from Tomáš Čejka (T.C.), Elizabeth L. Isaac (E.I.), and Ulrich Stobbe (U.S.). Ulf Büntgen (U.B.) and Martina Peter (M.P.) revised the manuscript.

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Table 1. Biogeographic characteristics of the three truffle excavation sites, for which we cannot provide further spatial details to secure the truffle sites from illegal harvesting.

	BG	RT	UL
Excavation date	10th Dec 2011	11th Dec 2011	28-29th Oct 2011
Location	Villingen-Schwenningen (GER)	Villingen-Schwenningen (GER)	Singen (GER)
Altitude (m a.s.l)	780	785	430
Geology	scree from Upper Jurassic Limestones	scree from Upper Jurassic Limestones	glacial moraine deposits
Soil type	calcareous shallow rendzina	calcareous shallow rendzina	shallow, poorly developed luvisol
Forest type	spruce forest single-storied even aged	beech forest single-storied even aged	mixed beech forest multi-storied uneven aged
Tree species	*Picea abies*	*Quercus robur* *Fagus sylvatica* *Carpinus betulus*	*Quercus robur* *Fagus sylvatica* *Carpinus betulus*
Shrub species	*Fagus sylvatica* *Corylus avellana* *Fraxinus excelsior* *Sambucus nigra* *Lonicera sp*	*Crataegus monogyna* *Fraxinus excelsior* *Ligustrum vulgare*	*Fraxinus excelsior* *Robinia pseudoacacia* *Crataegus monogyna* *Ligustrun vulgare*

Table 2. Truffle harvests (m = maturity in %), w = weight in g) from the three 1m × 1m excavation plots in UL, BG, RT, together with the reference dog harvests in the vicinity of the excavation plots (nd = no data).

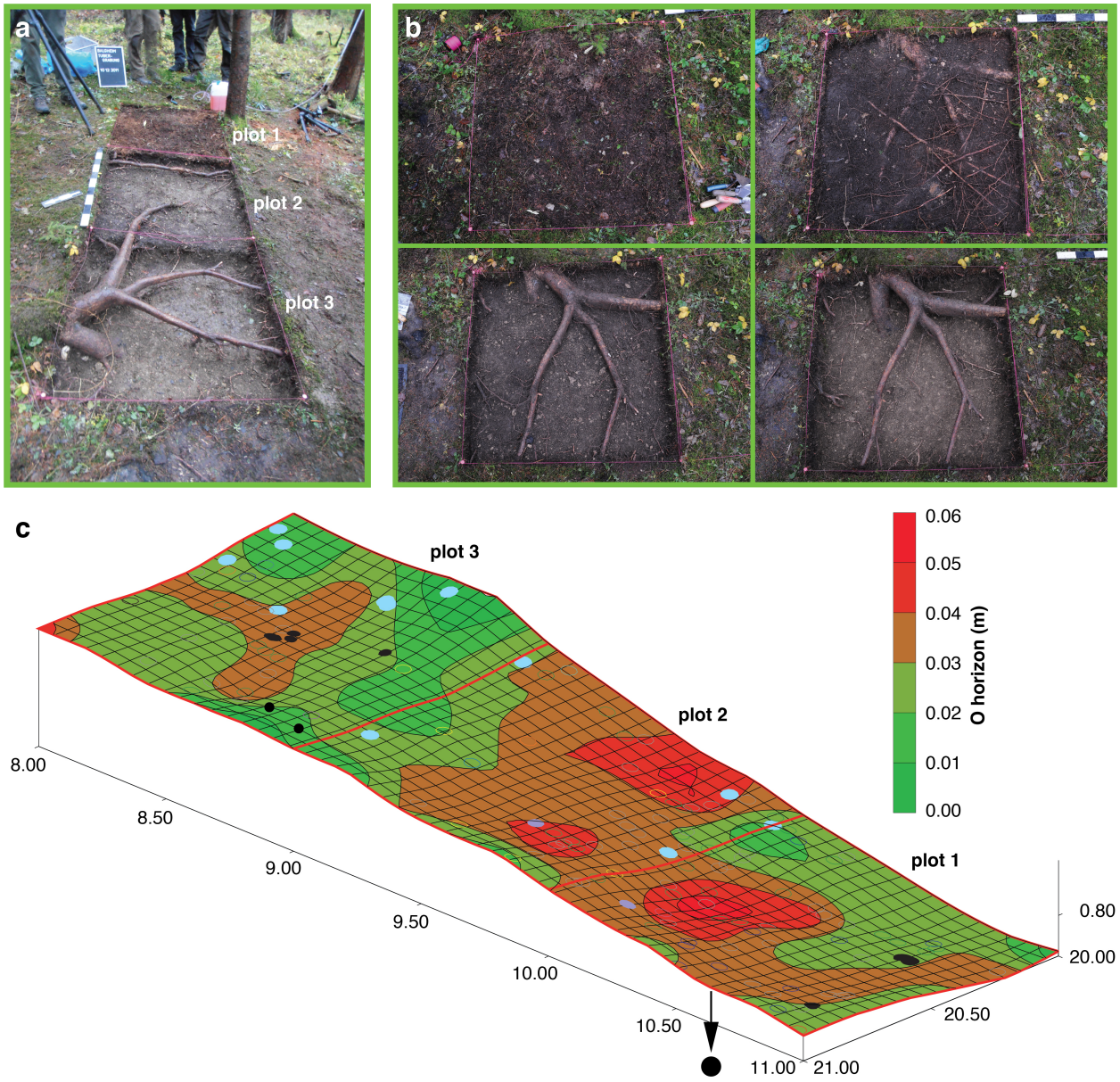
	UL1		UL3		UL4		UL external		BG1		BG3		BG external		RT1		RT external	
	m	w	m	w	m	w	m	w	m	w	m	w	m	w	m	w	m	w
1	87	103	88	12.7	88	8.5	nd	54.0	100	17.1	88	82.2	100	0.9	100	28.0	100	31.1
2	75	23.9	85	35.7	83	18.8	nd	33.0	100	6.4	82	4.8	100	6.6	100	31.5	100	39.1
3	0	0.1	84	25.3	51	14.7	nd	17.0	84	10.9	60	10.3	88	56.2	100	2.8	93	3.3
4	0	0.3	82	53.7	0	0.6	nd	9.0	75	32.2	57	1.1	87	25.5	91	10.5	90	7.4
5	nd	49.7	0	0.6	0	0.6	nd	25.0	51	13.9			86	11.5	72	4.2	88	3.9
6			0	0.5			nd	7.0	49	5.4			86	36.2	71	4.3	87	27.5
7							nd	23.0	30	6.4			79	31.0	53	1.2	79	15.7
8							nd	16.0					72	19.2	31	1.1	70	7.1
9							nd	2.0					67	32.8	6	6.0		
10							nd	24.0							0	1.2		
11							nd	4.0							0	0.1		
12							nd	18.0							0	0.2		
13							nd	7.0							0	0.2		
14							nd	7.0										
15							nd	3.0										
16							nd	24.0										
min	**0**	**0**	**0**	**1**	**0**	**1**	**nd**	**2**	**30**	**5**	**57**	**1**	**67**	**1**	**0**	**0**	**0**	**3**
mean	**41**	**35**	**57**	**21**	**45**	**9**	**nd**	**17**	**70**	**13**	**72**	**25**	**85**	**24**	**48**	**7**	**88**	**17**
median	**38**	**24**	**83**	**19**	**51**	**8**	**nd**	**17**	**75**	**11**	**71**	**8**	**86**	**25**	**53**	**3**	**89**	**12**
max	**87**	**103**	**88**	**54**	**88**	**19**	**nd**	**54**	**100**	**32**	**88**	**82**	**100**	**56**	**100**	**31**	**100**	**39**
SD	**47**	**49**	**44**	**21**	**43**	**8**	**nd**	**14**	**27**	**9**	**16**	**39**	**11**	**17**	**43**	**11**	**29**	**14**

Table 3. Multilocus genotypes (MLGs) identified in truffle fruitbodies and ectomycorrhizal root tips (ECMs) in the three study sites Ueberlingen (UL), Balgheim (BL) and Rietheim (RT). The number of truffles and ECMs carrying the respective MLG is given. Only samples of which data from all 11 nuclear simple sequence repeat (nSSR) loci and the mating type were available are shown and were included in the analyses of Psex to test for true clones. Psex values and significance were calculated with 1000 permutations for each site/population separate as well as for all samples together. A significant Psex value suggests that an MLG can be considered as a true clone.

MLG	Location	n (total)	n (fruitbodies)	n (ECMs)	Psex (all)	Level	Psex (site/population)	Level
RT02	Rietheim	1	1		-	-	-	-
RT03	Rietheim	1	1		-	-	-	-
RT01	Rietheim	63	11	52	9.1E-15	***	1.7E-01	ns
BG02	Balgheim	2	2	0	8.6E-09	***	0.0E+00	***
BG01	Balgheim	145	17	128	3.3E-15	***	2.6E-03	**
UL01	Ueberlingen	46	8	38	0.0E+00	***	0.0E+00	***
UL03	Ueberlingen	2	2	0	8.7E-01	ns	8.5E-01	ns
UL02	Ueberlingen	3	3	0	1.3E-03	**	0.0E+00	ns
UL07	Ueberlingen	28	9	19	9.9E-15	***	0.0E+00	***
UL04	Ueberlingen	2	2	0	2.3E-07	***	0.0E+00	***
UL05	Ueberlingen	2	2	0	8.3E-08	***	0.0E+00	***
UL08	Ueberlingen	1	1	0	-	-	-	-
UL06	Ueberlingen	5	5	0	3.5E-01	ns	3.21E-05	***

661 **Table 4.** Number of ectomycorrhizal (ECM) root tips showing a truffle-like morphotype
662 analysed, and number and percentage of truffle ECMs genetically identified in the six excavation
663 plots at different soil horizons.

Site	Plot	Horizon	Nb truffle ECM	Nb ECM other species	total Nb ECM analysed	% truffle ECMs
Balgheim	B1	0-5	20	25	45	44%
Balgheim	B2	0-5	0	10	10	0%
Balgheim	B3	0-5	1	18	19	5%
Balgheim	B1	10-15	61	1	62	98%
Balgheim	B2	10-15	30	2	32	94%
Balgheim	B3	10-15	4	13	17	24%
Balgheim	B1	20-25	29	2	31	94%
Rietheim	R1	0-5	18	17	35	51%
Rietheim	R1	10-15	45	7	52	87%
Ueberlingen	Ue1	0-5	25	39	64	39%
Ueberlingen	Ue2	0-5	16	37	53	30%
Ueberlingen	Ue3	0-5	16	34	50	32%
Ueberlingen	Ue1	10-15	40	42	82	49%
Ueberlingen	Ue2	10-15	26	36	62	42%
Ueberlingen	Ue3	10-15	23	21	44	52%
Ueberlingen	Ue1	20-25	20	46	66	30%



664

665 **Fig. 1.** (**a**) Excavation plots BG1-3, (**b**) various excavation stages of our eco-archaeological
666 experiment, and (**c**) a digital example of an excavation profile, including a surface model,
667 isolines of the O horizon three times exaggerated. The black arrow refers to the fruitbody found
668 at circa 20–25 cm soil depth.

669

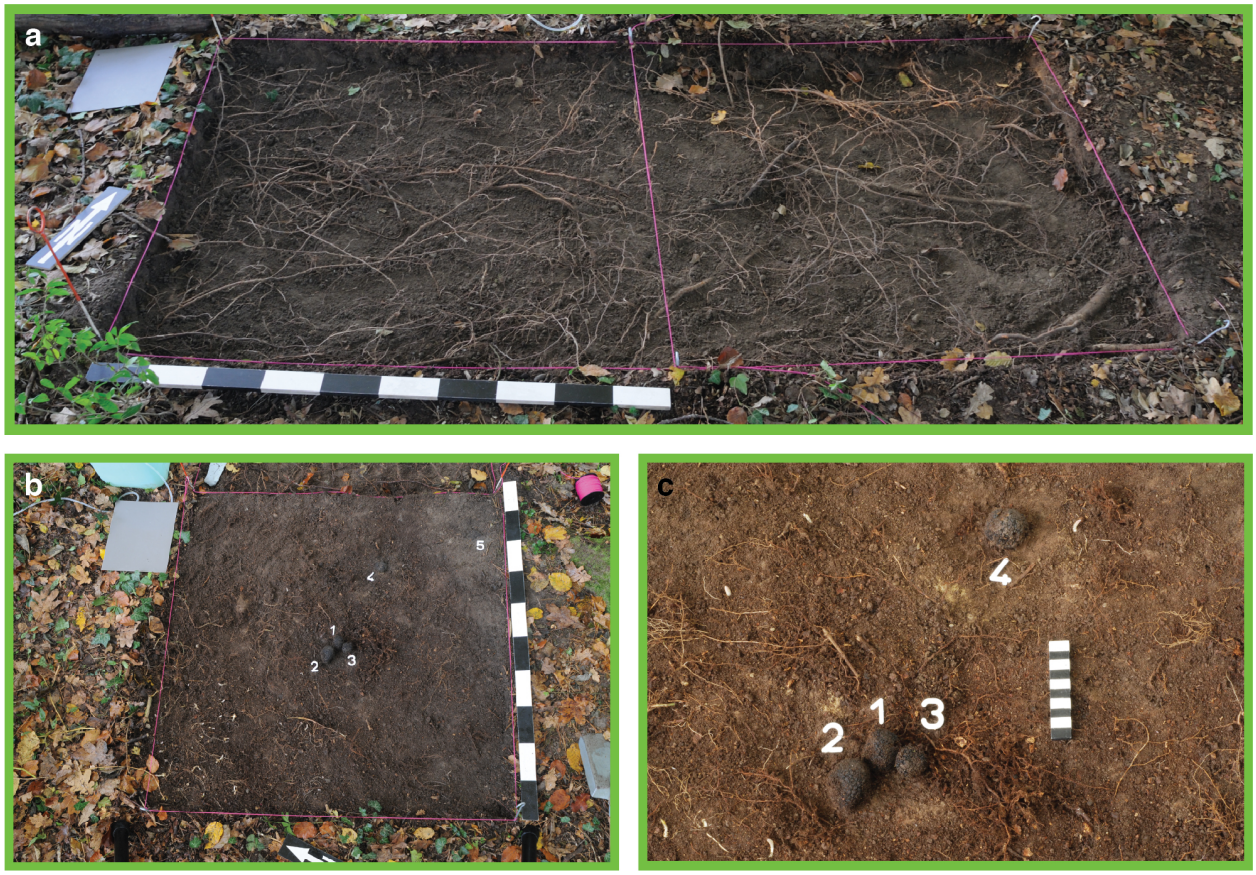
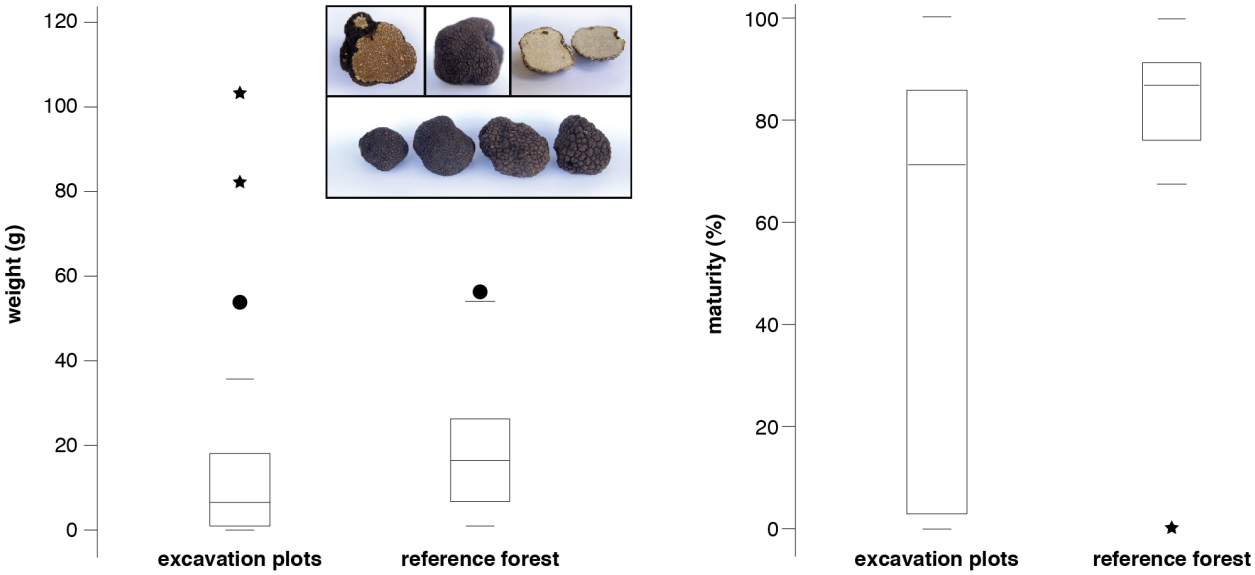


Fig. 2. (**a-c**) Eco-archaeological setup at different excavation steps, showing the location of truffle fruitbodies in relation to tree roots.

674

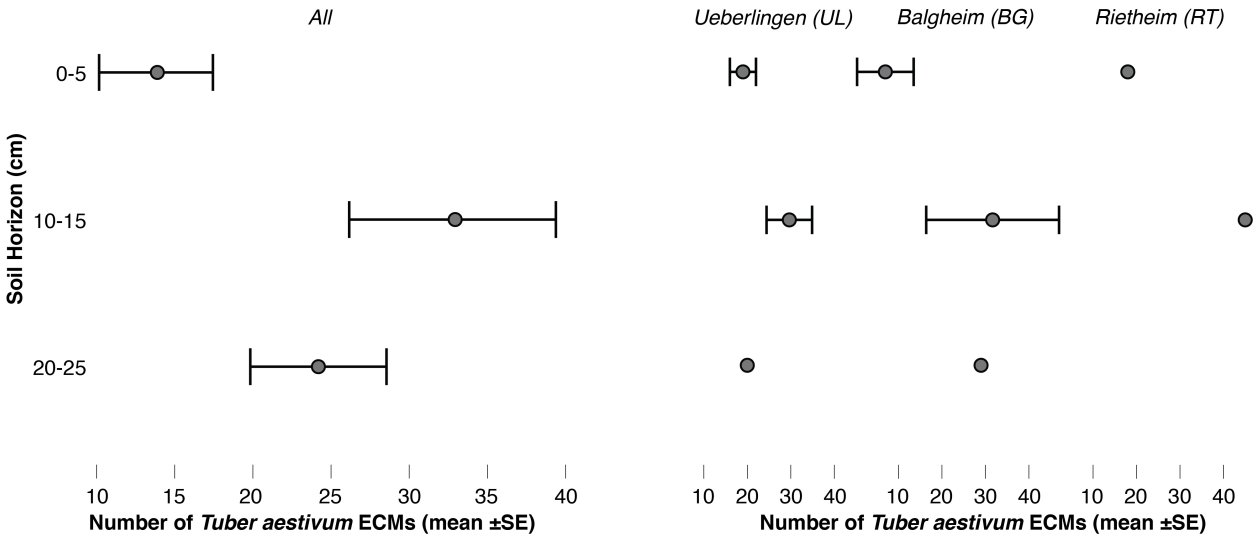
675 **Fig. 3.** Characteristics and stages of truffle growth. (**a**) An example of a very small and very
676 young truffle at maturation stage 0% from RT1, which is usually not detected by truffle dogs. (**b**)
677 An almost decomposed truffle at maturation stage 100% from RT1. (**c**) Rare example of an
678 upward growing fruitbody that surrounds an oak root from UL1.

679

680 

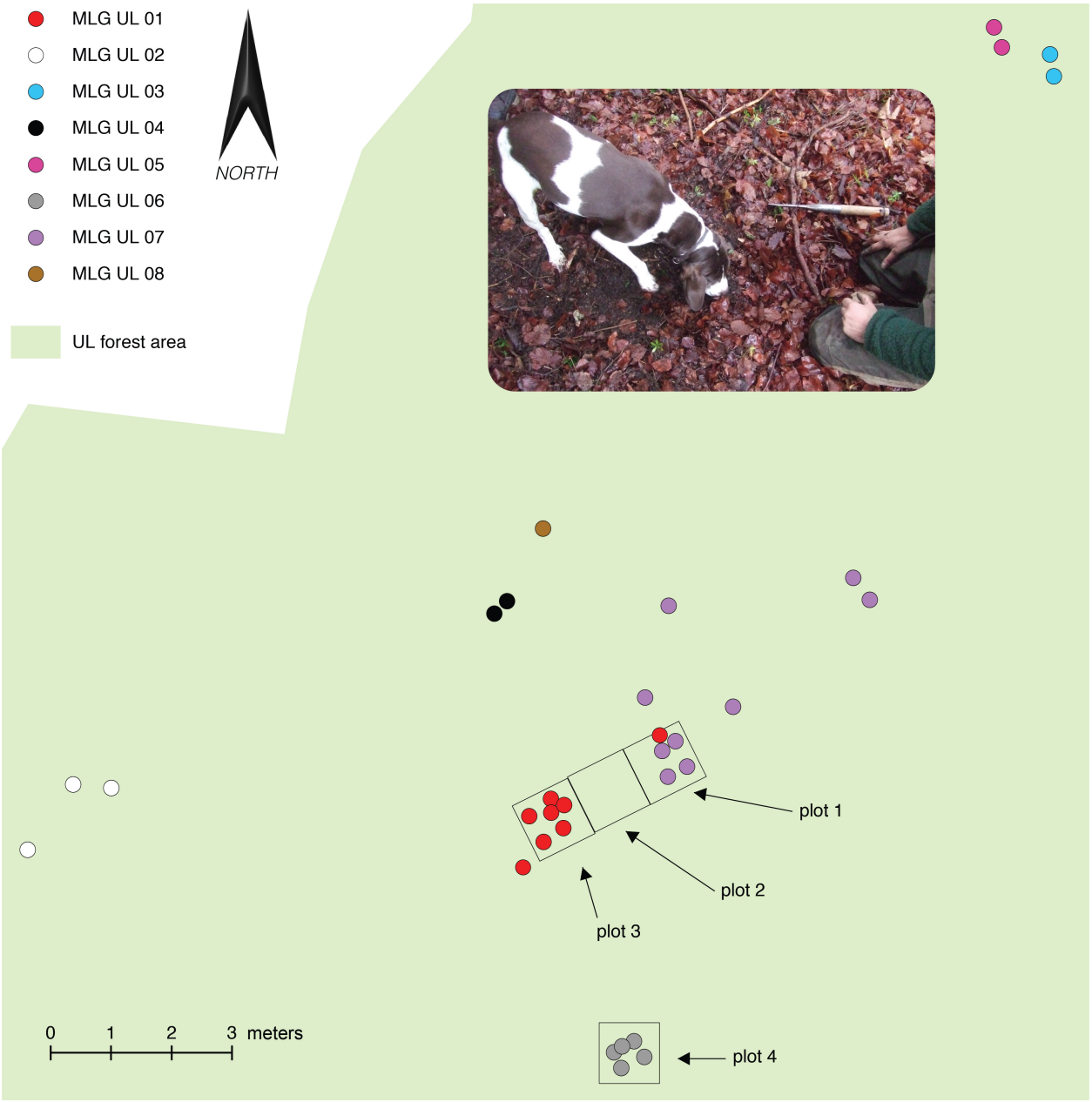
681 **Fig. 4.** Boxplots of the weight and maturity of truffle fruitbodies from the excavation plots and

682 the surrounding reference forest sites.



683

684 **Fig. 5.** Distribution of the belowground truffle ectomycorrhizas (ECMs) on fine roots along
685 vertical gradients of three soil horizons at 0–5 cm, 10–15 cm and 20–25 cm depth, averaged over
686 all three locations (left) and separately for the three locations (right).



687

688 **Fig. 6.** Schematic representation of an excavations plot (black squares) and the reference forest
689 (green shading) at the UL site. The multilocus genotype (MLG) mapping of each fruitbody is
690 indicated by coloured dots, and the inset photo shows a trained truffle dog indicating the
691 presence of a fruitbody in the reference forest.