Mycorrhizal fungi and invertebrates: Impacts on Tuber melanosporum ascospore dispersal and lifecycle by isopod mycophagy

Article *in* Elsevier - Food Webs Journal · October 2022

DOI: 10.1016/j.fooweb.2022.e00260

CITATIONS
0

READS
27

2 authors, including:

Paul Thomas
University of Stirling
37 PUBLICATIONS **483** CITATIONS

SEE PROFILE

Food Webs 33 (2022) e00260

Contents lists available at ScienceDirect

Food Webs

journal homepage: www.elsevier.com/locate/fooweb

Mycorrhizal fungi and invertebrates: Impacts on *Tuber melanosporum* ascospore dispersal and lifecycle by isopod mycophagy

Paul W. Thomas [a,b,*], Heathcliff W. Thomas [b]

[a] Faculty of Natural Sciences, University of Stirling, FK9 4LA Stirling, UK
[b] Mycorrhizal Systems Ltd, Lancashire PR25 2SD, UK

ARTICLE INFO

Keywords:
Mycorrhiza
Mycophagy
Truffle cultivation
Oniscus asellus
Tuber melanosporum (perigord truffle)
Isopod

ABSTRACT

The icon ectomycorrhizal (EcM) species, *Tuber melanosporum*, requires mycophagy for ascospore dispersal. Isopods are often found within fruitbodies and to explore why, *Oniscus asellus* were presented with *T. melanosporum* as a food source. Fruitbodies were consumed at a rate of 4.0 mg per isopod, over 24 h. Most of the recovered faecal pellets contained ascospores after 12 h. Gut-transit inflicted little mechanical damage to ascospores, and the majority were still contained with an ascus 30 h post feeding. Further, ascospores were observed in faecal pellets 18 days after consumption. Combined, the results suggest a previously overlooked role for isopods in EcM spore dispersal. The impacts for EcM ecology and the role of isopods in *Tuber* spp. lifecycles, including mating type distribution, is discussed alongside the emerging threat of climate change and how such knowledge can inform management by custodians of relevant habitat types.

1. Introduction

Within the animal kingdom, mycophagy is a commonly observed behaviour. From grizzly bears (Mattson et al., 2002) to white-footed mice (Maser and Maser, 1987), a huge range of species have been recorded as engaging in some level of mycophagy of an equally as diverse list of fungi species. Of the vertebrates, the class Mammalia is most often associated with mycophagy (Fogel and Trappe, 1978; Urban, 2016) but this feeding behaviour is also well documented in bird species, who may act as effective long-range dispersal agents (Elliott et al., 2019a; Caiafa et al., 2021) and has even been recorded in reptiles (Elliott et al., 2019b; Cooper and Vernes, 2011).

Mycophagy may be incidental or intentional, and spore dispersal is the most often cited benefit to the consumed species. Incidental mycophagy in coprophilous species may co-occur with adaptations from relatively small fruitbodies to a large spore size, representing quick maturation but also high resilience and germination competence of spores (Halbwachs and Bässler, 2020). Transit through a digestive tract here provides a spatial but also nutritionally focused distribution mechanism. Mycophagy may also support spore dispersal prior to consumption. For example, epigeous fungi are known to be collected by Eurasian red squirrels and cached in trees, to be eaten later. This process transports the fruitbody to an elevated position and in one study the average height transfer was 1–10 m with horizontal transfer distances of ~35 m (Lee, 2002). Such behaviour enhances the spore distribution potential of the fruitbody without transfer through the digestive tract. However, the most documented process of mycophagy aiding spore dispersal is through intentional consumption and animal movement (Fogel and Trappe, 1978; Urban, 2016; Elliott et al., 2019a; Caiafa et al., 2021; Elliott et al., 2019b; Cooper and Vernes, 2011) where spores may pass through the gut, undamaged. This process is well documented and for many hypogeal fruiting species that have lost the ability to forcibly eject spores, it may be the primary dispersal mechanism (Trappe, 2009). Moreover, many hypogeal fruiting species and especially those from the *Tuber* genus (hereinafter, termed truffles) have a symbiotic and principally ectomycorrhizal (EcM) lifestyle, in which the fungi interact with the roots of a plant partner and provide nutrients in return for photosynthetically derived carbon (Smith and Read, 2010). In these truffle species, deposition of ascospores in faecal matter within close proximity to the root system of a potential mutualistic plant partner, may be beneficial. The development of hypogeal fruiting in truffles may have arisen in response to a desiccating environment (Trappe and Claridge, 2005) and many species appear to have adaptations to facilitate consumption. The most widely acknowledged of these include the release of chemical attractants to stimulate mycophagy (Pacioni et al., 2015; Splivallo et al., 2011).

* Corresponding author at: Faculty of Natural Sciences, University of Stirling, FK9 4LA Stirling, UK.
E-mail address: paul.thomas@stir.ac.uk (P.W. Thomas).

https://doi.org/10.1016/j.fooweb.2022.e00260
Received 6 June 2022; Received in revised form 30 September 2022; Accepted 2 October 2022
Available online 6 October 2022

The relationship between truffles and vertebrates has been widely explored and appears to be largely mutualistic (Fogel and Trappe, 1978; Urban, 2016; Elliott et al., 2019a; Caiafa et al., 2021; Elliott et al., 2019b; Cooper and Vernes, 2011). However, a wide range of invertebrates are also known mycophagists and the relationship here is less clear. For example, in one study with plant pathogenic fungi, the viability of post-digestion spores differed depending on the consuming species (Hyder et al., 2009). Further, work with Collembolans has shown that they can transport a startling array of fungal propagules via gut transit and adhesion to appendages, many of which have been shown to be viable through post-transport culturing (Anslan et al., 2016). However, this viability may be confined to mycelial fragments as a separate study with 16 fungi species and *Ceratophysella denisana* (Collembola: Hypogastruridae) showed that 93–100% of spores were damaged during passage through the digestive tract (Nakamori and Suzuki, 2010). All fungi species were EcM basidiomycetes and the spore coat had been damaged, with the contents spilled. Further, the gut passage time was so short that most of the spores (post-transit) were deposited on the fruitbody. Thus, mycophagy by Collembolans may not be a viable distribution method for EcM despite some plant pathogenic species having previously shown to maintain viability after gut transit (Dromph and Borgen, 2001). Further evidence of EcM spore dispersal by invertebrates is scant (Kobayashi et al., 2017; Lilleskov and Bruns, 2005; Ori et al., 2021) and is often not addressed in mycophagy discussion articles (Fogel and Trappe, 1978; Urban, 2016; Horton, 2017).

Truffle harvesters often report invertebrate damage at the time of collection (Rosa-Gruszecka et al., 2017) and isopods are often found within hollowed areas of such hypogeal fruitbodies (author observation). However, no EcM spores have ever been documented as having been consumed or transported by isopods (*Oniscidea*), although spores of arbuscular mycorrhiza (AM) species have (Vašutová et al., 2019). This is very surprising for two reasons. Firstly, isopods are recognized as having a key regulating role in decomposition and nutrient cycling, feeding on soil fungi and litter (Yang and Li, 2020) and may therefore be effective and important agents of fungal propagule dispersal (Vašutová et al., 2019). Secondly, several studies have reported recovery of AM spores from faecal pellets. In one study, around 50% of recovered spores appeared viable and some isopods were noted as having little else in their digestive systems but AM fungi (Rabatin and Stinner, 1988). There are two reasons why the small number of studies that have addressed fungi spores in faecal matter of isopods may not have identified EcM spores. Firstly, it is possible that isopods may not browse EcM fruitbodies and secondly, there may not have been access to EcM fruitbodies in the studies in which mycophagy was the focus. In terms of the relationship between truffles and isopods, although it is unknown if they are consuming the fruitbodies directly, we hypothesize that as known mycophagists (Crowther et al., 2013) this is indeed the case, and further, they have the potential to spatially distribute spores.

In order to explore a potential role of invertebrates, and specifically isopods, as a spore dispersal mechanism for hypogeal EcM species, a feeding experiment was devised. *Oniscus asellus* were presented with *Tuber melanosporum* (the Périgord truffle) as a food source, and faecal pellets were collected at timed intervals and assessed for their presence/absence of ascospores. *Tuber melanosporum* was chosen as one of the most widely appreciated and cultivated hypogeal EcM species (Doménech and Barreda, 2014) and so that the results could have an added benefit in helping to inform management practices for truffle cultivators and custodians of naturally producing truffle woods.

2. Materials and methods

On the 24th of March 2021, 10 isopods were collected from under branches of a fallen and decaying horse chestnut (*Aesculus hippocastanum*) tree on the Isle of Bute (Scotland, UK) and were identified as the common woodlouse (*O. asellus*), by way of gross morphological features (Gregory, 2021). Isopods were transferred to one container at room temperature, with the addition of damp tissue as a water source and with the inclusion of leaf litter. On the 25th of March 2021 at 7.30 am, isopods were transferred to a single clean container, with damp tissue and 0.47 g of fresh *T. melanosporum* fruitbody, identified by morphological means (Thomas et al., 2019). Thereafter, every hour, all faecal pellets were removed from the container and analysed. Faecal pellets were placed on a slide with a drop of water and gently crushed with a coverslip so that the contents were well dispersed and easily observed with a compound microscope. The presence or absence of ascospores within each faecal pellet was recorded. After 15 h, at 22.30 pm, at which point two consecutive sampling points had returned a result of 100% of all faecal pellet samples having the presence of ascospores, collection of faecal pellets ceased.

After a further 9 h and 15 min, a full 24 h and 15 min since the truffle food source was added, it was removed and weighed. At this point, isopods were transferred to clean individual containers containing only: a small piece of damp kitchen roll (for moisture), one dry oak leaf and the addition of a food source which included a small piece of apple and a small piece of Basidiomycota fungi fruitbody, of various species. The food source was added to support gut transit of existing contents and as a proxy for normalized feeding behaviour. Food sources were regularly removed and changed, as they degraded, over the study period. Thereafter, every 24 h, all faecal pellets were collected from each individual container and observed with a compound microscope, as described above. The presence or absence of ascospores within each faecal pellet was recorded. After 18 consecutive days, due to laboratory constraints, the experiment was terminated, and isopods were carefully returned to their exact collection site.

To observe the impact of mycophagy on asci rupturing, some faecal pellets were analysed in greater depth. This occurred at two timepoints: 30 h after the addition and 30 h after the removal of truffle as a food source. The first 10 faecal pellets after each timepoint were removed and prepared as previously described, for microscopic analysis. Ruptured asci are hard to identify as the ascospores are released and remnants of the original ruptured ascus may be hard to locate (or too degraded to locate). Therefore, ascospores were counted, with a target of ~100 per faecal pellet, and the location in or out of asci was recorded. The number of asci and the ascospores per asci were also recorded. A targeted number of ascospores were used for pellet observations, so that asci degradation could be observed independent of changes in ascospore density of faecal pellets.

3. Results

The first stage of the experiment involved the addition of truffle as a food source, with faecal pellets collected hourly from this timepoint. A linear regression revealed no significant relationship between elapsed time and the hourly number of faecal pellets recovered ($R^2 = 0.04729$, $F_{1,13} = 0.6453$, $p = 0.44$) and so duration of presentation of truffle as a food source had no impact on faecal pellet production. However, the relationship (Table 1) between elapsed time and number of recovered faecal pellets that contained ascospores was significant ($R^2 = 0.3810$, $F_{1,13} = 8.002$, $p = 0.014$) and most of the recovered faecal pellets contained ascospores 12 h or later (see Fig. 1A). The piece of truffle introduced as a food source weighed 0.47 g, and 24 h and 15 min later when the truffle was removed, the weight had decreased by 0.04 g, presenting an average feeding rate of 4.0 mg of truffle per isopod within a 24 h period.

The second stage of the experiment involved the removal of truffle as a food source, with faecal pellets collected every 24 h (daily) from this timepoint. A linear regression revealed no significant relationship between elapsed time and the daily number of faecal pellets recovered ($R^2 = 0.1217$, $F_{1,16} = 2.218$, $p = 0.16$) suggesting that the absence of truffle as a food source had no impact on faecal pellet production. However, the percentage of recovered pellets in which ascospores were observed decreased from 100% at days one and two to 27% at day 18 (Fig. 1B),

Table 1
Statistical analysis results for unpaired *t*-tests of different observations 30 h after truffle had been added as a food source (AD) and 30 h after truffle had been removed as a food source (RE). Numbers in parenthesis gives the average figure per faecal pellet and individual t-tests were carried out on the following measurements for each pellet: number of asci, ascospores in asci, free ascospores (not seen in asci), ascospores per ascus and total recorded ascospores. Linear regression results for total number of faecal pellets observed (PellQ) and number of faecal pellets that contained ascospores, vs elapsed time, are also displayed. Linear regression results for 'Hour' observations represent hours after truffle was added as a food source, and for 'Day' observations represent days after truffle was removed as a food source. The 95% Confidence interval (CI) represents the difference between means for unpaired t-tests and of the slope, for linear regression. Significant results with $p < 0.05$ marked with an asterisk (*).

	Mean diff.	95% CI	*p* value	R^2
Unpaired t-test				
Asci, RE (7) vs. AD (30.3)	−23.27	−28.02 to −18.52	<0.01*	-
Ascospores in asci, RE (20.7) vs. AD (85.9)	−65.18	−77.10 to −53.27	<0.01*	-
Free ascospores, RE (70.7) vs. AD (7.2)	63.55	46.68 to 80.41	<0.01*	-
Ascospores per ascus, RE (2.8) vs. AD (2.9)	−0.06	−0.73 to 0.60	0.84	-
Total ascospores, RE (91.5) vs. AD (93.1)	−1.64	−20.39 to 17.11	0.86	-
Linear regression				
PellQ total vs. Hour	-	−0.21 to 0.10	0.44	0.0474
PellQ with ascospores vs. Hour	-	0.05 to 0.34	0.01*	0.3810
PellQ total vs. Day	-	−0.41 to 0.07	0.16	0.1217
PellQ with ascospores vs. Day	-	−0.86 to −0.35	<0.01*	0.6115

and the relationship between elapsed time and the number of recovered faecal pellets (Table 1) that contained ascospores was significant (R^2 = 0.6115, $F_{1,16}$ = 25.19, $p \leq 0.001$). *O. asellus* may therefore transport ascospores of *T. melanosporum* over at least 18 days post feeding and within the digestive tract. Superficial damage to ascospores within this study (observed as cracked or fragmented ascospores) was noted as being almost non-existent (Fig. 2).

To explore the impact of digestion on ascus degradation, faecal pellets were collected 30 h after *T. melanosporum* had been added as a food source and 30 h after removal as a food source and a target number of ~100 ascospores per pellet was selected for analysis, regardless of ascospore load of the faecal pellet. The actual number of observed recorded ascospores per pellet did not differ ($t_{(20)} = 0.1820$, $p = 0.86$) between the truffle added (93) or truffle removed groups (91).

Observations of the first 10 faecal pellets collected 30 h after *T. melanosporum* had been added as a food source revealed a significantly higher ($t_{(20)} = 10.2220$, $p \leq 0.001$) number of whole asci per pellet (30) when compared to 30 h after *T. melanosporum* had been removed as a food source (7). Consequently, faecal pellets collected 30 h after *T. melanosporum* had been added as a food source revealed a significantly higher number of ascospores observed in asci (86 vs 21) when compared to 30 h after *T. melanosporum* had been removed as a food source ($t_{(20)} = 11.4112$, $p \leq 0.001$) and a significantly lower number of free ascospores (7 vs 71; $t_{(20)} = 7.8589$, $p \leq 0.001$). The truffle-added group therefore had 7.2% of ascospores recorded as free ascospores, whereas the truffle removed group had 75.7%. The number of ascospores per ascus was not significantly different between the truffle added or truffle removed groups, revealing no interaction of degradation speed and ascus size (2.9 vs 2.8; $t_{(20)} = 0.1996$, $p = 0.84$) (Fig. 2 and Table 1). However, the number of pellets recovered in the first portion of the experiment was 50 over a 15 h period, projected forward this would give an estimated total pellet count of 80 over a 24 h period, or 8 per isopod. Conversely, when pellets were collected daily, after the truffle had been removed, the average number of pellets collected per 24 h was 10.7 or 1.1 per isopod. There are several factors that may explain this (see discussion).

4. Discussion

This is the first study that has conclusively shown gut transit of *T. melanosporum* ascospores by arthropods and as far as we are aware, the first to show EcM spore transport by isopods. The significance of our reported findings is explored in detail below.

4.1. Mycophagy, gut transit time and impact on ascospores

The duration after presentation or removal of *T. melanosporum* as a food source did not reduce or increase faecal pellet production, suggesting no significant influence on forage intensity. However, there was a large decline in the number of faecal pellets recovered per 24 h when collected daily vs hourly. This may be explained by increased coprophagy, where pellets were available for longer in-between daily collection periods, or it may be indicative of higher feeding and defecation rates when truffle was present. Nevertheless, here we show for the first time that fruitbodies of *T. melanosporum* are a readily accepted food source by the terrestrial isopod *O. asellus*, although preference when compared to other food sources remains to be elucidated. Mycophagy occurs rapidly and four hours after the presentation of truffle, complete ascospores may be recovered from faecal pellets. After 14 h, all recovered pellets were found to contain ascospores. An isopod feeding rate of 4.0 mg per 24 h was observed, and although experiments occurred in a humid environment some of the observed weight loss may be caused by moisture loss from the fruitbody. Further, this feeding rate is around 4× that reported previously (Bílá et al., 2014; Des Marteaux et al., 2020), although this may be an artifact of different food sources used between studies with different contributing evaporative loss rates. Interestingly, even at 30 h after the initial presentation of truffle, the majority of excreted ascospores are found to still be contained within an ascus (Fig. 2). Here, the ascus seems to be providing a high degree of mechanical protection and this shield may also be relevant for observed resilience to fire events (Glassman et al., 2016) desiccation (Bonito et al., 2012) and prolonged flood events (Thomas, 2021) in members of the *Tuber* genus. This insight of prolonged ascus integrity suggests that the enclosed ascospores are undamaged and viability is likely to still be high post transit. The nutritional benefit of mycophagy to the isopods must therefore be derived from non-ascospore components of the fruitbody, such as the mycelial mass. This raises the question of whether *O. asellus* may also be grazers of EcM vegetative mycelium in the soil or aggregations as mycorrhizal root tips. Although we were unable to locate published evidence of isopods feeding directly on EcM mycelium, the results presented here along with reported feeding on saprotrophic mycelia by isopods (Crowther et al., 2013) suggests that there may be a further interaction between *O. asellus* and *T. melanosporum* via mycelial grazing. This is a theoretical point, but other invertebrates have been shown to preferentially graze different EcM species (Kanters et al., 2015) with ecological significance (LeFait et al., 2019) and so the ecological interaction of *O. asellus* and *T. melanosporum* would benefit from further exploration.

The finding that the majority of ascospores are excreted complete in a protective ascus, 30 h after the presentation of truffle as a food source, suggests that isopods may also be a significant ascospore dispersal mechanism of *T. melanosporum*, with most of the faecal pellet composed of a mass of ascospores at this stage. Here we have also observed that a full 18 days after the removal of truffle as a food source, 27% of recovered faecal pellets may still contain complete and intact ascospores. At this stage the ascospore load is reduced and the majority of recovered ascospores are no longer in ascus, but they do appear to be physically intact and not superficially damaged. The loss of ascus may be

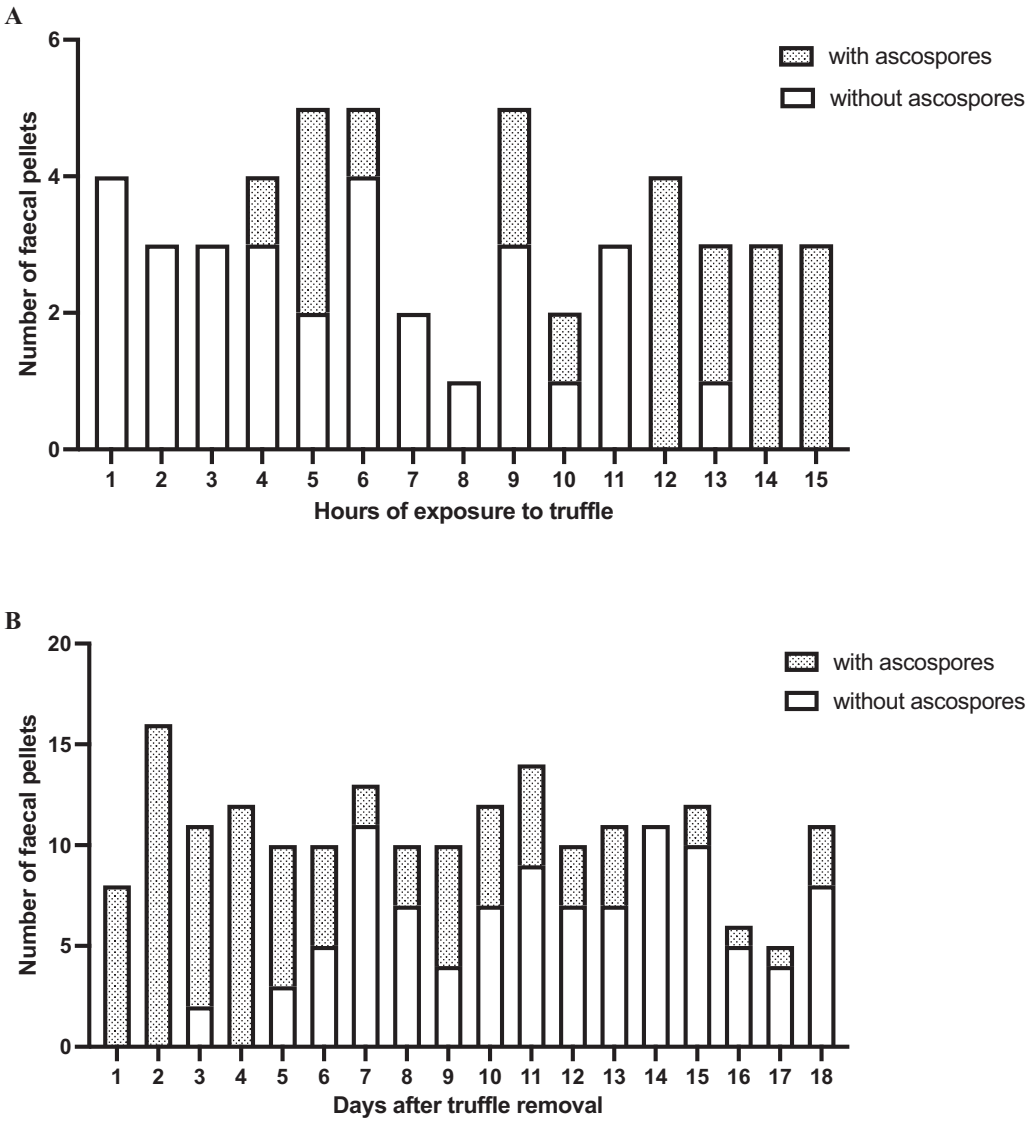

Fig. 1. Number of *Oniscus asellus* faecal pellets and the presence or absence of *T. melanosporum* ascospores displayed by timepoint after truffle had been added as a food source (A). Most of the recovered pellets contained *T. melanosporum* ascospores 12 h or later. The number of faecal pellets and the presence or absence of *T. melanosporum* ascospores displayed by timepoint after truffle had been removed as a food source is also displayed (B). The majority of faecal pellets contained ascospores up to five days post truffle removal.

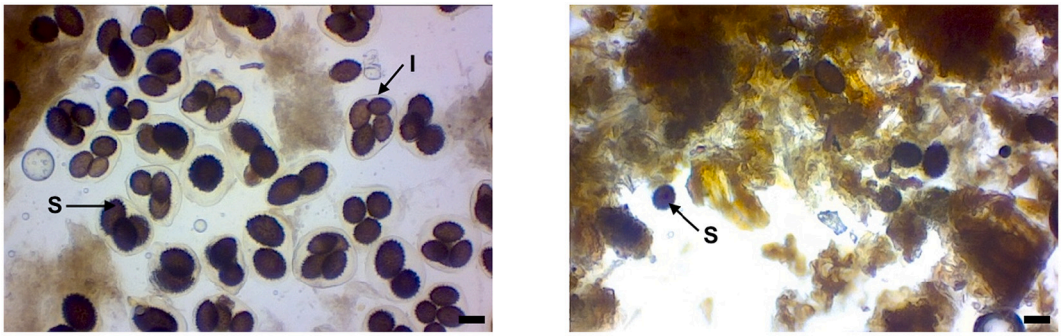

Fig. 2. Images of faecal pellet contents 30 h after truffle had been added as a food source (left) where the contents display a high concentration of ascospores (S) which are largely contained in complete asci (I). The second image (right) displays contents of a faecal pellet 30 h after truffle had been removed as a food source with no complete spore-containing asci observable and a low concentration of ascospores (S). Note the dark melanized pigmentation of ascospores which appear intact and undamaged. Scale bar = 20 μm.

indicative of the longer gut transit time directly or possibly by increased coprophagy, and the undamaged appearance of ascospores is surprising. Melanisation of the ascospores may afford some protection from harsh environmental conditions such as passage through a digestive tract (Garnica et al., 2007; Halbwachs et al., 2015) and release from the ascus is a prerequisite for successful growth, post-germination. Indeed, work with the slug species *Derocera invadens* (Ori et al., 2021), the domestic pig *Sus scrofa* (Piattoni et al., 2014) and the common house mouse *Mus musculus* (Ori et al., 2021) has shown that for the related species *Tuber aestivum*, asci are almost totally destroyed during digestion but the ability of the ascospores to produce mycorrhiza is higher when compared to ascospores that have not passed through a digestive tract. In both studies, ascospore walls were found to be partly degraded and this combined with the removal of the ascus was thought to be influential in the improved formation of mycorrhizas (Ori et al., 2021; Piattoni et al., 2014). However, as all faecal matter in these studies were macerated in the laboratory prior to analysis, it may be that an interplay of digestion or digestion artefacts and post-transit maceration was an important component in the observed release from ascus and ascospore wall degradation. Nevertheless, the improved post-digestion mycorrhization observed in these studies (Ori et al., 2021; Piattoni et al., 2014) suggests that the viability of the ascospores after passage through the digestive tract of *O. asellus* is likely to be high, but robust empirical evidence is needed.

4.2. Role in ascospore dispersal and the implications for tuber lifecycle

Although there have been many studies on isopod foraging behaviour (Dias et al., 2012; Dixie et al., 2015; Ott et al., 2012) the simple question as to how far they may range and over what time frame, is unknown. The surprisingly long gut transit time of 18 days observed within this study suggests a potentially significant role in fungi spore dispersal, although observations are needed to explore the spatial significance and rule out coprophagy in these observations. Ori et al. (2021) suggested that slugs may have an overlooked role in the dispersion and reproduction of many EcM hypogeal as well as epigeal mushrooms, and here we suggest that it is invertebrates in general that have been overlooked. Invertebrates have been shown to transport AM propagules, but few studies have addressed their role in the dispersal of viable EcM propagules (Kobayashi et al., 2017; Lilleskov and Bruns, 2005; Ori et al., 2021) and much work needs to be done (Vašutová et al., 2019). Here we have shown that isopods may be effective dispersal agents and this adds to an emerging picture of invertebrates as short-distance fungi dispersal mechanisms with mammals having increased importance as longer-range dispersal mechanisms (Fig. 3). Further, we hypothesize that predation of grazing invertebrates by larger species may facilitate increased spatial distribution of the consumed spores (Fig. 3). Although, it should also be noted that some invertebrates may travel significant distances unaided (Brouwers and Newton, 2009).

Spore dispersal in EcM species is not only a means of developing new mycorrhiza and genetically diverse colonies in space and time, but it is also potentially crucial in the production of further fruitbodies. This latter point is particularly interesting in the context of *T. melanosporum*, a heterothallic species in which sexual development is controlled by the mating type (MAT) locus where there exists one of two alternative *MAT* genes (Martin et al., 2010; Rubini et al., 2011a). As these loci contain different genes and are not allelic, the two versions of a MAT locus (*MAT1–1* and *MAT1–2*) are known as idiomorphs (Rubini et al., 2011a). Within the fruitbody the vegetative component is only formed of one mating type, but either of the two idiomorphs can contribute this maternal component or provide the paternal contribution to fruitbody production (Rubini et al., 2011a; Rubini et al., 2011b). Within truffle producing areas, it is often the case that the maternal genotype found within fruitbodies is also identified on adjacent collected *Tuber* EcM but the paternal partner is much harder to locate, contributing to few fruitbodies and showing a low degree of temporal persistence (Rubini et al., 2011b; Murat et al., 2013; Taschen et al., 2016; De la Varga et al., 2017). Consequently, there is a growing consensus between authors that germinating ascospores may be the source of the paternal partner (Taschen et al., 2016; De la Varga et al., 2017). The hypogeal nature of *T. melanosporum*, combined with a lack of spore-ejective tissue, presents a dependence on mycophagy for spore dispersal. Although mammals are the assumed primary vectors, invertebrates are also crucial in this process. The potentially long gut retention times, high faecal ascospore loads, deposition in an undamaged state and in a location near to potential plant-partner root systems, presents invertebrate mycophagy as having a significant role in spatial distribution of genetic material to not only form new EcM colonies but to facilitate the sexual production of further fruitbodies (Fig. 3). This hypothesis is further supported by Ori et al. (2021) who highlighted that populations of *Tuber* spp. are characterized by a strong isolation by distance, and so physically close individuals are also genetically close (Murat et al., 2013; Taschen et al., 2016; De la Varga et al., 2017). This observation does not correlate with medium-long ascospore transit by movement of wild mammals which

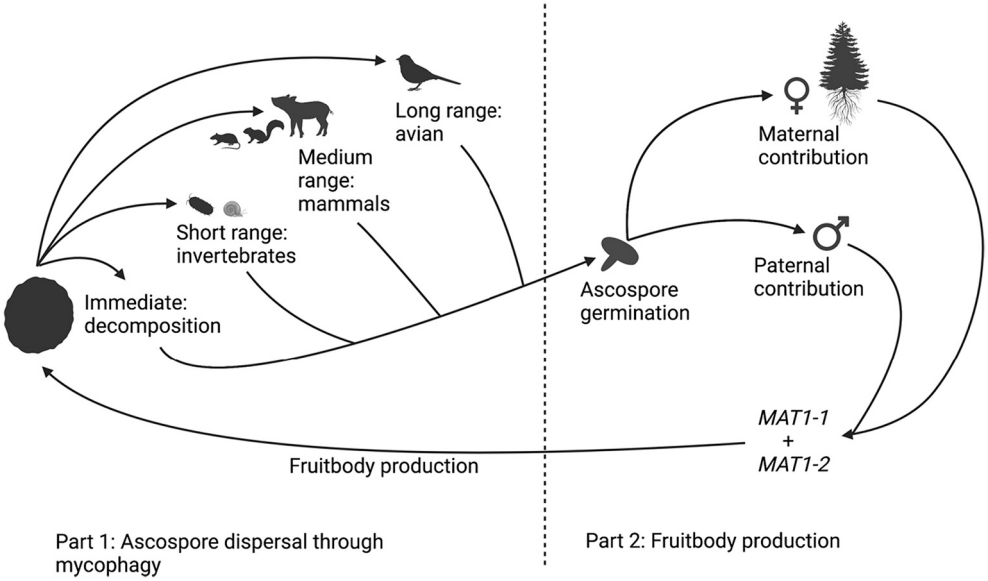

Fig. 3. The distribution of ascospores in hypogeal EcM. Distribution may be in the immediate area from decomposition of the fruitbody or over short distances through mycophagy by invertebrates, and medium distances by mammals or long distances by birds. Avian dispersal may be through mycophagy or through predation of mycophagous animals. After gut transit, ascospores germinate and then may either form new mycorrhiza and the maternal component of future fruitbody production or contribute as the paternal partner. The two idiomorphs (known as *MAT1–1* and *MAT1–2*) combine to produce further fruitbodies. See discussion.

would mix genotypes over long distances, but it does fit dispersal by invertebrates with a shorter foraging range (Ori et al., 2021). We hypothesize that this isolation by distance is likely to be stronger in fruiting areas in which mammals have been historically excluded or persecuted, when compared to those in which wild-mammal populations have been historically high. Regardless, the role of invertebrates in the spread of genetic material is of relevance to truffle cultivators and managers of truffle-producing woodland, where management methods can be altered to facilitate such activity. Further, the importance of invertebrates in sexual reproduction may be relevant to other heterothallic EcM or non-EcM fungi species (Ni et al., 2011).

4.3. Role in truffle producing sites and likely impacts of climate change

The high potential importance of *O. asellus*, and possibly invertebrates in general, in further fruitbody production of *T. melanosporum* (see 4.3) highlights the need for such organisms to be considered in the management of truffle production grounds. This is especially important in commercial production sites, where other vectors of ascospore dispersal, such as a mammal species, may be purposely excluded with fencing or trapping/poisoning (Chevalier and Sourzat, 2012). In such cases a balance may be important in having enough predation to facilitate ascospore dispersal and the sexual production of further fruitbodies and having too much that significant quantities of the harvest are damaged (Rosa-Gruszecka et al., 2017). The alternative is to spread ascospores manually to replicate the role of dispersal agents and this is an approach that has been shown to have merit (Murat et al., 2016; Garcia-Barreda et al., 2020) although it is not always feasible. Further, a suggested benefit of the COVID-19 pandemic is that the observed reduced harvesting pressure may benefit future production by increasing the ascospore load within the soil (Büntgen et al., 2021) although such impacts may be greater where there is a higher concentration of mycophagous animals.

Isopods are recognized as having a key role in nutrient recycling of woodland habitats (Yang and Li, 2020) and based on the evidence presented here, they may also be important in both the temporal and spatial distribution of EcM species. As such, they may have a significant and previously unknown role in EcM ecology. However, the impact of climate change is accelerating, and this has been widely explored, both in terms of the potential impacts on several EcM species (Boddy et al., 2014; Thomas and Büntgen, 2019), plant-partner distribution and functioning (Dyderski et al., 2018) and soil nutrient cycling (Jansson and Hofmockel, 2020). There is also emerging evidence of the impacts on isopods. For example, decreases in rainfall and increasing drought events and temperatures reduce feeding time, forage attack rates and time spent walking of *O. asellus* (Dias et al., 2012; Ott et al., 2012). In parallel with increased mortality, drier conditions also result in body mass loss (Dias et al., 2012; Dixie et al., 2015) which may impact fecundity (Antoł and Czarnoleski, 2018). Anthropogenically driven climate change is associated with increasing drought events and temperatures in regions in which *O. asellus* is found (EEA, 2016) as well as those of the target EcM species in this study (EEA, 2016; Thomas and Büntgen, 2019). As spore dispersal vectors, the impact of these changes may be significant from an ecological perspective.

5. Conclusions

Mycophagy has an important role in the distribution across space and time of hypogeal EcM species. This is important for the establishment of new populations but also in fruitbody development of heterothallic species. EcM spore dispersal by animals had previously focused on vertebrates and primarily mammals but here we have shown that the role of invertebrates can be significant. Isopods readily consume fruitbodies of the truffle species *T. melanosporum* and in doing so can potentially transport ascospores for at least 18 days to be deposited in an undamaged state. The shorter foraging range of invertebrates vs mammals fits the previously reported strong isolation by distance of *T. melanosporum* populations. Here, based on the evidence we have presented, we suggest that isopods have a role in the ecology of *T. melanosporum* and likely other EcM species, also.

At the same time as the importance of invertebrates in EcM ecology is emerging, developing climate change is threating the fecundity and survival of their populations. An awareness of these changes along with the understanding of such ecological dynamics, can contribute to informed management practices by custodians of woods and truffle orchards, alike.

Declaration of Competing Interest

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

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

Acknowledgments

With thanks to I. J Thomas and L. E Thomas for their helpful contributions to experimental design and care of isopods, throughout.

References

Anslan, S., Bahram, M., Tedersoo, L., 2016. Temporal changes in fungal communities associated with guts and appendages of Collembola as based on culturing and high-throughput sequencing. Soil Biol. Biochem. 96, 152–159.
Antoł, A., Czarnoleski, M., 2018. Size dependence of offspring production in isopods: a synthesis. ZooKeys 801, 337.
Bílá, K., Moretti, M., de Bello, F., Dias, A.T., Pezzatti, G.B., Van Oosten, A.R., Berg, M.P., 2014. Disentangling community functional components in a litter-macrodetritivore model system reveals the predominance of the mass ratio hypothesis. Ecol. Evol. 4 (4), 408–416.
Boddy, L., Büntgen, U., Egli, S., Gange, A.C., Heegaard, E., Kirk, P.M., Kauserud, H., 2014. Climate variation effects on fungal fruiting. Fungal Ecol. 10, 20–33.
Bonito, G., Smith, M.E., Brenneman, T., Vilgalys, R., 2012. Assessing ectomycorrhizal fungal spore banks of truffle producing soils with pecan seedling trap-plants. Plant Soil 356 (1), 357–366.
Brouwers, N.C., Newton, A.C., 2009. Movement rates of woodland invertebrates: a systematic review of empirical evidence. Insect Conserv. Diver. 2 (1), 10–22.
Büntgen, U., Čejka, T., Trnka, M., Thomas, P.W., 2021. Rethinking a sustainable truffle sector under global crises. Erdkunde 75 (4), 345–348. https://doi.org/10.3112/erdkunde.2021.04.06.
Caiafa, M.V., Jusino, M.A., Wilkie, A.C., Díaz, I.A., Sieving, K.E., Smith, M.E., 2021. Discovering the role of Patagonian birds in the dispersal of truffles and other mycorrhizal fungi. Curr. Biol. 31, 5558–5570.
Chevalier, G., Sourzat, P., 2012. Soils and techniques for cultivating tuber melanosporum and tuber aestivum in Europe. In: Edible Ectomycorrhizal Mushrooms. Springer, Berlin, Heidelberg, pp. 163–189.
Cooper, T., Vernes, K., 2011. Mycophagy in the larger bodied skinks of the genera Tiliqua and Egernia: are there implications for ecosystem health? Austral. Zool. 35 (3), 681–684.
Crowther, T.W., Stanton, D.W., Thomas, S.M., A'Bear, A.D., Hiscox, J., Jones, T.H., Boddy, L., 2013. Top-down control of soil fun-gal community composition by a globally distributed keystone consumer. Ecology 94 (11), 2518–2528.
De la Varga, H., Le Tacon, F., Lagoguet, M., Todesco, F., Varga, T., Miquel, I., Murat, C., 2017. Five years investigation of female and male genotypes in périgord black truffle (tuber melanosporum Vittad.) revealed contrasted reproduction strategies. Environ. Microbiol. 19 (7), 2604–2615.
Des Marteaux, L.E., Kullik, S.A., Habash, M., Schmidt, J.M., 2020. Terrestrial isopods *Porcellio scaber* and *Oniscus asellus* (Crustacea: Isopoda) increase bacterial abundance and modify microbial community structure in leaf litter microcosms: a short-term decomposition study. Microb. Ecol. 80 (3), 690–702.
Dias, N., Hassall, M., Waite, T., 2012. The influence of microclimate on foraging and sheltering behaviours of terrestrial isopods: implications for soil carbon dynamics under climate change. Pedobiologia 55 (3), 137–144.
Dixie, B., White, H., Hassall, M., 2015. Effects of microclimate on behavioural and life history traits of terrestrial isopods: implica-tions for responses to climate change. ZooKeys 515, 145.
Doménech, S.R., Barreda, S.G., 2014. Black truffle cultivation: a global reality. Forest Syst. 23 (2), 317–328.
Dromph, K.M., Borgen, A., 2001. Reduction of viability of soil borne inoculum of common bunt (Tilletia tritici) by collembolans. Soil Biol. Biochem. 33 (12–13), 1791–1795.

Dyderski, M.K., Paź, S., Frelich, L.E., Jagodziński, A.M., 2018. How much does climate change threaten European forest tree species distributions? Glob. Chang. Biol. 24 (3), 1150–1163.
EEA (European Environment Agency), 2016. Climate change, impacts and vulnerability in Europe 2016. https://www.eea.europa.eu/publications/climate-change-impacts-and-vulnerability-2016 (accessed 23 Oct 2021).
Elliott, T.F., Jusino, M.A., Trappe, J.M., Lepp, H., Ballard, G.A., Bruhl, J.J., Vernes, K., 2019a. A global review of the ecological significance of symbiotic associations between birds and fungi. Fungal Divers. 98 (1), 161–194.
Elliott, T.F., Bower, D.S., Vernes, K., 2019b. Reptilian mycophagy: a global review of mutually beneficial associations between reptiles and macrofungi. Mycosphere 10 (1), 776–797.
Fogel, R., Trappe, J.M., 1978. Fungus consumption (mycophagy) by small animals. Northwest Sci. 52 (1), 1–31.
Garcia-Barreda, S., Marco, P., Martín-Santafé, M., Tejedor-Calvo, E., Sánchez, S., 2020. Edaphic and temporal patterns of tuber melanosporum fruitbody traits and effect of localised peat-based amendment. Sci. Rep. 10 (1), 1–9.
Garnica, S., Weiss, M., Walther, G., Oberwinkler, F., 2007. Reconstructing the evolution of agarics from nuclear gene sequences and basidiospore ultrastructure. Mycol. Res. 111 (9), 1019–1029.
Glassman, S.I., Levine, C.R., DiRocco, A.M., Battles, J.J., Bruns, T.D., 2016. Ectomycorrhizal fungal spore bank recovery after a severe forest fire: some like it hot. ISME J. 10 (5), 1228–1239.
Gregory, S., 2021. An Introduction to the Identification of the Woodlice (Isopoda: Oniscidea) occurring in Berkshire, Buckingham-shire and Oxfordshire. Available online: https://anhso.org.uk/wp-content/uploads/Fritillary/frit8-woodlice.pdf (accessed on 01 March 2021).
Halbwachs, H., Bässler, C., 2020. No bull: dung-dwelling mushrooms show reproductive trait syndromes different from their non-coprophilous allies. Mycol. Prog. 19 (8), 817–824.
Halbwachs, H., Brandl, R., Bässler, C., 2015. Spore wall traits of ectomycorrhizal and saprotrophic agarics may mirror their distinct lifestyles. Fungal Ecol. 17, 197–204.
Horton, T.R., 2017. Spore dispersal in ectomycorrhizal fungi at fine and regional scales. In: Biogeography of mycorrhizal symbiosis. Springer, Cham, pp. 61–78.
Hyder, N., Coffey, M.D., Stanghellini, M.E., 2009. Viability of oomycete propagules following ingestion and excretion by fungus gnats, shore flies, and snails. Plant Dis. 93 (7), 720–726.
Jansson, J.K., Hofmockel, K.S., 2020. Soil microbiomes and climate change. Nat. Rev. Microbiol. 18 (1), 35–46.
Kanters, C., Anderson, I.C., Johnson, D., 2015. Chewing up the wood-wide web: selective grazing on ectomycorrhizal fungi by collembola. Forests 6 (8), 2560–2570.
Kobayashi, M., Kitabayashi, K., Tuno, N., 2017. Spore dissemination by mycophagous adult drosophilids. Ecol. Res. 32 (4), 621–626.
Lee, T.H., 2002. Feeding and hoarding behaviour of the Eurasian red squirrel Sciurus vulgaris during autumn in Hokkaido, Japan. Acta Theriol. 47 (4), 459–470.
LeFait, A., Gailey, J., Kernaghan, G., 2019. Fungal species selection during ectomycorrhizal grazing by Collembola. Symbiosis 78 (1), 87–95.
Lilleskov, E.A., Bruns, T.D., 2005. Spore dispersal of a resupinate ectomycorrhizal fungus, Tomentella sublilacina, via soil food webs. Mycologia 97 (4), 762–769.
Martin, F., Kohler, A., Murat, C., Balestrini, R., Coutinho, P.M., Jaillon, O., Montanini, B., Morin, E., Noel, B., Percudani, R., Porcel, B., 2010. Périgord black truffle genome uncovers evolutionary origins and mechanisms of symbiosis. Nature 464 (7291), 1033–1038.
Maser, C., Maser, Z., 1987. Notes on mycophagy in four species of mice in the genus Peromyscus. Great Basin Natural. 308–313.
Mattson, D.J., Podruzny, S.R., Haroldson, M.A., 2002. Consumption of fungal sporocarps by Yellowstone grizzly bears. Ursus 95–103.
Murat, C., Rubini, A., Riccioni, C., De la Varga, H., Akroume, E., Belfiori, B., Paolocci, F., 2013. Fine-scale spatial genetic structure of the black truffle (tuber melanosporum) investigated with neutral microsatellites and functional mating type genes. New Phytol. 199 (1), 176–187.
Murat, C., Bonneau, L., De La Varga, H., Olivier, J.M., Fizzala, S., Le Tacon, F., 2016. Trapping truffle production in holes: a promising technique for improving production and unravelling truffle life cycle. Ital. J. Mycol. 45, 2531–7342.
Nakamori, T., Suzuki, A., 2010. Spore resistance and gut-passage time of macrofungi consumed by Ceratophysella denisana (Collembola: Hypogastruridae). Fungal Ecol. 3 (1), 38–42.
Ni, M., Feretzaki, M., Sun, S., Wang, X., Heitman, J., 2011. Sex in fungi. Annu. Rev. Genet. 45, 405–430.
Ori, F., Menotta, M., Leonardi, M., Amicucci, A., Zambonelli, A., Covès, H., Iotti, M., 2021. Effect of slug mycophagy on tuber aestivum spores. Fungal Biol. 125 (10), 796–805.
Ott, D., Rall, B.C., Brose, U., 2012. Climate change effects on macrofaunal litter decomposition: the interplay of temperature, body masses and stoichiometry. Philosoph. Trans. Royal Soc. B: Biol. Sci. 367 (1605), 3025–3032.
Pacioni, G., Rapino, C., Zarivi, O., Falconi, A., Leonardi, M., Battista, N., Maccarrone, M., 2015. Truffles contain endocanna-binoid metabolic enzymes and anandamide. Phytochemistry 110, 104–110.
Piattoni, F., Amicucci, A., Iotti, M., Ori, F., Stocchi, V., Zambonelli, A., 2014. Viability and morphology of tuber aestivum spores after passage through the gut of Sus scrofa. Fungal Ecol. 9, 52–60.
Rabatin, S.C., Stinner, B.R., 1988. Indirect effects of interactions between VAM fungi and soil-inhabiting invertebrates on plant pro-cesses. Agric. Ecosyst. Environ. 24 (1–3), 135–146.
Rosa-Gruszecka, A., Gange, A.C., Harvey, D.J., Jaworski, T., Hilszczański, J., Plewa, R., Hilszczańska, D., 2017. Insect-truffle interactions–potential threats to emerging industries? Fungal Ecol. 25, 59–63.
Rubini, A., Belfiori, B., Riccioni, C., Tisserant, E., Arcioni, S., Martin, F., Paolocci, F., 2011a. Isolation and characterization of MAT genes in the symbiotic ascomycete tuber melanosporum. New Phytol. 189 (3), 710–722.
Rubini, A., Belfiori, B., Riccioni, C., Arcioni, S., Martin, F., Paolocci, F., 2011b. Tuber melanosporum: mating type distribution in a natural plantation and dynamics of strains of different mating types on the roots of nursery-inoculated host plants. New Phytol. 189 (3), 723–735.
Smith, S.E., Read, D.J., 2010. Mycorrhizal Symbiosis. Academic Press.
Splivallo, R., Ottonello, S., Mello, A., Karlovsky, P., 2011. Truffle volatiles: from chemical ecology to aroma biosynthesis. New Phytol. 189 (3), 688–699.
Taschen, E., Rousset, F., Sauve, M., Benoit, L., Dubois, M.P., Richard, F., Selosse, M.A., 2016. How the truffle got its mate: insights from genetic structure in spontaneous and planted Mediterranean populations of tuber melanosporum. Mol. Ecol. 25 (22), 5611–5627.
Thomas, P., Büntgen, U., 2019. A risk assessment of Europe's black truffle sector under predicted climate change. Sci. Total Environ. 655, 27–34.
Thomas, P.W., 2021. Ectomycorrhiza resilience and recovery to extreme flood events in tuber aestivum and Quercus robur. Mycorrhiza 31 (4), 511–517.
Thomas, P.W., Elkhateeb, W.A., Daba, G., 2019. Truffle and truffle-like fungi from continental Africa. Acta Mycol 54 (2).
Trappe, J.M., 2009. Diversity, Ecology, and Conservation of Truffle Fungi in Forests of the Pacific Northwest, 772. US Department of Agriculture, Forest Service, Pacific Northwest Research Station.
Trappe, J.M., Claridge, A.W., 2005. Hypogeous fungi: Evolution of reproductive and dispersal strategies through interations with animals and plants. In: The Fungal Community-its Organizafion and Role in the Ecosystem. Taylor & Francis, Boca Raton, FL, USA, pp. 613–622.
Urban, A., 2016. Truffles and small mammals. In: True Truffle (Tuber spp.) in the World. Springer, Cham, pp. 353–373.
Vašutová, M., Mleczko, P., López-García, A., Maček, I., Boros, G., Ševčík, J., Kjøller, R., 2019. Taxi drivers: the role of animals in transporting mycorrhizal fungi. Mycorrhiza 29 (5), 413–434.
Yang, X., Li, T., 2020. Effects of terrestrial isopods on soil nutrients during litter decomposition. Geoderma 376, 114–546.