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The Release and Spread of Basidiospores of Red-Listed Wood-Decay Fungus *Fistulina hepatica* in Oak Stands

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Abstract: The aim of this study was to obtain a better understanding on short-distance basidiospore dispersal of the wood-decay fungus *Fistulina hepatica*, thereby providing valuable knowledge for the conservation management of this protected species. Specifically, the study was expected to reveal site-specific patterns of basidiospore release and spread in oak stands during one fruiting season under north European conditions. The trapping of fungal spores was carried out between August and October 2022 using passive spore traps placed in three oak stands (>200-year-old) in central Lithuania. The average daily temperature was recorded throughout the period of spore trapping. Collected samples were analyzed using high-throughput sequencing of fungal ITS2 rDNA. The results showed that the relative abundance of *F. hepatica* reads was influenced by the time of fruitbody maturation, but not by the average daily temperature. Although there was a certain variation among different study sites, the results showed that a great majority of *F. hepatica* spores were deposited within 50 m from the fruitbody, showing that the fungus to a large extent is dependent on local habitats for colonization.

Keywords: beef steak fungus; conservation management; protected species; *Quercus robur*; spore dispersal; wood-decay

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1. Introduction

Fungi represent one of the most diverse groups of organisms, accounting for a large proportion of species richness and being key players in ecosystem processes [1,2]. Until recently, fungi have been considered as a poorly studied group of organisms due to their mostly hidden nature and often sporadic and short-lived fruitbodies. However, a recent development of new research methods has provided new insights in fungal biology and ecology [3]. For example, DNA-based methods allow the detection and identification of fungal species directly in environmental samples. Such methods also open new possibilities for gathering new information needed for the conservation management of protected fungal species, which can be an important component of overall fungal biodiversity [4]. Recent observations suggest that the loss of fungal biodiversity can be as great as in other more well-studied groups of organisms [5]. The conservation of specific fungal species can be affected by their habitat requirements, which must be maintained and renewed to ensure their availability [6].

One example of such fungi is *Fistulina hepatica*, which is a wood-decay fungus that mainly develops on older trees of genera *Quercus* and *Castanea*. In rare cases, it is also found on trees of genera *Acer*, *Betula*, *Corylus*, or *Fagus* [7]. This fungus occurs in the temperate climate zone and follows the distribution of host trees [7–9]. Although the species is widespread, its abundance is not high due to the shortage of suitable habitats, i.e., old-growth trees. The species is red-listed and protected in many countries, including Poland, Lithuania, and some Scandinavian countries [10]. *Fistulina hepatica* is often found

in forests with old and naturally dying host trees, but it may also be found in urban environments with suitable habitats [11]. *Fistulina hepatica* usually infects old living trees that have reached their maturity, where it develops as a pathogen at the same time, causing brown rot (Figure 1) [7]. Interestingly, this fungus was not found in stem wounds of mid-age oaks, showing that it may take many years for the fungus to establish in wood, cause the rot, and eventually produce fruitbodies [12]. Indeed, the fungus can develop in a tree for several decades before the wood structure is heavily affected, i.e., wood becomes brittle and falls into individual prismatic particles (Figure 1). Wood decay usually develops in the central part of the stem and, in rare cases, can reach up to six meters in height [9,13]. As decay progresses, hollow cavities can appear in the lower part of the tree. It can also colonize and persist in fallen trees or stumps as a wood-decaying saprotroph [13].

Figure 1. Brown rot of *Quercus robur* wood caused by *Fistulina hepatica*.

Fruitbodies are annual and appear on tree stems, in hollows or old wounds, on roots by emerging from the ground, and quite often on stumps in the later stages of decay. Fruitbodies often emerge in the second half of the summer and develop until late autumn. The fruitbody is soft, red in color, and secretes red juice. Pores are circular, creamy to yellowish in color, and often with gutta drops. Basidiospores are reddish brown, 4–5.5 × 3–4 µm in size [14], and represent the main source of fungal infections, which often takes place via exposed wood [15].

The production and release of spores of different fungal species has been widely discussed in the literature [16,17]. Depending on the fungal species and their adaptation, spores can be spread in the form of aerosols, with water droplets, or using vectors such as insects or animals [18]. Nevertheless, wind is the most common mean that fungi use for the spread of their spores. Although many fungal species release a large quantity of

spores, these are usually deposited in a close vicinity to fruitbodies and only 0.8–2.2% of all spores are transported much further [19]. However, to estimate accurately how fungal spores disperse over long distances is often difficult [20]. For example, the spores of *Fusarium*, *Mycosphaerella*, and *Lobaria* were shown to travel distances of 40 to 400 m [21], while conidia of *Aspergillus sydowi* can travel thousands of kilometers [22]. The latter fungi are widespread and abundant in different environments, but the ability of protected and rare fungal species to spread over short and long distances can be more vital [23].

In many European countries, including Lithuania, there is only a limited number of old oaks suitable for the establishment of *F. hepatica*. Such oaks are often scattered both in forests and in agricultural landscapes, which limits the conservation management of *F. hepatica*. Even in larger oak stands, the distances between old oak trees can often reach more than a hundred meters, which may directly affect the spread and colonization of new habitats by this fungus. The aim of this study was to obtain a better understanding on short-distance basidiospore dispersal of the wood-decay fungus *F. hepatica*, which was expected to contribute to the conservation management of this protected species.

2. Materials and Methods

The spores of *F. hepatica* were trapped between August and October 2022, i.e., in the period when fruitbodies of *F. hepatica* are formed. The spore trapping was carried out in three oak stands, including Šilinė, Punia, and Dūkšos, which were known for the presence of *F. hepatica* (Figure 2).

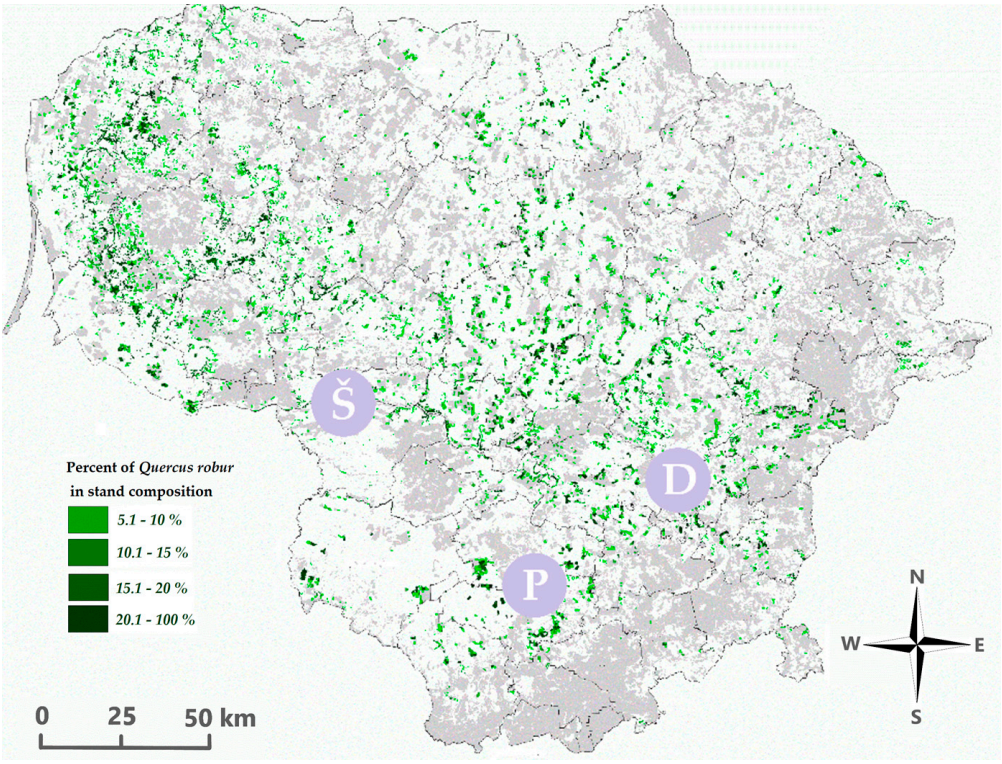

Figure 2. Map of Lithuania showing the position of study sites: P—Punia (N 54°30′50.1″, E 24°04′51.6″), D—Dūkštos (N 54°49′57.2″, E 24°57′15.0″), and Š—Šilinė (N 55°5′22.58″, E 22°57′13.85″). The map was reproduced with permission from FORESTGEN, www.forestgen.mi.lt (accessed on 5 September 2023).

The selected oak stands were more than 200 years old. The oak stands at Punia and Dūkštos were similar in tree species composition as these were dominated by *Q. robur* with a small admixture of *Picea abies* and *Alnus incana* and with *Corylus avellana*, *Sorbus* sp., and *Ulmus* sp. in the understory. The ground vegetation consisted of *Aegopodium podagraria*,

Urtica dioica, and *Carex* sp. At Šilinė, it was a pure old-growth oak stand with rare *Prunus padus* in the understory. The herbaceous cover consisted of *Corex* sp. Punia and Šilinė oak stands being on fertile binary soils of normal humidity, i.e., with groundwater deeper than 3 m from the surface. The Dūkštos oak stand was also on soils of normal humidity, but soil fertility was higher than in the other two oak stands of the study. The distance between individual study sites was >70 km (Figure 2). In all sites, the fruitbodies of *F. hepatica* were growing on the stems of living oaks at a height of 0.5–1.0 m from the ground. Fungal spores were collected using passive spore traps [24,25]. Each trap consisted of a 9 cm diameter Munktell filter paper (made of cotton stains, particle retention 5–6 µm, class 1F) (Ahlstrom-Munksjö, Stockholm, Sweden) that was sandwiched between two 10 × 10 cm stainless steel grills (mesh size 1 × 1 cm) that were attached horizontally to a 0.8 m long stick to anchor the trap to the ground (Figures 3 and 4). Spore traps were placed at the end of August, i.e., just after the emergence of *F. hepatica* fruitbodies.

Figure 3. Development of *Fistulina hepatica* fruitbody. (**A**)—7 September 2022 (15-day-old fruitbody); (**B**)—13 September (21-day-old); (**C**)—21 September (29-day-old); (**D**)—27 September (35-day-old); (**E**)—5 October (43-day-old); (**F**)—12 October (50-day-old); and (**G**)—19 October (57-day-old). Plastic sheets in pictures were sticky traps used for collection of wood-colonizing insects (not this study). They did not interfere with the sampling of fungal spores.

Figure 4. The passive spore trap with a filter paper in one of oak stands.

At each site, spore traps were placed at a distance of 5, 25, 50, 150, and 300 m from the fruitbody of *F. hepatica* (Figures 3 and 4). Spore traps were placed in the direction of prevailing winds, i.e., from west to east. In all sites, spore trapping was initiated on 23 August 2022 and filters with fungal spores were collected once a week [23]. The spore trapping was continued until the end of the fruiting season of *F. hepatica*, which was different in different sites. In Šilinė, the last spore sample was collected on 5 October; in Punia, on 27 September; and in Dūkštos, on 26 October. In total, there were 30 filters with spore samples collected in Šilinė, 25 in Punia, and 45 in Dūkštos, resulting in 100 samples altogether. During the sampling period, each site was regularly monitored (within a radius of 500 m) for the presence of other fruitbodies of *F. hepatica*, but such fruitbodies were not detected. Collected samples with fungal spores were labeled, packed in plastic bags, transported to the laboratory, and stored at −20 °C before further processing. At each site, information on air temperature and relative humidity was collected at one-hour intervals near the fruitbodies using UT330C (Uni-Trend Technology, Dongguan, China) temperature and humidity loggers.

2.1. DNA Extraction, PCR Amplification, and Sequencing

In the laboratory, samples with fungal spores were freeze-dried (Labconco FreeZone Benchtop Freeze Dryer, Cole-Parmer, Vernon Hills, IL, USA) at −60 °C for 24 h. For the isolation of DNA, half of each filter paper was taken, cut into smaller pieces, and placed into three 2 mL screw-cap centrifugation tubes together with three sterile glass beads, which were 3 mm in diameter. The remaining materials were stored at −20 °C as a backup. The isolation of DNA followed the study by Marčiulynas et al. [26]. The concentration of DNA was measured using a NanoDrop One spectrophotometer (Thermo Scientific, Rodchester, NY, USA) and adjusted to 10 ng/μL.

Amplification of the ITS2 rDNA region was carried out using a fungal specific primer gITS7 [27] and a universal primer ITS4 [28], both containing sample identification barcodes. PCR reactions were performed in 50 µL reactions and included 0.25 ng/µL of template DNA, 200 µM of dNTPs, 750 µM of MgCl2, 0.025 µM of polymerase (5 U/µL), (DreamTaq Green, Thermo Scientific, Waltham, MA, USA), and 200 nM of each primer. The PCR reactions were performed using an Applied Biosystems 2720 thermal cycler (Waltham, MA, USA) and the following parameters: 95 °C for 5 min, which was followed by 27 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and an extension at 72 °C for 30 s. The final extension step was at 72 °C for 7 min. The PCR products were examined on a 1.5% agarose gel stained with Nancy-520 (Sigma-Aldrich, Stockholm, Sweden). The purification of PCR products was conducted using 3 M sodium acetate (pH 5.2) (Applichem GmbH, Darmstadt, Germany) and a 96% ethanol mixture (1:2). The quantification of purified PCR products was carried out using a Qubit fluorometer 4.0 (Life Technologies, Hägersten, Sweden). High-throughput sequencing of the amplified samples, which were pooled in an equimolar mix, was achieved using the PacBio Sequel platform and two SMRT cells (SciLifeLab, Uppsala, Sweden).

2.2. Bioinformatics and Statistical Analysis

Produced sequences were quality filtered and clustered using the SCATA NGS sequencing pipeline (http://scata.mykopat.slu.se, accessed on 2 September 2023). Short sequences (<200 bp) and low-quality sequences were removed, while homopolymers were collapsed to three base pairs (bp) prior to clustering. Sequences missing barcodes or primers were also removed. After quality filtering, sequences were clustered into different OTUs using single-linkage clustering based on a 98.5% similarity. For each cluster, the most common genotype (true read) was used to represent each OTU. A consensus sequence was generated for clusters with two sequences. The GenBank (NCBI) database and Blastn algorithm were used for taxonomic identification of different OTUs. The following parameters were used for identification: sequence coverage of at least 80%, species level—98–100%, and genus level—94–97%. Sequences that did not meet these criteria remained unidentified. Correlation analysis was carried out to reveal the relationship between the abundance of high-quality sequences of *F. hepatica* and air temperature, and the abundance of high-quality sequences of *F. hepatica* and the distance from the fruitbody using SAS v. 9.4 (Cary, NC, USA).

3. Results and Discussion

Although high-throughput sequencing generated a large number of reads, in the present study, only reads representing *F. hepatica* were used in the downstream analyses. Taken together, the absolute abundance of *F. hepatica* reads was as follows: in Dūkštos, 338 (62.4%) reads; in Šilinė, 123 (22.7%) reads; and in Punia, 81 (14.9%) reads. Although the number of *F. hepatica* reads varied among different sites, their abundance largely depended on the distance from the fruitbody (Figure 5). In the Punia and Dūkštos sites, 90.1% and 66.7% of all sequence reads were detected 5 m away from the fruitbody, respectively. In the Šilinė site, 60.2% of *F. hepatica* reads were detected at a distance of 50 m from the fruitbody, 36.6%—at a distance of 25 m, and 3.2%—at a distance of 5 m (Figure 5). At the distance of 150 m and 300 m from the fruitbody, there were only 0.6–3.7% of reads detected at the Dūkštos or Punia sites and no reads were detected at the Šilinė site. Such results may suggest that the structure of the stand and the density of the understory vegetation had only a minor effect on the dispersal of *F. hepatica* spores at each site. For example, in the Šilinė oak stand, which had no understory, sequence reads of *F. hepatica* were not found at a distance larger than 50 m from the fruitbody, while in the more dense oak stands of Dūkštos and Punia, sequence reads were found at much larger distances from the fruitbody (Figure 5). The latter may suggest that a great majority of *F. hepatica* spores were deposited within a distance of 50 m from their fruitbodies. This may also suggest that the spread of *F. hepatica* spores can be considered sufficient only within short distances from the fruitbody,

i.e., within the same stand. In addition, as some spores are usually not viable, this may further limit their spread and colonization of new habitats [29].

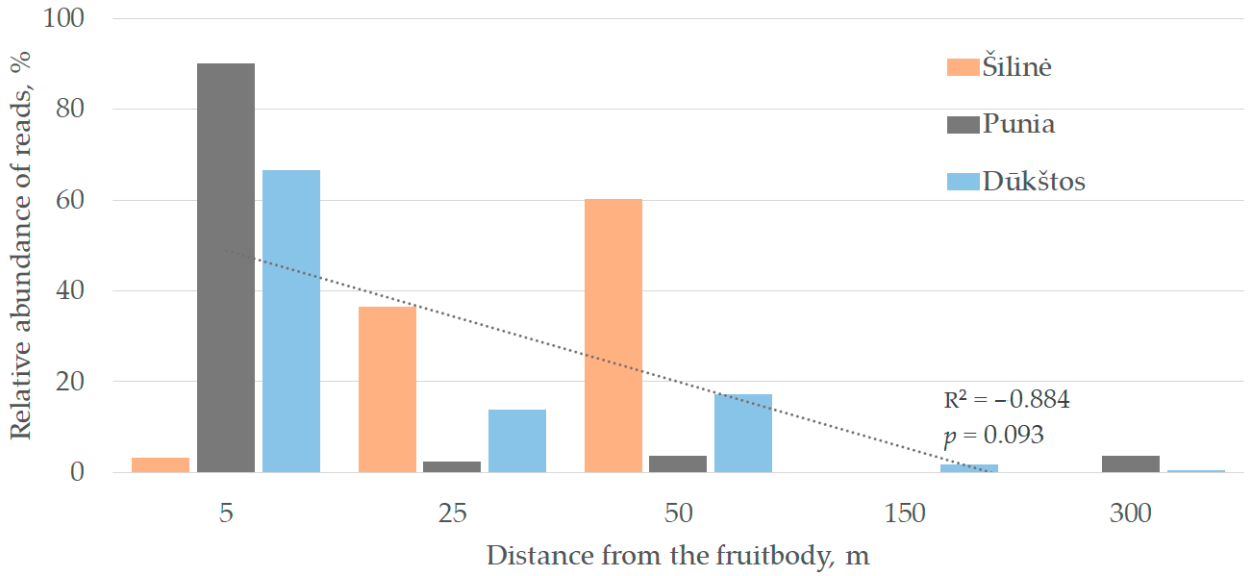

Figure 5. Relative abundance of *Fistulina hepatica* sequence reads at different distances from the fruitbody. All sampling time points are combined.

Although there was no significant correlation between the number of sequence reads and the distance from the fruitbody (Figure 5), in agreement with other studies, the results showed that there is a gradient of spore deposition from the fruitbody [30]. Stenlid [31] showed that the gradient of spore deposition of *Heterobasidion annosum* (Fr) Bref., which is a common root-rot pathogen in temperate coniferous forests, is very steep within the first 100 m from the fruitbody. However, under extreme atmospheric conditions, its spores can travel up to 500 km.

Sequence reads of *F. hepatica* were detected for the first time on 30 August, showing the release and spread of *F. hepatica* spores taking place in low abundances soon after the emergence of the fruitbody (Figure 6). In central Slovakia, the spores of *F. hepatica* were detected from the beginning of July until the first half of November, showing that sporulation takes place throughout the entire developmental period of the fruitbody [32]. We found that *F. hepatica* sporulated most intensively during the period of four weeks, i.e., from the end of September to the middle of October (Figure 6). However, there were certain differences at different study sites as at Šilinė and Punia, the largest number of *F. hepatica* sequence reads was detected on 27 September, while in Dūkštos, it was on 12 October. These differences were mainly due to the decay of fruitbodies as after 27 September, at both Šilinė and Punia, the fruitbodies decomposed and a further sampling of spores was not possible. At Dūkštos, fruitbodies of *F. hepatica* decomposed at the end of October, i.e., one month later. Nuss [33] showed that under favorable conditions, sporulation takes place for almost five weeks. Although Nuss [33] found that the period between the emergence of the fruitbody and its maturation and intensive sporulation was about two weeks, our observations showed that such a period was much longer, i.e., about 28 days (Figures 3 and 6), which can probably be due to local environmental conditions. It was shown that intensive autumn sporulation of polypore fungi usually ends at average temperatures below 15 °C [33]. The results of the present study showed that the most intensive sporulation of *F. hepatica* occurred when the average daily temperature was around 10 °C (Figure 6). Nevertheless, there was no significant correlation between the number of detected sequence reads of *F. hepatica* and the average daily temperatures ($p > 0.05$).

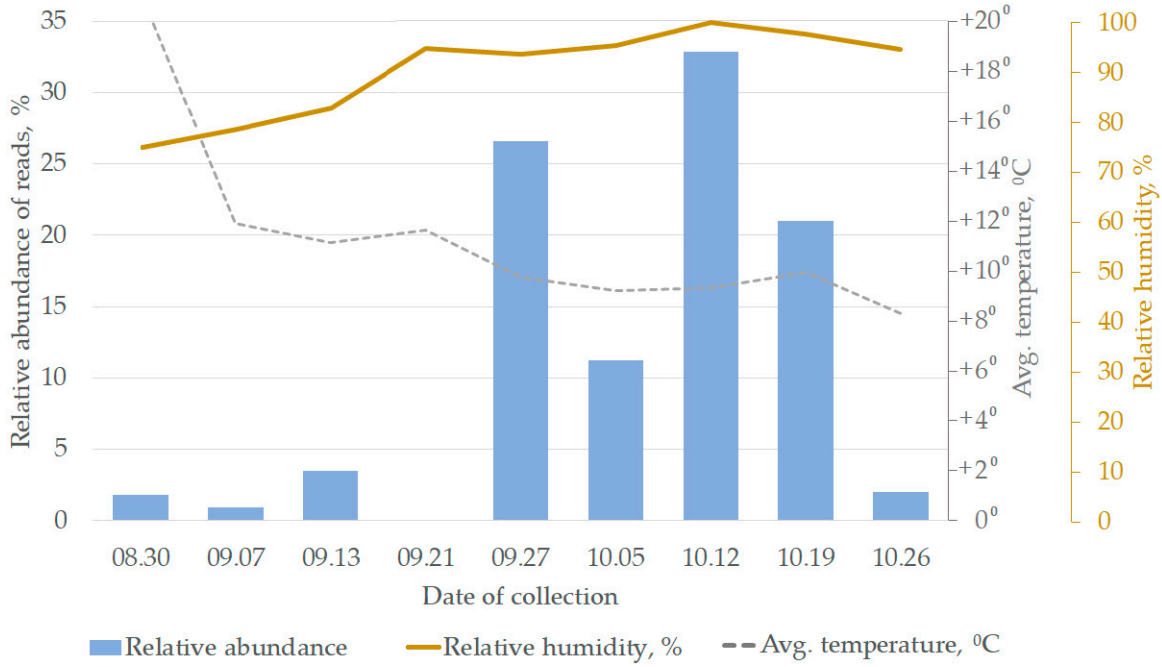

Figure 6. Relative abundance of *Fistulina hepatica* sequence reads, average daily temperature, and relative air humidity detected at different time points. All study sites and distances from the fruitbody are combined.

In contrast to the average daily temperature, the relative air humidity showed a significant positive correlation ($r = 0.62$, $p < 0.05$) with the number of detected *F. hepatica* sequence reads (Figure 6). It was shown that air humidity may have a varying effect on the production and release of spores in different fungal species. For example, a high relative humidity favours the formation and release of *Ganoderma* sp. spores [34,35], but the opposite is observed for *Aspergillus fumigatus* and *Penicillium* sp. spores, which reach their minimum when the relative air humidity exceeds 70% [36]. The abundance of *Cladosporium* sp. spores in the air was shown to positively correlate with temperature but negatively correlate with relative air humidity and rainfall [37,38]. Taken together, the results suggest that the release of *F. hepatica* spores is most dependent on fruitbody maturation and relative air humidity as in our study, where the period of massive release of *F. hepatica* spores coincided with the period of fruitbody maturation and higher relative air humidity, but not with the changes in temperature. After reaching the peak, a sharp decrease in the abundance of *F. hepatica* sequence reads, and thus spores, is likely to be associated with a rapid decomposition of soft fruitbodies of *F. hepatica* [33,39].

4. Conclusions

The results demonstrated that a great majority of *F. hepatica* spores were deposited with a distance of 50 m from the fruitbody, showing that this protected species to a large extent depends on local habitats for colonization, while the long-distance dispersal is likely to be very occasional. The release of *F. hepatica* spores took place during the entire period of fruitbody development, but the extent of spore release was most dependent on the state of fruitbody maturation and a relative air humidity, but not on the average daily temperature. To increase the abundance of *F. hepatica* in oak stands, conservation measures should be directed to the creation of new habitats (e.g., exposed oak wood) in a close vicinity to its fruitbodies.

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formal analysis, A.M. (Adas Marčiulynas) and A.M. (Audrius Menkis); investigation, A.M. (Adas Marčiulynas); resources, A.M. (Adas Marčiulynas) and A.M. (Audrius Menkis); data curation, A.M. (Adas Marčiulynas) and A.M. (Audrius Menkis); writing—original draft preparation, A.M. (Adas Marčiulynas); writing—review and editing, A.M. (Audrius Menkis); visualization, A.M. (Adas Marčiulynas) and A.M. (Audrius Menkis); supervision, A.M. (Audrius Menkis). All authors have read and agreed to the published version of the manuscript.

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