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Entomophthoralean fungi overwinter with the bird cherry-oat aphid on bird cherry trees

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ABSTRACT

In Scandinavia, the bird cherry-oat aphid *Rhopalosiphum padi* overwinter as eggs on the bird cherry tree *Prunus padus*. Branches of *P. padus* were collected at the late February / early March from 17 locations in Norway over a three-year period. We found 3599 overwintering aphid eggs, 59.5% of which were dead. Further, a total of 879 overwintering fungus-killed cadavers were observed. These cadavers were found close to bud axils, where overwintering eggs were also usually attached. Cadavers were infected with either *Zoophthora* cf. *aphidis* or *Entomophthora planchoniana*. All the fungal-killed cadavers were filled with overwintering structures of *Z.* cf. *aphidis* (as resting spores) or *E. planchoniana* (as modified hyphal bodies). We found a significant negative correlation between eggs and cadavers per branch. However, both numbers of eggs and cadavers varied greatly between years and among tree locations. This is the first report of *E. planchoniana* overwintering in *R. padi* cadavers as modified hyphal bodies. We discuss whether *P. padus* may act as an inoculum reservoir for fungi infecting aphids in cereals in spring.

1. Introduction

Winter in temperate and subarctic climates represents a bottleneck for many species, especially for poikilothermic organisms such as insects and fungi. Consequently, several overwintering strategies have been naturally selected to overcome cold conditions and lack of resources: for example, antifreeze agents are synthesized in structures adapted to overwintering; or behavioural avoidance is used, such as overwintering in buffering environments, *e.g.*, under snow cover (*e.g.,* Snider et al., 2000; Nielsen et al., 2003; Bale & Hayward, 2010; Kunert et al., 2019). Successful overwintering is critical for establishing new populations in spring and has implications for all organisms sensitive to temperature changes. In agroecosystems, this is particularly significant for pests and their natural enemies. Indeed, pest pressure and the need for biological control are dictated partly by the quantity and quality of local reservoirs, both of which are products of the overwintering success of organisms at both trophic levels.

The bird cherry-oat aphid, *Rhopalosiphum padi* (Hemiptera: Aphidoidea), is one of the major pests in Europe on cereals (Blackman and Eastop, 2007). In northern Europe, it is completely holocyclic and occurs on different plant hosts over the year. During summer, *R. padi* feeds on cereals and other grasses (Poaceae) (Finlay and Luck, 2011), and during autumn and spring it feeds on the bird cherry, *Prunus padus* (Rosales: Rosaceae) (Leather, 1992). At the beginning of autumn, aphids produce alate gynoparae and males that migrate to their winter host (Leather, 1992). They choose bird cherry trees that will maximise the fitness of their offspring (Leather, 1986; Kurppa, 1989). The males slowly mature and the gynoparae females produce oviparae females that can mate and produce overwintering eggs containing glycerol as a cryoprotectant or antifreeze agent that confers cold resistance to -40 °C (Sömme, 1969). Oviparae females seem to locate themselves randomly within a tree (Leather, 1981a), although their choice of egg-laying site is non-random (Leather, 1981b).

Oviparae females compete for the best egg-laying sites: sheltered

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locations in the bud axils (a behavioural avoidance strategy) (Leather, 1992). When population densities are high, females will also lay eggs in the cracks of branches (Kurppa, 1989). At first, egg mortality is density-dependent, and only sheltered, well-attached eggs survive the first difficult winter conditions (Leather, 1992). Even among those eggs laid in optimal sites, 3% egg mortality per week was estimated, increasing to 6.5% per week in early spring due to the increased activity of natural enemies. Total winter survival of eggs was estimated to be around 30% (Leather, 1980; 1983). At the beginning of spring, eggs hatch, and *R. padi* establishes new colonies on unfurling bird cherry leaves. New fundatrices produce 2–3 wingless generations on the tree, after which alates are produced that migrate to cereals or other graminaceous plants (Hansen, 2006). Scandinavian studies have shown a correlation between the number of overwintering eggs on bird cherry trees at the end of winter and *R. padi* population size the following summer on cereals (*e.g.*, Leather, 1983). In Norway and in Finland, bird cherry trees have been monitored for *R. padi* eggs for more than 20 years. From this prevalence, an estimate of spring *R. padi* pressure is then provided by a plant protection decision support system (in Norway: https://www.vips-landbruk.no/ and in Finland: https://maatalousinfo.luke.fi/fi/cms/kasvinterveys/fi/kasterveys/arkisto/vanhat-tuomikirvaennusteet/).

Entomopathogenic fungi in the order Entomophthorales are an important group of natural enemies of *R. padi.* Infection rates of up to 90% have been reported in aphid populations (Elkassabany et al., 1992; Hatting et al., 2000; Chen and Feng, 2004; Barta and Cagáň, 2004; 2007; Jensen et al. 2008). Several fungal species have been documented as infecting *R. padi*, namely: *Pandora neoaphidis*, *Entomophthora planchoniana*, *Conidiobolus obscurus*, *Neozygyites fresenii*, *Zoophthora aphidis*, *Z. radicans*, and *Z. occidentalis* (Nielsen and Steenberg, 2004; Barta and Cagáň, 2004; Barta 2009; Manfrino et al., 2014). Entomophthoralean fungi are mainly biotrophic with a close relationship to their hosts (*e.g.*, Pell et al., 2010). They can form several overwintering structures: 1) resting spores in the host or in the ambient environment (*e.g.*, Klingen et al., 2008; Duarte da Silveira et al., 2013; Hajek et al., 2018), 2) loricoconidia (thick-walled conidia) found in soil (Nielsen et al., 2003), 3) modified hyphae –with various shapes from irregular to club shaped and slightly thicker cell wall– present either in cadavers or in soil (Keller, 1987), 4) semi-latent hyphal bodies in their live, hibernating hosts (Klingen et al., 2008; Eilenberg et al., 2013). The formation of overwintering structures is induced by many factors, including change in host morph, food quality, day length and temperature (reviewed in Hajek et al., 2018). Further, activation and germination of overwintering structures may be induced by cues from the host itself (Nielsen et al., 2003; Duarte da Silveira et al., 2013; Hajek et al., 2018). Previous studies of fungus-killed *R. padi* on bird cherry trees reported cadavers with conidia only (no overwintering structures) and mainly in autumn (Nielsen and Steenberg, 2004; Barta and Cagáň, 2004). Hajek et al. (2018) hypothesized that entomophthoralean fungi would benefit from overwintering close to where the host is active in spring since it would optimise the probability of reaching the host and initiating primary fungal infections in spring populations. Hence, we hypothesised that entomophthoralean fungi of *R. padi* overwinter on or in the vicinity of bird cherry trees.

The aim of this study was, therefore, to answer the following research questions: 1) Do entomophthoralean fungi overwinter in *R. padi* on bird cherry trees? 2) If so, what are their overwintering structures? 3) Which fungal species are present in the overwintering aphid population, and how prevalent are they? 4) Does the prevalence of *R. padi* eggs and fungus-killed cadavers vary between years, latitude, or tree locations?

2. Materials and methods

2.1. Field sampling

Ten branches of bird cherry located at the borders of cereal fields in Norway were collected between the end of February and beginning of March from 17 locations (Fig. 1). Thirteen locations were monitored in this way over three years (2017–2019), two locations for two years (2017–2018), and two were sampled only in 2019 (Table 1). Branches were sampled from the last annual shoots from the tree crown and transported to the laboratory where they were either immediately examined for cadavers and eggs or kept in cold storage (4–7 °C) until examination to avoid hatching of eggs. Over three years, 450 branches were examined for overwintering *R. padi* eggs and for fungus-killed overwintering cadavers as described below.

2.2. Aphid and fungal species identification

Eighty-three fungus-killed overwintering cadavers collected in 2018 were cut in half laterally to separate the abdomen into two equal parts (Table 2). One part was used for molecular identification and the other for morphological observation.

2.2.1. Molecular identification

DNA was extracted from cadavers in 2 mL safe-lock Eppendorf tubes. Tissues were disrupted by first shaking at 30 Hz for 1 min on a mixer mill with one 3 mm tungsten carbide beads (Qiagen, Cat No. 69997) and 180 µL ATL buffer, followed by the addition of 20 µL proteinase K and incubation at 56 °C overnight. DNA was then extracted from the homogenized samples using Blood and Tissues kits from Qiagen (ID: 69504) following the protocol according to the manufacturer.

2.2.1.1. Aphid identification. Aphids were identified by amplifying and sequencing the cytochrome oxidase I (COI) region using the primers HCO2198 (TAAACTTCAGGGTGACCAAAAAATCA) and LCO1490 (GGTCAACAAATCATAAA GATATTGG) (Folmer et al. 1994). PCR reactions were carried out in 25 µL volumes, each with 200 µM of each dNTP, 0.4 µM of each primer, 1X of PCR Buffer without $MgCl_2$, 1.5 mM of MgCl2, 1 U/rxn of Platinum DNA polymerase (ThermoFisher Scientific ID: 10966026) and 3 µL of extracted DNA (undiluted) from our samples. The PCR amplification was carried out with initial denaturation for 3 min at 94 °C, followed by 6 cycles with denaturation for 30 *sec* at 94 °C, annealing for 30 *sec* at 45 °C, extension for 1 min at 72 °C, followed by 35 cycles with denaturation for 30 *sec* at 94 °C, annealing for 1 min at 51 °C, extension for 1 min at 72 °C and a final extension for 10 min at 72 °C. The products were kept at 12 °C until further analysis.

2.2.1.2. Fungi identification. Fungal species were identified by amplifying and sequencing the large subunit (LSU) region of rDNA using the Entomophthoromycota-specific primers nu-LSU-0018-5« (5«-GTAGTTATTCAAATCAAGCAAG) (Jensen and Eilenberg, 2001) and nu-LSU-0805-3« (5«-CATAGTTCACCATCTTTCGG) (Kjøller and Rosendahl, 2000). The PCR reactions were carried out in 50 µL volumes each with 200 µM of each dNTP, 0.5 µM of each primer, 10 µL of Phusion HF Buffer with $MgCl_2$, 0.02 U/µL of Phusion DNA polymerase (ThermoFisher Scientific ID: F530S) and 3 µL of extracted DNA (undiluted) from our samples. The PCR amplification was carried out with initial denaturation for 30 *sec* at 98 °C, followed by 35 cycles with denaturation for 30 *sec* at 98 °C, annealing for 30 *sec* at 55 °C, extension for 30 *sec* at 72 °C and a final extension for 10 min at 72 °C.

PCR amplification was verified by gel electrophoresis (Agarose from Sigma, A9539, 1 %, 90 Voltage for 40 min duration) with intercalant (Ethidium bromide from VWR, E406-5 mL), and successfully amplified products were purified and Sanger-sequenced in the forward and reverse directions by Eurofins Genomics (Cologne, Germany).

For each cadaver, consensus sequences for the insect COI region and the fungal LSU were generated using Geneious v. 9 (Biomatters ApS, Denmark). Megablast searches of the insect COI sequences against the NCBI non-redundant nucleotide collection were used to identify the aphid cadavers. A best match of > 99% identity to an aphid reference

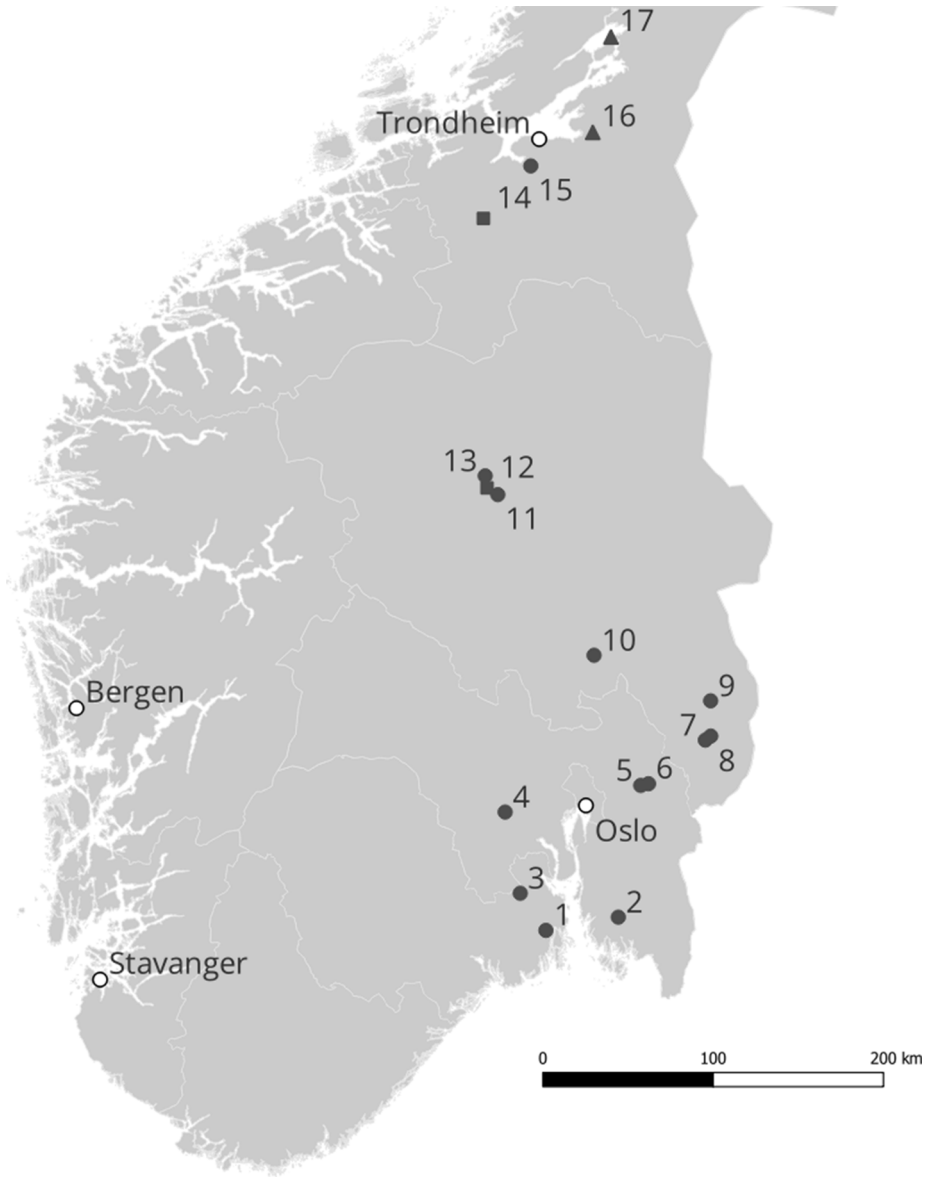

Fig. 1. Bird cherry (*Prunus padus)* branch collection sites for *Rhopalosipum padi* overwintering eggs and fungus-killed *R. padi* cadavers. Tree locations were sampled in 1 year (triangle), over 2 years (square), or over 3 years (round). The sites are numbered according to an increasing order along the latitudinal gradient. Figure drawn by Robert Banfi.

sequence was required for positive identification to species level. Regarding fungi identification, we used the taxonomy listed in MycoBank as of the 1st of June 2023. Within- and between-group sequence similarity was calculated for the fungal LSU sequences from our samples using the PID2 calculation from the BioStrings package in R (Pagès et al., 2018). Fungal LSU sequences from the cadavers were combined with entomophthoralean sequences retrieved from GenBank to generate a data matrix for phylogenetic analysis. A single representative for each unique sequence variant among the fungal LSU sequences was included in the data matrix. Sequences were aligned using MAFFT v. 7 (Katoh and Toh, 2008), and the resulting alignment was manually verified. Bayesian analyses were conducted in Mr. Bayes version 3.2.2 (Ronquist et al., 2012). Two independent runs of four Markov Chain Monte Carlo chains with 5.0×10^6 generations each were made under a GTR + I + G model, with trees sampled every 1000^{th} generation. A final standard deviation of < 0.01 for the split frequency was taken as an indication that convergence had been achieved. The first 25% of sampled trees were discarded as burn-in and posterior probabilities for each node of the 50% majority rule consensus tree were recorded.

2.2.2. *Morphological identification*

Morphological observations were conducted by mounting half of the cadaver in lactic acid cotton blue (0.075% cotton blue in 50% lactic acid), and fungal structures were observed and measured under a compound microscope (200-400X). Aphid species and morph (adult or nymph) were identified under stereo microscope (50-80X) according to Blackman and Eastop (2007). No fungus-killed cadavers were found in 2019 and, unfortunately, no fungus-killed cadaver samples from 2017 were kept for identification purposes.

2.3. *Counting Rhopalosiphum padi overwintering eggs and cadavers*

In the laboratory, overwintering *R. padi* eggs and fungus-killed cadavers per branches were counted under a stereomicroscope (50-80X). Open, empty, and flat *R. padi* eggs were recorded as dead, while full and shiny black eggs were recorded as alive.

Table 1
Average number (±SD) of *Rhopalosiphum padi* overwintering eggs, egg mortality, and overwintering fungus-killed *R. padi* cadavers per year and per tree. Ten branches were collected and examined per tree over three years (2017, 2018 and 2019). The tree locations are ordered in the increasing latitudinal gradient. Average number of eggs and cadavers: black bold: > 1; red bold: > 10. Egg mortality: black bold > 80 %; blue bold: < 50 %.

Trees		2017			2018			2019		
Nb	*Name*	*Eggs*	*Egg mortality (%)*	*Cadavers*	*Eggs*	*Egg mortality (%)*	*Cadavers*	*Eggs*	*Egg mortality (%)*	*Cadavers*
1	*Stokke*	0 ± 0	0 ± 0	**2.1** ± 2.5	0.9 ± 0.9	**91.7** ± 3.5	**22.2** ± 7.2	0.6 ± 0.8	75 ± 18.7	0 ± 0
2	*Øsaker*	1.0 ± 1.2	**100** ± 0	0 ± 0	**8.9** ± 9.1	**97.5** ± 0.6	0.8 ± 1.9	**1.9** ± 2.1	53.3 ± 1.9	0 ± 0
3	*Lardal*	**1.8** ± 1.1	**95.8** ± 1.2	**13.2** ± 16.4	**9.7** ± 4.6	49.5 ± 10.9	0.6 ± 1.3	**2.9** ± 3.4	*69.4 ± 12.3*	0 ± 0
4	*Buskerud*	0 ± 0	0 ± 0	0 ± 0	0.1 ± 0.3	**100** ± 0	**3** ± 3.5	**2.2** ± 2.9	**83.3** ± 11.1	0 ± 0
5	*Blaker*	**4.9** ± 5.4	62.5 ± 23.4	**1.3** ± 3.8	**12.6** ± 12.1	45.1 ± 5.8	**5.2** ± 5.4	0.8 ± 1.7	**100** ± 0	0 ± 0
6	*Auli*	**4.0** ± 3.5	**100** ± 0	0 ± 0	**1.1** ± 2.1	54.3 ± 20.7	**1.1** ± 1.6	0.4 ± 1.0	**100** ± 0	0 ± 0
7	*Leirud*	0.7 ± 0.9	**100** ± 0	0 ± 0	**30.5** ± 17.9	54.8 ± 5.8	0.7 ± 1.1	**18.1** ± 8.9	**45.2** ± 3	0 ± 0
8	*Brandval*	0.9 ± 1.0	**91.7** ± 3.5	**18.9** ± 13.3	**9.8** ± 5.7	71.2 ± 10	**15.3** ± 16.3	**2.4** ± 2.4	*56.2 ± 15.7*	0 ± 0
9	*Kirkenær*	**3.6** ± 3.2	68.6 ± 11.2	0.2 ± 0.4	**11** ± 5.9	**89** ± 1.6	0.8 ± 1.9	**8.0** ± 5.1	72.5 ± 3.2	0 ± 0
10	*Apelsvoll*	**5.1** ± 4.0	74.5 ± 6	0 ± 0	**31.6** ± 28.8	57 ± 4.8	0.6 ± 0.8	0.2 ± 0.6	50 ± 0	0 ± 0
11	*Suleng*	**9.1** ± 9.0	51.2 ± 5.5	0 ± 0	**34.3** ± 11.7	51.2 ± 2.1	0.5 ± 1.0	1.0 ± 1.0	**13.9** ± 4	0 ± 0
12	*Vinstra*	**5.4** ± 5.8	**87.4** ±2.6	0 ± 0	**101.3** ± 26.6	50.9 ± 0.8	1.0 ± 1.3	-	-	-
13	*Storøya*	**2.0** ± 1.9	77.1 ± 11.8	0 ± 0	**29.1** ± 21.1	73.4 ± 1.9	0.3 ± 0.5	**1.1** ± 1.4	55 ± 16	0 ± 0
14	*Meldal*	-	-	-	**8.6** ± 9.3	52.1 ± 12.1	0.2 ± 0.6	0.7 ± 1.6	65 ± 0.2	0 ± 0
15	*Meeggen*	0.6 ± 0.7	**80** ± 16	0 ± 0	0.5 ± 0.8	66.7 ± 5.6	0 ± 0	0.1 ± 0.3	**100** ± 0	0 ± 0
16	*Stjørdal*	-	-	-	-	-	-	0 ± 0	0 ± 0	0 ± 0
17	*Grønnesby*	-	-	-	-	-	-	0.5 ± 0.8	**100** ± 0	0 ± 0

– = no collection of branches.

Table 2
Number of *Rhopalosiphum padi* fungus-killed cadavers found on bird cherry branches at the late February / early March 2018 with the number of identified molecularly individuals per species. The number of *R. padi* cadavers per biological stage are separated between nymph, adult, and unknown stage. The tree locations are ordered in the increasing latitudinal gradient.

Cherry tree location in 2018			*Number of* R. padi *fungus-killed cadavers*				*Biological stage of* R. padi *cadavers*		
*#**	*Name*	*GPS coordinates*	*Total collected*	*Identified molecularly*	*Entomophthora planchoniana*	*Zoophthora* cf. *aphidis*	*Nymph*	*Adult*	*Unknown*
1	Stokke	N59.25931, E10.29269	222	17	0	17	7	0	10
2	Øsaker	N59.319436, E11.042608	8	0	0	0	0	0	0
3	Lardal	N59.45765, E10.03441	6	3	0	3	3	0	0
4	Buskerud	N59.887397, E9.88924	30	11	0	11	8	0	3
5	Blaker	N60.010612, E11.321309	52	10	0	10	9	0	1
6	Auli	N60.018079, E11.403685	10	5	0	5	4	0	1
7	Leirud	N60.235676, E12.023339	7	4	1	3	4	0	0
8	Brandval	N60.255749, E12.08266	153	15	0	15	14	0	1
9	Kirkenær	N60.441597, E12.099202	8	4	2	2	2	0	2
10	Apelsvoll	N60.704855, E10.869095	6	3	3	0	1	1	1
11	Suleng	N61.56300, E9.855402	5	3	3	0	1	0	2
12	Vinstra	N61.59912, E9.73751	9	4	1	3	4	0	0
13	Storøya	N61.66299, E9.71835	3	2	1	1	1	1	0
14	Meldal	N63.02119, E9.73070	2	2	0	2	1	0	1
15	Meeggen	N63.294316, E10.29285	0	0	0	0	0	0	0
16	Stjørdal	N63.463413, E11.031647	-	-	-	-	-	-	-
17	Grønnesby	N63.962843, E11.285051	-	-	-	-	-	-	-
	TOTAL		*521*	*83*	*11*	*72*	*59*	*2*	*22*

#*= Location number as on map in Fig. 1.
– = no collection of branches.

2.4. Statistical analysis

We excluded tree locations and years without any aphid or fungus-killed cadavers and tested for overdispersion of the number of eggs (dead and alive) on branches from the 16 tree locations with the function "dispersiontest" from R package "AER" (Kleiber and Zeileis, 2008). Since our data were overdispersed, we used zeroinflated count model with Poisson distribution through the "zeroinfl" function from the "pscl" package (Zeileis et al, 2008) to test the correlation between number of eggs and number of overwintering fungus-killed *R. padi* cadavers, the influence of year and the latitudinal coordinates of the tree locations. We compared the different years to each other with estimated marginal means (post hoc analysis, R package "emmeans", Lenth 2017).

3. Results

3.1. Description and identification of fungus-killed overwintering aphid

3.1.1. Aphid identification and description

Insect COI sequences were successfully generated for 14 of the 83 aphid cadavers investigated, all of which were positively identified as *R. padi* in BLAST searches. Among the 83 cadavers studied, 71.1% were nymphs, 2.4% were adults and 26.5% were not possible to identify to aphid stage due to the lack of crucial anatomical parts such as corniculae

(Table 2). No anatomical evidence of wings was observed on any of the fungus-killed cadavers.

3.1.2. Fungi identification and description

The fungal LSU sequences represented two distinct groups with > 99% sequence similarity that corresponded to two fungus-killed cadaver morphotypes (see below) (Fig. 2). Between-group sequence similarity was 77%. In the phylogenetic analysis, all fungal LSU sequences from the cadavers nested within the Entomophthorales, which formed a distinct, highly supported monophyletic clade (96% Bayesian posterior probability [BPP]). The distribution among trees of all molecularly identified cadavers are presented in Table 2.

The fungal LSU sequences from 72 cadavers formed a distinct clade (100% BPP) grouped within other *Zoophthora* species and was sister to an undescribed species of *Zoophthora* infecting *Eurois occulta* (Lepidoptera: Noctuidae, where only resting spores have been found, Avery and Post, 2013) (Fig. 2). All of these fungal-killed *R. padi* cadavers belonged to the same morphotype when observed under stereo- and compound microscopes. The cadavers were black, dry, and hard to break without immersing the body in a liquid (Fig. 3A, B). Legs, antennae and corniculae, when still attached to the body, were usually black but some individuals had brown legs. Many holdfasts extended from the abdomen and thorax of the aphid and attached the cadaver body to the branch. The aphid body (thorax and abdomen) was filled with resting spores. Under the microscope, these resting spores appeared smooth hyaline and spherical with an average diameter of 39.2 μm ± 2.59 (mean ± SE) (range: 30 – 45 μm) covered by a rough dark brown episporum (Fig. 4A, B, D).

The fungal LSU sequences corresponding to the other 11 cadavers formed a strongly supported (100% BPP) monophyletic clade with representative sequences of *E. planchoniana* that were distinct from other *Entomophthora* species, and the fungus is identified as *E. planchoniana* (Fig. 2). These overwintering *R. padi* cadavers formed a second morphotype and contained hyphal bodies only (no resting spores). These cadavers were brown, dry, and varied from very hard to relatively easy to break. Legs, antennae and corniculae, when still attached to the body, were brown or yellowish (Fig. 3C). No holdfasts were present (Fig. 3D), and the cadaver was attached to the branch by being intertwined with the branch trichomes. The aphid body (thorax and abdomen) was filled with "modified" hyphal bodies, which differed from the normal hyphal bodies of the species by being of different shape and having slightly thicker cell walls. Ten cadavers were filled with modified hyphal bodies of heterogenous shape and length (Fig. 4C), while one cadaver was filled with homogenous rod-shaped hyphal bodies (Fig. 4E). The homogeneous rod-shaped hyphal bodies had a mean length of 41.69 μm ± 5.07 (mean ± SE) (range: 37.5–50 μm) and a

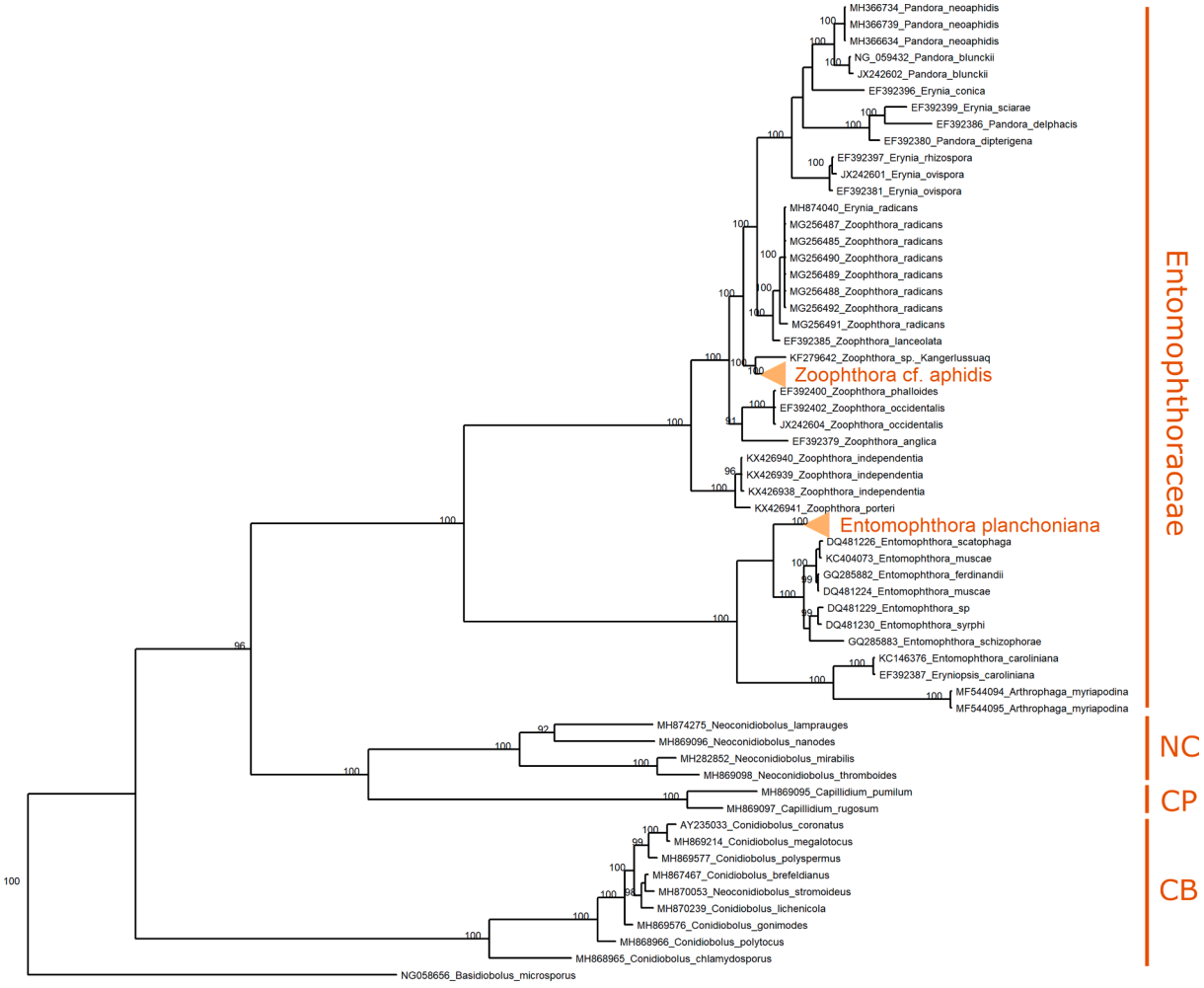

Fig. 2. Phylogenetic tree proposed by Bayesian inference for the fungal LSU sequences of two cadaver morphologies (in orange). The fungal LSU sequences from fungus-killed *Rhopalosiphum padi* cadavers with resting spores formed a distinct clade sister to an unidentified species of *Zoophthora* from a resting spore infected *Eurois occulta* (Lepodoptera: Noctuidae). Fungus-killed *R. padi* cadavers with hyphal bodies formed a monophyletic clade with sequences of *Entomophthora planchoniana*. The numbers close to the branches indicate the posterior probability values. NC: Neoconidiobolaceae, CP: Capillidiaceae, CB: Conidiobolaceae. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

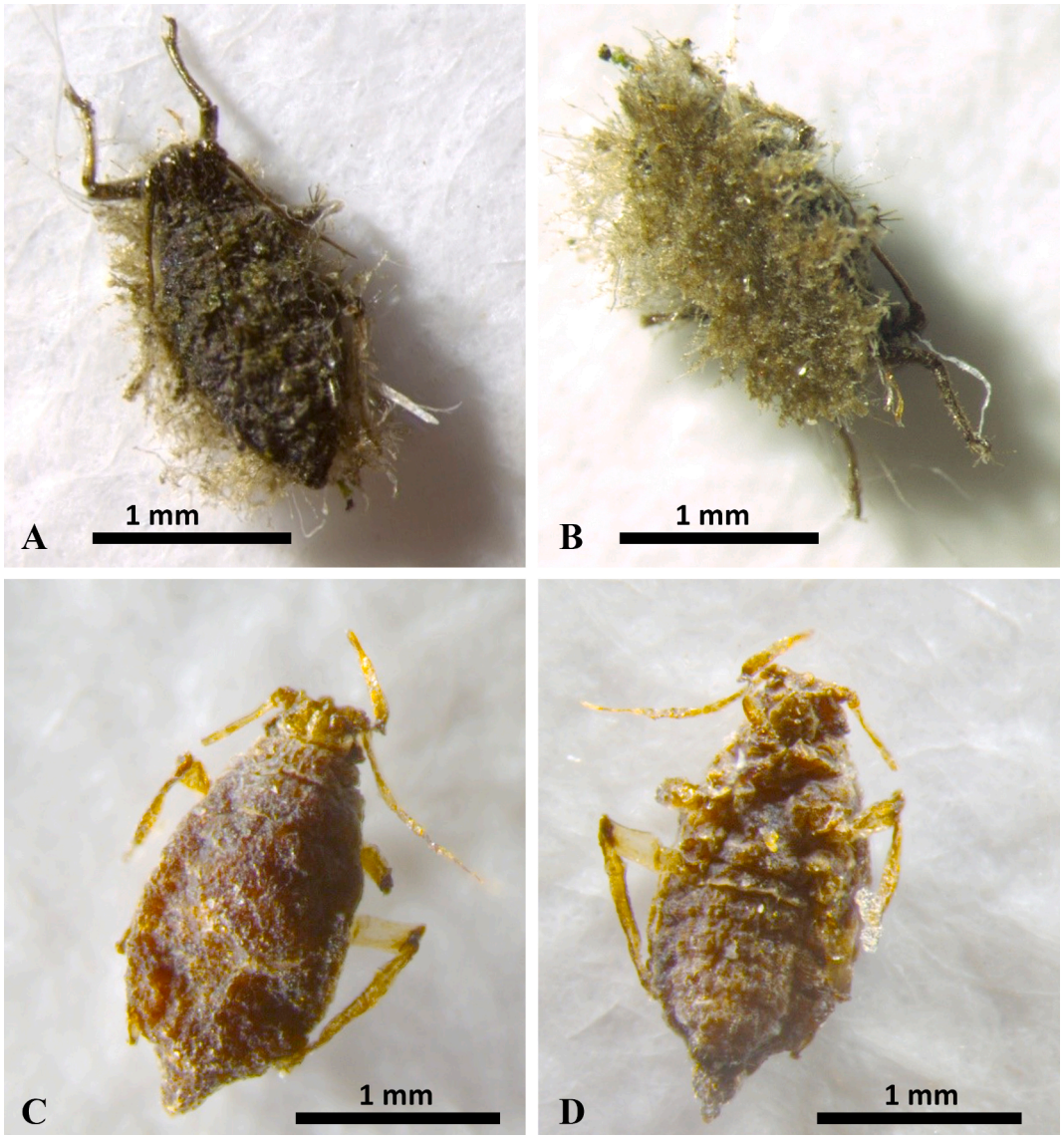

Fig. 3. A) and B) Overwintering fungus-killed *Rhopalosiphum padi* cadaver filled with resting spores of *Zoophthora* cf. *aphidis* A) Dorsal face of the cadaver, B) Ventral face showing many holdfasts intertwine with trichomes from the tree, which attached the cadaver to the *Prunus padus* branch. C) and D) Overwintering fungus-killed *R. padi* cadaver filled with modified hyphal bodies of *Entomophthora planchoniana* C) dorsal face, and D) ventral face of the cadaver showing no holdfasts. Photo: Stéphanie Saussure.

mean diameter of 17.99 μm ± 2.98 (mean ± SE) (range: 12.5 – 22.5 μm).

3.2. Overwintering eggs and fungus-killed cadavers per branch

We observed a total of 879 cadavers and 3599 overwintering *R. padi* eggs. Fungus-killed *R. padi* cadavers were found close to bud axils, where overwintering *R. padi* eggs are also usually observed (Fig. 5A-C). When the density of cadavers was high, some were also found on the branch between buds (Fig. 5D). Two trees sampled had no eggs or cadavers during one year. On trees with eggs and/or cadavers, the percentage of branches per tree with only *R. padi* eggs varied from 46.7 to 58.5% per tree; between 0 and 36.7% had a mix of overwintering eggs and cadavers, and 0 to 10% had only fungal cadavers.

The number of overwintering eggs per branch increased from south to north, peaking at around 61.6°N (Fig. 6A). However, the number of eggs per branch peaked at different latitudes depending on the year. A similar pattern was observed when dead and live eggs were considered separately but this is not illustrated. As for the number of cadavers per branch, they were found in relatively large numbers below 60.7°N and only in low numbers at higher latitudes (Fig. 6B).

The overall number of overwintering eggs per branch was negatively correlated to the number of overwintering fungus-killed cadavers ($F = 8.191$, $df = 1$, $p = 0.004$) (Fig. 7A). The number of overwintering eggs was significantly different between years ($F = 74.042$, $df = 2$, $p < 0.001$). More precisely, 2018 was significantly different from 2017 and 2019 ($p < 0.001$ for both comparisons), with a higher number of eggs found in 2018. However, egg numbers in 2017 and 2019 were not significantly different from each other ($p = 0.663$) (Fig. 7A). In total, 59.5% of the total overwintering aphid eggs were dead, and a big variability in egg mortality per branch was observed. No general trend in egg mortality rate along the latitudinal gradient was found (Fig. 7B).

3.3. Variability between bird cherry tree locations

We observed a high variability between bird cherry tree locations and years in the average number of *R. padi* eggs per branch, and the average number of overwintering fungus-killed *R. padi* cadavers per branch (Table 1). No fungus-killed *R. padi* cadavers were found on any

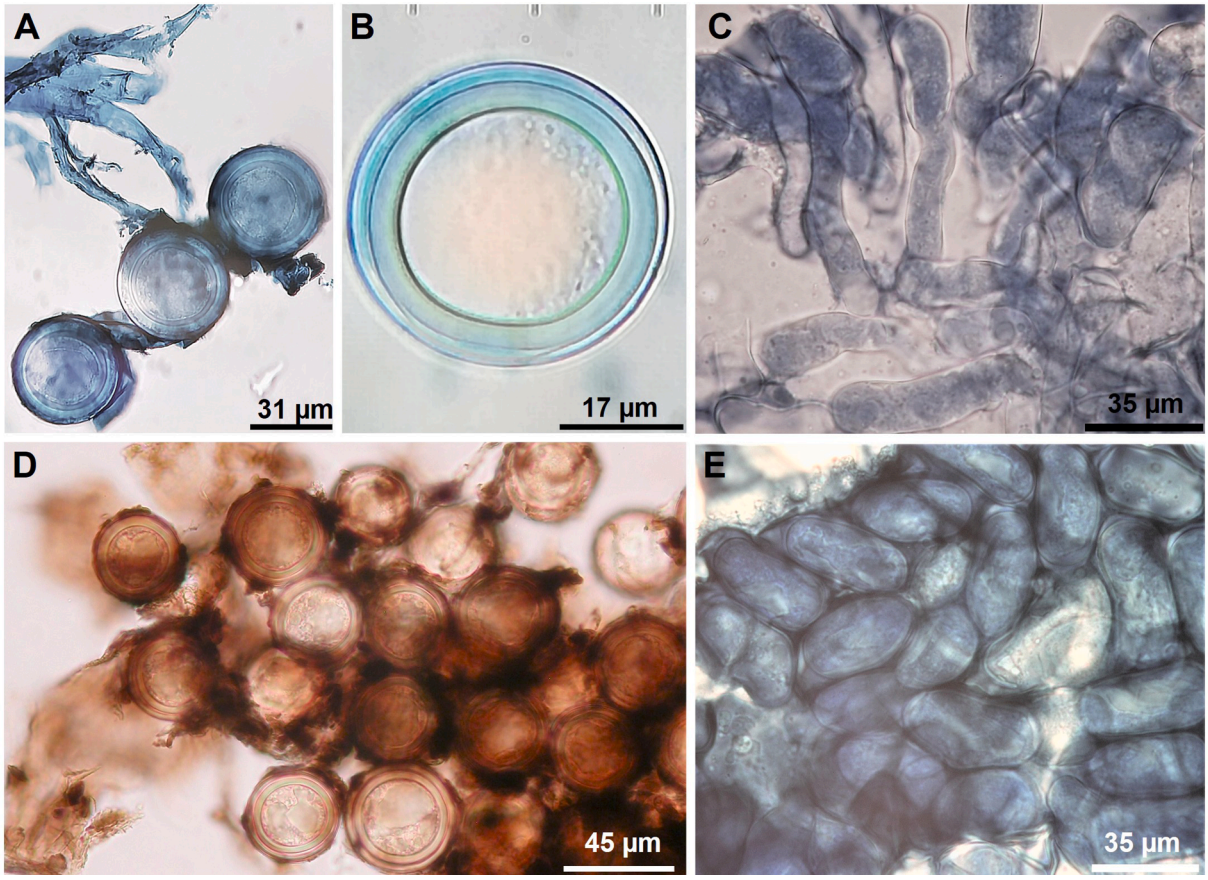

Fig. 4. A, B and D) Pictures of resting spores of *Zoophthora* cf. *aphidis* A) Resting spores with epispore and intertwined with rhizoids, B) Smooth and hyaline resting spore without epispore showing two thick walls and D) uncoloured mass of resting spores showing a dark brown and rough epispore covering smooth and hyaline spores. C and E) Pictures of overwintering modified hyphal bodies of *Entomophthora planchoniana*. C) Hyphal bodies of different shapes; D) Rod shaped hyphal bodies. Photo: Karin Westrum and Stéphanie Saussure.

tree in 2019. Almost all tree locations in 2018 had a mix of *R. padi* eggs and fungus-killed *R. padi* cadavers. The number of cadavers observed ranged from 0 to 222 per 10 branches on one tree. In 2017, only a few tree locations had overwintering fungus-killed cadavers. The variability in cadaver and egg numbers between years among the trees can be summarized as follows: 1) nine trees consistently had predominantly overwintering *R. padi* eggs (Fig. 8A), 2) one tree consistently had predominantly overwintering fungus-killed *R. padi* cadavers (Fig. 8B), 3) five trees had predominantly overwintering fungus-killed cadavers one year and predominantly overwintering eggs the following year (Fig. 8C) in 2018 only, two trees had a significant mix of overwintering cadavers and eggs simultaneously (Fig. 8D).

4. Discussion

This study demonstrates that two fungal species in the order Entomophthorales form overwintering structures in *R. padi* cadavers on bird cherry trees, presumably to survive the winter. One species exclusively formed resting spores in its host's body. This species clustered in between the other *Zoophthora* species. Seven *Zoophthora* species are known to be pathogenic to aphids (*Z. aphidis, Z. phalloides, Z. radicans, Z. canadensis, Z. occidentalis, Z. orientalis, Z. anhuiensis*) of which only *Z. aphidis, Z. radicans* and *Z. phalloides* have been reported to infect *R. padi* (Keller, 1991; Nielsen et al., 2001; Barta and Cagáň, 2006; Barta, 2009; Manfrino et al., 2014). Based on our phylogenetic analysis, the resting-spore forming species detected here is distinct from *Z. phalloides* and *Z. radicans*. The resting spores we observed are morphologically quite consistent with Keller's (1991) account of *Z. aphidis* infecting *R. padi*, which describes black cadavers filled with resting spores, which are round with a diameter of 34.8–46.6 µm (29–55 µm) and a "rough, black episporium, [which] separated easily from hyaline, smooth spore". The resting spores observed in this study were in the range observed by Keller (30–45 µm) but presented a rough dark brown epispore. However, the lack of a reference DNA sequence from a known isolate of *Z. aphidis* precludes unequivocal confirmation of the species identification, and we hereafter refer to this fungus as *Zoophthora* cf. *aphidis*. Even though several *Zoophthora* species have been found infecting *R. padi* on *P. padus*, *Zoophthora* infections are usually not recorded on *R. padi* when feeding on cereals in Europe (Nielsen et al., 2001; Barta and Cagáň, 2006), and *Zoophthora* species have until now not been considered as an important natural enemy of cereal aphids in Europe. Further, both of the latter studies were conducted in Denmark and Slovakia, respectively, and both the prevalence and importance of *Zoophthora* may vary with geographic location. This needs to be studied further, however, since in our study, 87% of overwintering fungi observed belonged to *Zoophthora* cf. *aphidis*.

The other fungal species identified from *R. padi* was *E. planchoniana* overwintering as hyphal bodies within cadavers. *Entomophthora planchoniana* is a common fungus infecting aphids in cereals and may cause epizootics (Barta and Cagáň, 2006; Ben Fekih et al., 2015; Hatting et al., 2000). It has also been reported on *Sitobion avenae* in Norway (Sletteng 2014) but not on *R. padi*. Keller (1991) found *E. planchoniana* infecting *R. padi* and reported that it produced both primary conidia and resting spores. Keller (1987) also reported that *E. planchoniana* overwinters as modified hyphal bodies inside the oviparae of the sapling sycamore aphid *Drepanosiphum acerinum*. However, our *R. padi* cadavers were

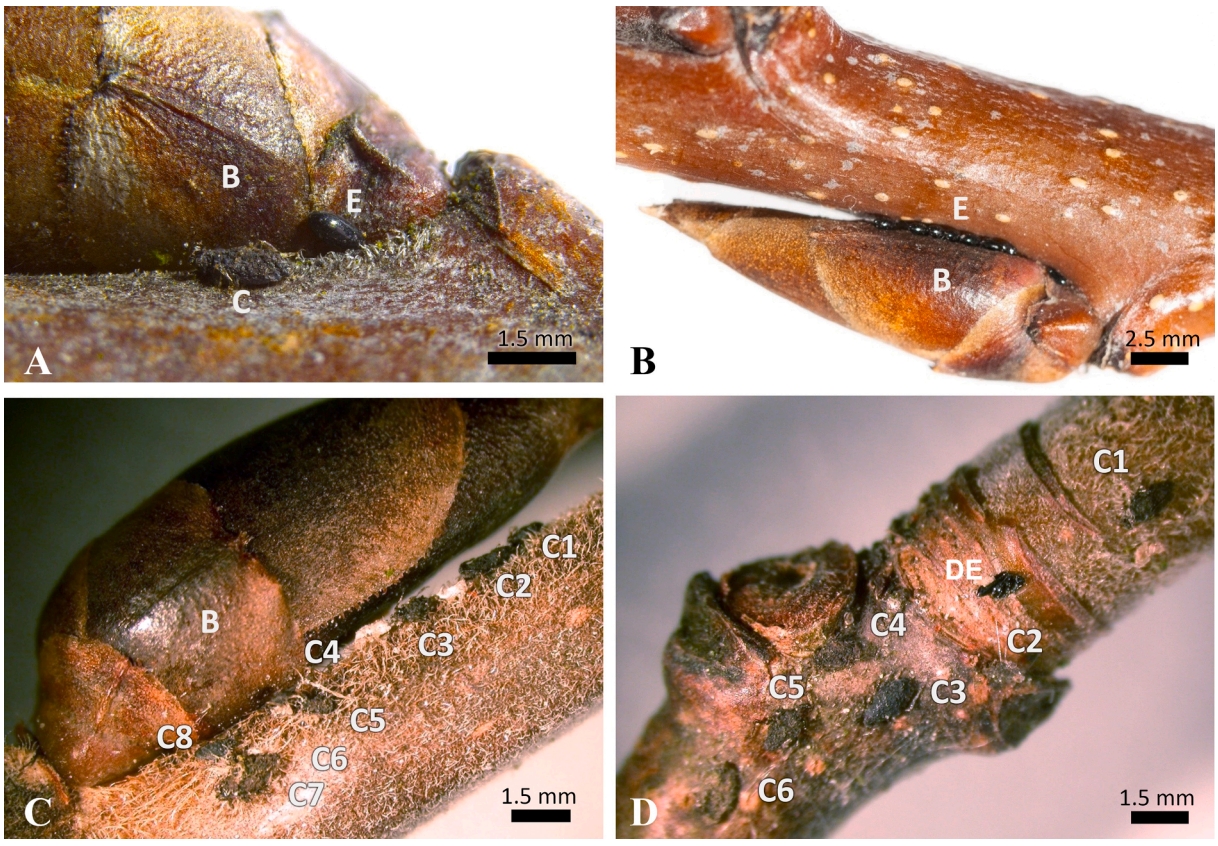

Fig. 5. Typical micro-location for overwintering fungus-killed *Rhopalosiphum padi* cadavers and overwintering aphid egg close to bird cherry (*Prunus padus*). A) one fungus-killed *R. padi* cadaver next to one overwintering aphid egg at a *P. padus* bud axil on last annual shoot. B) Twelve overwintering eggs close to the bud axils. C) Eight fungus-killed *R. padi* cadavers close to a *P. padus* bud axil. D) When most of the bud axils are already overcrowded, cadavers were found on the branches between buds. On this picture we can see a dead egg between buds. Legend: C: cadavers, E: alive eggs, DE: dead egg, B: bud. Photo: Erling Fløistad and Stéphanie Saussure.

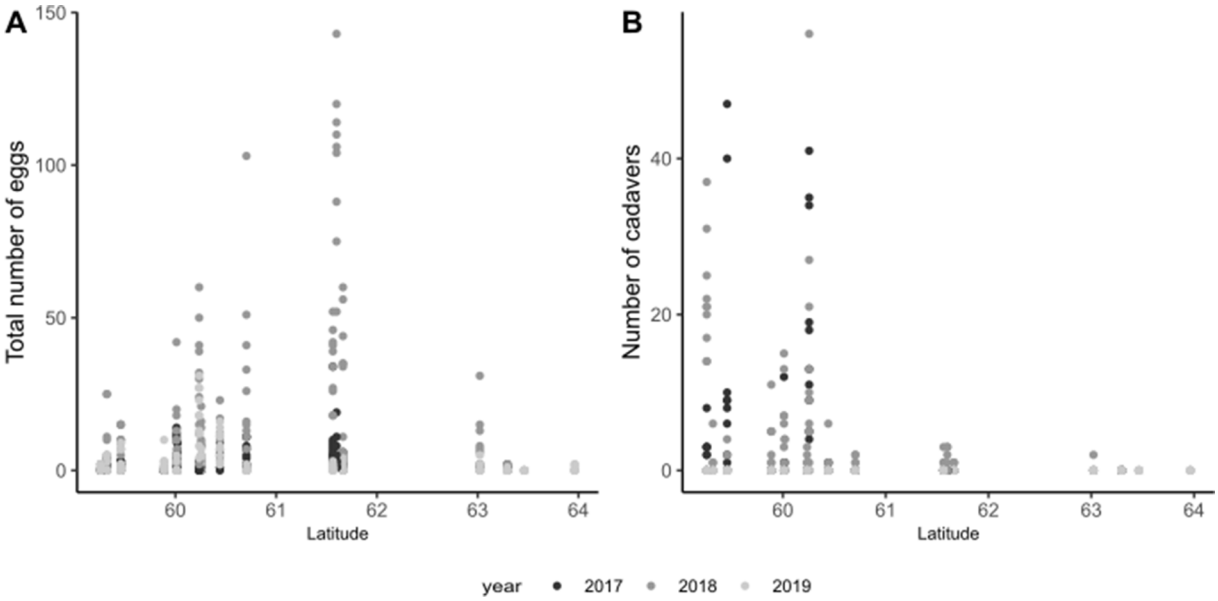

Fig. 6. Distribution of A) the total number of overwintering eggs (dead and alive), and B) the total number of cadavers along the latitudinal gradient of the tree locations over the three years of the survey. The decimal numbers of the North GPS coordinates were used.

filled with hyphal bodies only, and this is the first report of *E. planchoniana* overwintering in *R. padi* cadavers as hyphal bodies. The *E. planchoniana* modified hyphal bodies in *D. acerinum* described by Keller (1987) have various shapes (from rod to straight shaped or even slightly bent) and were embedded in undefined material. Their cell walls were also slightly thicker than those of the usual hyphal bodies. Keller

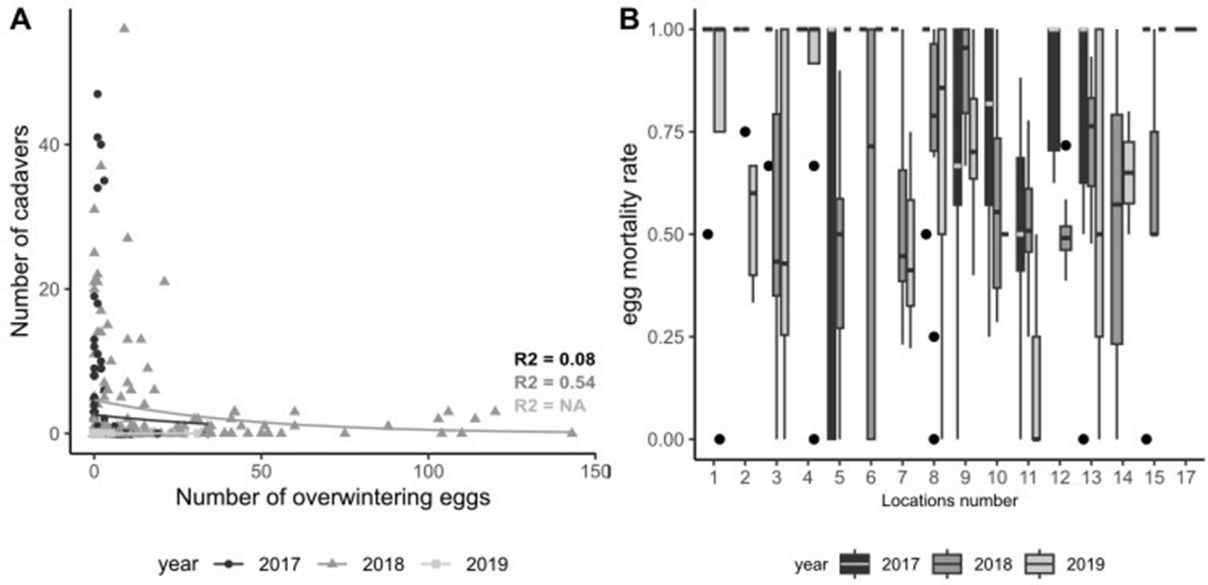

Fig. 7. A) Correlation between numbers of *Rhopalosiphum padi* eggs and overwintering fungus-killed *R. padi* cadavers per bird cherry (*Prunus padus*) branch in 2017, 2018 and 2019. Ten branches were collected and examined from 17 bird cherry tree locations. Both live and dead eggs are included. The Maximum likelihood squared ratio is given for each year. B) distribution of the egg mortality over the latitudinal gradient (expressed as the ordered number of the tree locations for aesthetic purpose) per year.

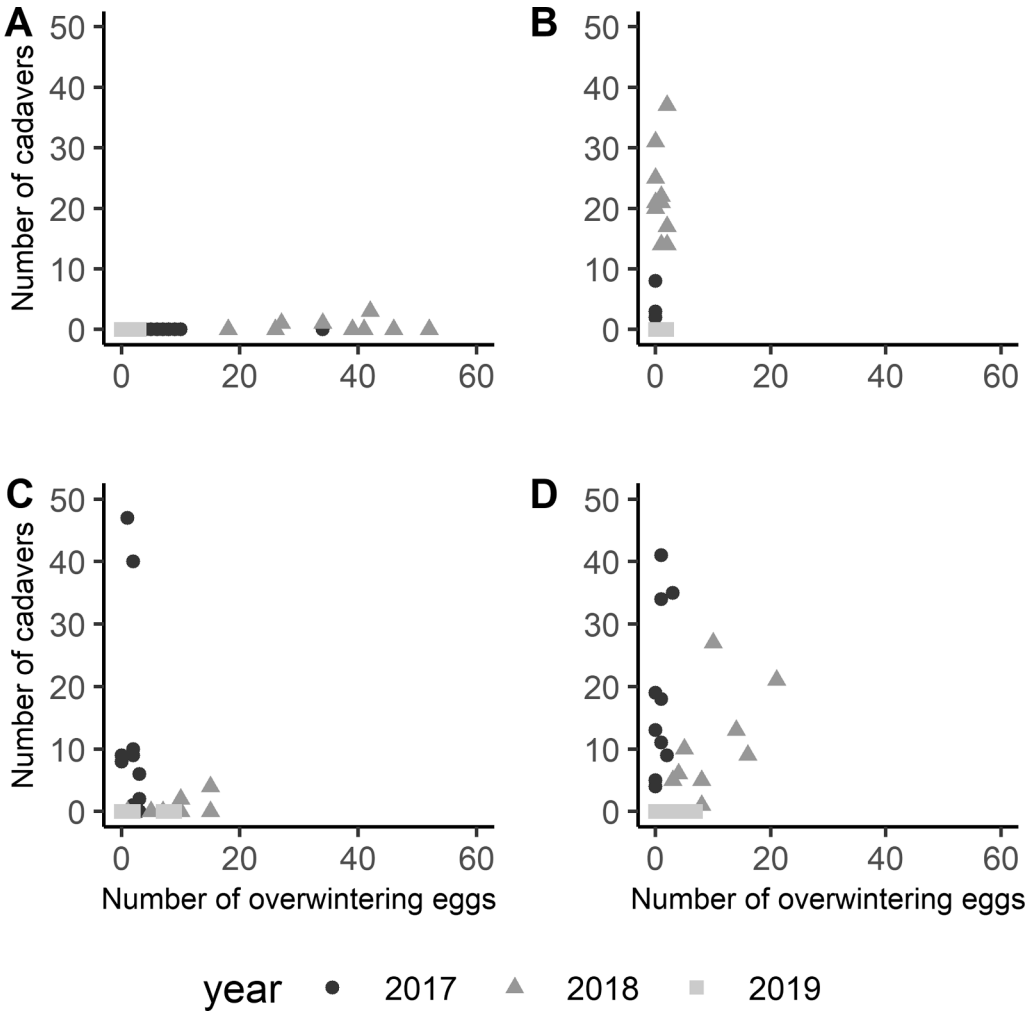

Fig. 8. Numbers of *Rhopalosiphum padi* overwintering eggs and fungus-killed cadavers per bird cherry branch over three years. A) Tree in Suleng with almost only overwintering eggs during 3 years. Aphids may escape fungi. B) Tree in Stokke with almost only overwintering cadavers during the 3 years. Fungi might not infect new aphid populations in spring the following year on the tree. C) Tree in Lardal with mostly overwintering cadavers one year (2017) and mostly overwintering eggs the following year (2018 and 2019). Fungi may infect new spring population of aphids after one year of delay. D) Tree in Brandval showing the same dynamics between aphid and fungi populations, plus a mix of both populations during the same year in 2018. During this year, fungi could potentially infect new spring aphid population after their first winter.

only measured the rod-shaped hyphal bodies, which have a mean length of 47.3 – 48.5 μm (29 – 68 μm) and a mean diameter of 15.9 – 16.5 μm (12 – 21 μm). Our observations correspond to the description given for *E. planchoniana* by Keller (1987). In addition, the size of the rod-shaped hyphal bodies we observed falls within the range indicated by him.

Barta and Cagáň (2006) listed 33 entomophthoralean species in nine genera infecting aphids. Further, Barta and Cagáň (2004) and Nielsen and Steenberg (2004) reported in total six entomophthoralean species infecting *R. padi* in autumn on bird cherry trees. In these studies, no overwintering structures were observed, however, it may indicate that more species than observed in our study could potentially overwinter on the bird cherry.

Our study showed an overall negative correlation between numbers of overwintering eggs and cadavers per branch, which can explain the low infection pressure on spring *R. padi* generations seen by Nielsen and Steenberg (2004) and Barta and Cagáň (2004). Further, the newly hatched fundatrices feed on unfurling leaves, and live in galls that they induce (Leather and Dixon, 1981). Hence, they are effectively protected from airborne conidia or sporulating, overwintering cadavers outside galls. Aphids could potentially have a higher probability of infection by entomophthoralean fungi just prior to migrating to grasses. Moreover, our study showed that aphids were typically killed during their nymphal stage (71.1% of our samples). Males and gynoparae are alate individuals colonizing bird cherry trees at adulthood; the only nymphal stages produced on the bird cherry are oviparae (*e.g.*, Leather, 1992). This would suggest that the observed nymph fungus-killed cadavers were all oviparae. Therefore, we hypothesise that the observed negative correlation may be due to aphid mortality from fungal infections either at nymphal stage before oviparae females and males could mate, or at adulthood impeding mating or shortening life expectancy of oviparae, consequently reducing overall reproduction and egg laying. This may imply a reduction of the pest inoculum available during the next spring. Cadavers of both fungal species were attached to branches on bud axils, where oviparae lay their eggs. Keller (1987) also found infected *D. acerinum* filled with overwintering hyphal bodies of *E. planchoniana* at the same micro-location as *D. acerinum* overwintering eggs. Further, Byford and Ward (1968) observed that aphids infected by *E. planchoniana* on plum trees, *Prunus domestica* (Rosales: Rosaceae), die in different locations on the tree depending on whether fungus-killed cadavers produced resting spores (located on bark crevices) or conidia (located on leaves). Entomopathogenic fungi are known to modify host behaviour in many ways (*e.g.*, Roy et al., 2006; Trandem et al., 2015). We, therefore, speculate that these fungi could modify *R. padi* behaviour to increase the likelihood of their host dying on egg-laying sites, which might in turn increase the likelihood of the fungus surviving and infecting the new aphid populations in spring. By extension, there may be a competition between healthy oviparae females and entomophthorales-infected nymphs for the best micro-locations on a branch, in addition to the documented intra-specific competition among oviparae females for the best egg-laying sites close to buds (Leather, 1992).

In our study, the number of fungus-killed cadavers was important only until 60.7°N, while the total number of overwintering *R. padi* eggs increased with the latitude until peaking at 61.6°N, when the fungi prevalence was already significantly reduced. In Norway, cereals are grown mainly at southern latitudes (*e.g.*, Akershus or Hedmark), however, a considerable amount of spring cereals is produced in the Trøndelag region (from 62.2°N to 65.1°N) (Statistisk Sentralbyrå, 2023), where only a few cadavers were observed. Consequently, we can hypothesize that biological control realised by the fungi on the bird cherry in autumn was not important in Trøndelag; however, aphids also appear to overwinter in lower densities farther north. Eggs and cadavers numbers peaked at different latitudes depending on the year in our sampling. This could be explained by several factors such as climatic conditions, population cycles, and cereal production variability and needs to be studied further.

The overall egg mortality recorded in our study (59.5 %) was in the range of the average mortality rate previously observed [48.1 – 87.5%] (Leather 1992). Leather (1980, 1992, 1993) listed several factors potentially influencing the egg mortality: 1) when oviparae lay eggs in sub-optimal locations (not at buds' axil), these eggs are more vulnerable to rough conditions and might be dislodged by rain and wind, 2) the length of the winter period is then considered as the major factor causing egg mortality, 3) in spring, natural enemies such as predatory arthropods and birds can consume eggs. In our study, we did not monitor the initial eggs number at the end of autumn to compare it to the eggs number at the end of winter. Therefore, the mortality we observed is only partial as not considering the missing and dislodged eggs.

The proportion of branches with only fungus-killed cadavers was very low. However, the situations with a mixed population of eggs and fungus-killed cadavers were highly variable between years, branches, and tree locations. The high variability in numbers of overwintering eggs and cadavers between years may be explained by several factors, namely: 1) climatic conditions during the previous summer/autumn (*e.g.*, Steinkraus, 2006; Finlay and Luck, 2011); 2) different susceptibility among the host aphid lineages to fungal infection; and 3) fungal isolates with different virulence. A discussion of the two last factors mentioned is presented in Eilenberg et al. (2019). The high variability in eggs and fungus-killed cadavers observed within and between tree locations may be explained by the behaviour of *R. padi*. Indeed, gynoparae select trees on which they land (Archetti and Leather, 2005; Leather, 1986). Later, oviparae express significant exploratory movements within the tree (Leather, 1986) and select egg-laying sites (Leather, 1992).

The high variation in eggs and fungus-killed cadavers between trees, years and probably also branches may lead to different annual epidemiological patterns based on the following: 1) When only *R. padi* eggs are present, the bird cherry tree may be considered only as an overwintering site for *R. padi,* 2) When only fungi are present, the bird cherry tree may be considered as an overwintering site for fungi only, 3) When both *R. padi* eggs and entomopathogenic fungi are present, fungi may infect *R. padi* the following spring. Over several years, if *R. padi* eggs and fungal pathogens overwinter on the same location, but during different winters, the dormant fungi will be able to infect the aphid host in the following spring.

Resting spores are not infective structures, but when exposed to favourable conditions, they germinate and produce infective germ conidia (*e.g.*, Hajek et al., 2018). Overwintering hyphal bodies are not infective either but produce conidiophores that may produce infective conidia when exposed to favourable conditions (*e.g.*, Keller, 1987). In spring, *R. padi* fungal infection levels are usually low (Nielsen and Steenberg, 2004; Barta and Cagáň, 2004). This low spring fungal activity on *P. padus* led Barta and Cagáň (2004) to conclude that fungal infected *R. padi* was not an important inoculum of entomophthoralean fungi for summer populations of aphids in cereals. However, in our study, some trees harboured many fungal overwintering cadavers: up to 222 cadavers on 10 branches from one tree (the one located in Stokke in 2018). Because we only sampled low percentages of the total tree crown and only the last year's shoots, we may hypothesise that fungus-killed cadavers in the whole habitat can represent a significantly larger population. Further, *E. planchoniana* did not produce holdfasts, and some cadavers may have fallen to the ground more easily during winter. Therefore, we may underestimate its prevalence, and the soil below the tree canopy may contain overwintering fungal structures as well. We, therefore, suggest that under proper conditions 1) entomophthoralean fungi can significantly decrease *R. padi* overwintering population and consequently the following spring aphid infestation and, 2) fungus-killed *R. padi* cadavers can represent an important entomophthoralean reservoir and consequently an important inoculum the following spring.

More practically, our study argues for including counts of fungal-killed cadavers in the Nordic monitoring programs, in addition to the existing monitoring of *R. padi* overwintering eggs on the bird cherry tree. The number of cadavers per branch can provide an indication of

biological control in autumn on the winter host of the pest. It also gives us an indication of potential biological control in the following spring and summer. However, as mentioned earlier, the genus *Zoophthora* is almost never observed on cereal aphids feeding on cereals, even though it makes up 87% of our samples in 2018. Therefore, the important autumnal natural enemy, *Zoophthora* cf. *aphidis,* most likely completes its biological cycle on a different host in a different habitat. Further research is needed to learn more about this species to potentially incorporate it into IPM strategies. *Entomophthora plachoniana*, on the other hand, is commonly found in cereals, therefore, it may be more straightforward to incorporate into IPM strategies. However, further research is needed to fully assess potential fungal natural enemy threshold above which we can assume cereal aphids will not represent a major pest in spring. Future experiments should try and estimate the probability of these overwintering cadavers to initiate diseases in aphid spring population and its influence on the consequent aphid pest pressure in cereals.

5. Conclusion

Entomophthorales overwinter in *R. padi* on its winter host, the bird cherry tree. Overwintering modified hyphal bodies of *E. planchoniana* and resting spores of *Zoophthora* cf. *aphidis* were recorded. Fungus-killed *R. padi* cadavers were attached to bud axils at the same micro-location as overwintering eggs. We found a negative correlation between aphid overwintering eggs and fungus-killed cadavers and high variation between years and bird cherry tree locations. Some tree locations hosted only eggs or cadavers, while others hosted a mix of both. Therefore, fungal infection of spring *R. padi* populations is probably highly variable. If trees harbor only overwintering fungus-killed cadavers one year and only aphid eggs the following year and the fungus remains infective, the persisting cadavers may remain as an inoculum reservoir even after a one-year delay. We, therefore, suggest that entomophthoralean fungi can significantly reduce overwintering aphid population, and that *P. padus* may act as an inoculum reservoir for these two fungal species.

Declaration of Competing Interest

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

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References

Archetti, M., Leather, R., S.,, 2005. A test of the coevolution theory of autumn colours: colour preference of *Rhopalosiphum padi* on *Prunus padus*. Oikos 110, 339–343. https://doi.org/10.1111/j.0030-1299.2005.13656.x.

Avery, M., Post, E., 2013. Record of a *Zoophthora sp.* (Entomophthoromycota: Entomophthorales) pathogen of the irruptive noctuid moth *Eurois occulta* (Lepidoptera) in West Greenland. J. Invertebr. Pathol. 114 (3), 292–294. https://doi.org/10.1016/j.jip.2013.09.005.

Bale, J.S., Hayward, S.A.L., 2010. Insect overwintering in a changing climate. J. Exp. Biol. 213 (6), 980–994. https://doi.org/10.1242/jeb.037911.

Barta, M., 2009. Entomophthoralean fungi associated with aphids in woody plants in the Arboretum Mlyňany SAS. Folia oecologia 36, 1–7. ISSN 1336–5266.

Barta, M., Cagáň, L., 2004. A potential role of *Rhopalosiphum padi* (Linnaeus) colonies on winter host, *Padus avium* (Linneus), as an inoculum source of fungal diseases of cereal aphids in agricultural landscape. Acta Fytotechnica et Zootechnica 7, 5.

Barta, M., Cagáň, L., 2006. Aphid-pathogenic entomophthorales (their taxonomy, biology, and ecology). Biologia 61. https://doi.org/10.2478/s11756-007-0100-x.

Barta, M., Cagáň, L., 2007. Natural control of *Diuraphis noxia* and *Rhopalosiphum maidis* (Aphidoidea) by parasitic entomophthorales (Zygomycota) in Slovakia. Cereal Res. Commun. 35, 89–97. https://doi.org/10.1556/CRC.35.2007.1.11.

Ben Fekih, I., Boukhris-Bouhachem, S., Bechir Allagui, M., Jensen, A.B., Eilenberg, J., 2015. First survey on ecological host range of aphid pathogenic fungi (Phylum Entomophthoromycota) in Tunisia. Annales de la Société entomologique de France (NS) 51, 140–144.

Blackman, R.L., Eastop, V.F., 2007. Aphids on the world's crops: an identification and information guide (No. Ed. 2). John Wiley & Sons Ltd.

Byford, W.J., Ward, L.K., 1968. Effect of the situation of the aphid host at death on the type of spore produced by *Entomophthora* spp. Trans. Br. Mycol. Soc. 51, 598–600. https://doi.org/10.1016/S0007-1536(68)80033-6.

Chen, C., Feng, M., 2004. Observation on the initial inoculum source and dissemination of entomophthorales-caused epizootics in populations of cereal aphids. Sci. China C Life Sci. 47, 38. https://doi.org/10.1360/02yc0261.

Duarte da Silveira, V., Westrum, K., Lopes Ribeiro, A.E., Guedes Corrêa Gondim Junior, M., Klingen, I., Delalibera Júnior, I., 2013. Abiotic and biotic factors affecting resting spore formation in the mite pathogen *Neozygites floridana*. Int. J. Microbiol. https://doi.org/10.1155/2013/276168.

Eilenberg, J., Thomsen, L., Jensen, A.B., 2013. A third way for entomophthoralean fungi to survive the winter: slow disease transmission between individuals of the hibernating host. Insects 4, 392–403. https://doi.org/10.3390/insects4030392.

Eilenberg, J., Saussure, S., Ben Fekih, I., Jensen, A.B., Klingen, I., 2019. Factors driving susceptibility and resistance in aphids that share specialist fungal pathogens. Curr. Opin. Insect Sci. https://doi.org/10.1016/j.cois.2019.05.002.

Elkassabany, N.M., Steinkraus, D.C., McLeod, P.J., Correll, J.C., Morelock, T.E., 1992. *Pandora neoaphidis* (Entomophthorales: Entomophthoraceae): A potential biological control agent against *Myzus persicae* (Homoptera: Aphididae) on Spinach. J. Kansas Entomol. Soc. 65, 196–219. http://www.jstor.org/stable/25085351.

Finlay, K.J., Luck, J.E., 2011. Response of the bird cherry-oat aphid (*Rhopalosiphum padi*) to climate change in relation to its pest status, vectoring potential and function in a crop–vector–virus pathosystem. Agr Ecosyst Environ 144, 405–421. https://doi.org/10.1016/j.agee.2011.08.011.

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R., 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotech, 3, 294–299.

Hajek, A., Steinkraus, D., Castrillo, L., 2018. Sleeping beauties: horizontal transmission via resting spores of species in the Entomophthoromycotina. Insects 9, 102. https://doi.org/10.3390/insects9030102.

Hansen, L.M., 2006. Models for spring migration of two aphid species *Sitobion avenae* (F.) and *Rhopalosiphum padi* (L.) infesting cereals in areas where they are entirely holocyclic. Agric. For. Entomol. 8, 83–88. https://doi.org/10.1111/j.1461-9563.2006.00289.x.

Hatting, J.L., Poprawski, T.J., Miller, R.M., 2000. Prevalences of fungal pathogens and other natural enemies of cereal aphids (Homoptera:Aphididae) in wheat under dryland and irrigated conditions in South Africa. BioControl 45, 179–199.

Jensen, A.B., Eilenberg, J., 2001. Genetic variation within the insect-pathogenic genus *Entomophthora*, focusing on the *E. muscae* complex, using PCR—RFLP of the ITS II and the LSU rDNA. Mycol. Res. 105, 307–312. https://doi.org/10.1017/S0953756201003434.

Jensen, A.B., Hansen, L.M., Eilenberg, J., 2008. Grain aphid population structure: no effect of fungal infections in a 2-year field study in Denmark. Agric. For. Entomol. 10 (3), 279–290. https://doi.org/10.1111/j.1461-9563.2008.00383.x.

Katoh, K., Toh, H., 2008. Recent developments in the MAFFT multiple sequence alignment program. Brief. Bioinform. 9, 286–298.

Keller, S., 1987. Observations on the overwintering of *Entomophthora planchoniana.* J. Invertebr. Pathol. 50, 333–335. https://doi.org/10.1016/0022-2011(87)90103-0.

Keller, S., 1991. Arthropod-pathogenic Entomophthorales of Switzerland. II. *Erynia, Eryniopsis, Neozygites, Zoophtora* and *Tarichium*. Sydowia 43, 39–122.

Kjøller, R., Rosendahl, S., 2000. Detection of arbuscular mycorrhizal fungi (Glomales) in roots by nested PCR and SSCP (Single Stranded Conformation Polymorphism). Plant and Soil 226, 189–196.

Kleiber, C., Zeileis, A., 2008. Applied econometrics with R. Springer Science & Business Media, New York.

Klingen, I., Wærsted, G., Westrum, K., 2008. Overwintering and prevalence of *Neozygites floridana* (Zygomycetes: Entomophthorales) in hibernating females of *Tetranychus urticae* (Acari: Tetranychidae) under cold climatic conditions in strawberries. Exp. Appl. Acarol. 46, 231–245. https://doi.org/10.1007/s10493-008-9178-2.

Kunert, A.T., Pöhlker, M.L., Tang, K., Krevert, C.S., Wieder, C., Speth, K.R., Hanson, L.E., Morris, C.E., Schmale III, D.G., Pöschl, U., Fröhlich-Nowoisky, J., 2019. Macromolecular fungal ice nuclei in *Fusarium*: effects of physical and chemical processing. Biogeosciences 16 (23), 4647–4659. https://doi.org/10.5194/bg-16-4647-2019.

Kurppa, S., 1989. Predicting outbreaks of *Rhopalosiphum padi* in Finland. In Annales Agriculturae Fenniae 28, 333–347.

Leather, S.R., 1980. Egg survival in the bird cherry-oat aphid, *Rhopalosiphum padi*. Entomol. Exp. Appl. 27, 96–97. https://doi.org/10.1111/j.1570-7458.1980.tb02951.x.

Leather, S.R., 1981a. Reproduction and survival: a field study of the gynoparae of the bird cherry-oat aphid, *Rhopalosiphum padi* (Homoptera; Aphididae) on its primary host *Prunus padus*. Annales Entomologici Fennici 47, 131–135.

Leather, S.R., 1981b. Factors affecting egg survival in the bird cherry-oat aphid, *Rhopalosiphum padi*. Entomol. Exp. Appl. 30 (197), 199.
Leather, S.R., 1983. Forecasting aphid outbreaks using winter egg counts: An assessment of its feasibility and an example of its application in Finland. Z. Angew. Entomol. 96, 282–287. https://doi.org/10.1111/j.1439-0418.1983.tb03670.x.
Leather, S.R., 1986. Host monitoring by aphid migrants: do gynoparae maximise offspring fitness? Oecologia 68, 367–369. https://doi.org/10.1007/BF01036740.
Leather, S.R., 1992. Aspects of aphid overwintering (Homoptera: Aphidinea: Aphididae). Entomologia Generalis 17, 101–113. https://doi.org/10.1127/entom.gen/17/1992/101.
Leather, S.R., Dixon, A.F.G., 1981. Growth, survival, and reproduction of the bird-cherry aphid, *Rhopalosiphum padi*, on its primary host. Ann. Appl. Biol. 99, 115–118. https://doi.org/10.1111/j.1744-7348.1981.tb05136.x.
Lenth, R., 2017. Emmeans: estimated marginal means, aka Least-Squares Means. R Package Version 0.9.1.
Manfrino, R.G., Hatting, J.L., Humber, R., Salto, C.E., Lopez Lastra, C.C., 2014. Natural occurrence of entomophthoroid fungi (Entomophthoromycota) of aphids (Hemiptera: Aphididae) on cereal crops in Argentina. Ann. Appl. Biol. 164, 151–158. https://doi.org/10.1111/aab.12089.
Nielsen, C., Hajek, A.E., Humber, R.A., Bresciani, J., Eilenberg, J., 2003. Soil as an environment for winter survival of aphid-pathogenic Entomophthorales. Biol. Control 28, 92–100. https://doi.org/10.1016/S1049-9644(03)00033-1.
Nielsen, C., Steenberg, T., 2004. Entomophthoralean fungi infecting the bird cherry-oat aphid, *Rhopalosiphum padi*, feeding on its winter host bird cherry, *Prunus padus*. J. Invertebr. Pathol. 87, 70–73. https://doi.org/10.1016/j.jip.2004.05.003.
Nielsen, C., Eilenberg, J., Harding, S., Oddsdottir, E., Halldórsson, G., 2001. Geographical distribution and host range of Entomophthorales infecting the green spruce aphid *Elatobium abietinum* Walker in Iceland. J. Invertebr. Pathol. 78, 72–80. https://doi.org/10.1006/jipa.2001.5045.
Pagès, H., Aboyoun, P., Gentleman, R., DebRoy, S., 2018. Biostrings: Efficient manipulation of biological strings. R package version 2.48.0.
Pell, J.K., Hannam, J.J., Steinkraus, D.C., 2010. Conservation biological control using fungal entomopathogens. BioControl 55, 187–198. https://doi.org/10.1007/s10526-009-9245-6.
Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012. MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61, 539–542. https://doi.org/10.1093/sysbio/sys029.
Roy, H.E., Steinkraus, D.C., Eilenberg, J., Hajek, A.E., Pell, J.K., 2006. Bizarre interactions and endgames: entomopathogenic fungi and their arthropod hosts. Annu. Rev. Entomol. 51, 331–357. https://doi.org/10.1146/annurev.ento.51.110104.150941.
Sletteng, C., 2014. Aphids and their natural enemies (Hymenoptera and Entomophthorales) in cereals - Population dynamics, prevalence, and effect of insect pathogenic fungi. Norwegian University of Life Sciences. Department of Ecology and Natural Resource. MSc Thesis Management. https://static02.nmbu.no/mina/studier/moppgaver/2014-Sletteng.pdf.
Snider, C.S., Hsiang, T., Zhao, G., Griffith, M., 2000. Role of ice nucleation and antifreeze activities in pathogenesis and growth of snow molds. Phytopathology 90 (4), 354–361.
Sömme, L., 1969. Mannitol and glycerol in overwintering aphid eggs. Norsk Entomologisk Tidsskrift 16, 107–111.
Statistisk Sentralbyrå, 2023. Cereals and oil seeds, area and yields. https://www.ssb.no/en/jord-skog-jakt-og-fiskeri/jordbruk/statistikk/korn-og-oljevekster-areal-og-avlinger (accessed 22 June 2023).
Steinkraus, D.C., 2006. Factors affecting transmission of fungal pathogens of aphids. J. Invertebr. Pathol. 92, 125–131. https://doi.org/10.1016/j.jip.2006.03.009.
Trandem, N., Bhattarai, U.R., Westrum, K., Knudsen, G.K., Klingen, I., 2015. Fatal attraction: Male spider mites prefer females killed by the mite-pathogenic fungus *Neozygites floridana*. J. Invertebr. Pathol. 128, 6–13. https://doi.org/10.1016/j.jip.2015.04.002.
Zeileis, A., Kleiber, C., Jackman, S., 2008. Regression models for count data in R. J. Stat. Softw. 27 (8). http://www.jstatsoft.org/v27/i08/.