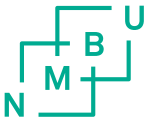

Norwegian University of Life Sciences
Faculty of Biosciences
Department of Plant Sciences

Philosophiae Doctor (PhD)
Thesis 2019:70

Interactions between the cereal aphid pests *Sitobion avenae* and *Rhopalosiphum padi* and their fungal natural enemies

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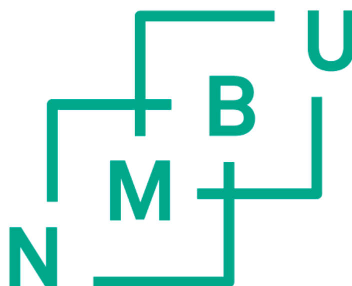

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À ma famille,

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Abstract

In agronomy, efficient plant protection strategies are required to reduce pest pressure and increase crop yield. Pesticide application has been the dominant method for plant protection for almost a century. However, the over-reliance on pesticides has several detrimental consequences. Therefore, alternative plant protection strategies have been developed and promoted to reduce pesticide use. An overall approach to using alternative strategies is Integrated Pest Management (IPM), defined as a sustainable approach to manage pests by combining eight principles (according to the EU regulation that Norway implemented in 2015) in a way that minimizes economic, environmental and health risks. These include the use of natural enemies and the manipulation of their trophic interactions with pests in order to protect the crop yield (*i.e.* biological control).

In Europe, two main aphid species cause damage in cereals such as winter wheat: the English grain aphid *Sitobion avenae* and the bird cherry-oat aphid *Rhopalosiphum padi*. Both species have many natural enemies, among which are entomopathogenic fungi in the sub-phylum Entomophthoromycotina. In this fungal group, the most interesting species for biological control of aphids are *Pandora neoaphidis* and *Entomophthora planchoniana*. As for any host-pathogen interactions, three important groups of factors are important potential drivers for an epidemic development: host population, pathogen population and environment. Together they are called the disease triangle. The aim of this PhD thesis was to identify important drivers of the disease triangle influencing (1) the success of fungal infection of aphids in cereals and (2) the capacity of the fungus to spread in these aphid populations.

In this thesis I first reviewed, the factors driving the aphid host susceptibility or resistance to fungal pathogens by considering the model system composed of *S. avenae*, *R. padi* and *P. neoaphidis*, *E. planchoniana*. Aphid behaviour and ecological niche preferences, host origin of the fungal isolate (from which host species has it been collected), aphid morph and presence of endosymbiotic bacteria are among the principal potential factors influencing the success of the fungal infection. Finally, I hypothesize that these aphid pathogenic fungi follow their host during their entire life cycle and therefore follow their spatial distribution.

I tested this hypothesis in a study on *R. padi*. *Rhopalosiphum padi* overwinter on the bird cherry tree *Prunus padus*, where it lays overwintering eggs. Fungus-killed cadavers, filled with overwintering structures, were found in the same microhabitat as *R. padi* eggs. *Zoophthora* sp. overwintered as resting spores, while *E. planchoniana* overwintered as modified hyphal bodies. There was a significant negative correlation between number of overwintering eggs and cadavers per branch. Number of both eggs and cadavers varied greatly between years and geographical locations. I discussed the potential role of *P. padus* as a reservoir for fungi infecting aphids in cereals.

In a laboratory study, I studied the potential cross-infection of three *P. neoaphidis* isolates (from one *S. avenae* population in Norway) between *S. avenae* and *R. padi*. Moreover, the effect of the fungal isolates on aphid mortality and fecundity at three different temperatures relevant for Norwegian conditions were studied. Our results showed that cross-infection is possible but potentially asymmetric. In effect, *P. neoaphidis* kills more *S. avenae* than *R. padi* and also kills *S. avenae* faster. A significant variability was found between the three fungal isolates in virulence and sub-lethal effect on aphid fecundity. The higher the temperature, the higher the mortality of fungal infected aphids. However, temperature did not consistently affect the time needed to kill the host or the effect on fecundity. Our findings are important for understanding and modelling *P. neoaphidis* epizootiology in aphid pests of cereals.

Finally, a modelling approach was used to investigate the epizootiology of *P. neoaphidis* infecting *S. avenae* on winter wheat. A mechanistic tri-trophic model was built that includes a high aphid population in order to overcome any potential host density threshold. Twelve parameters related to the fungus' biology and climatic conditions were allowed to vary in order to identify those most important for aphid and fungus populations and potential biological control. Three parameters were identified as crucial: (1) fungus transmission efficiency, (2) humidity threshold level that triggers fungal sporulation and (3) the weather (temperature and humidity). The longevity of fungus-killed cadavers (how long they may represent an inoculation source) was very important for the fungus population dynamic in this model. Interestingly, the proportion of infected aphids colonising the wheat field was the most important parameter to reduce the yield loss due to the biological control.

Sammendrag

For å redusere skader på planter og for å øke avlingene i landbruket trenger vi effektive plantevernstrategier. Bruk av kjemiske plantevernmidler har vært den plantevernmetoden som har vært mest brukt i nesten ett århundre. Den avhengigheten av kjemiske plantevernmidler har hatt flere uheldige effekter. Alternative plantevernstrategier er derfor blitt utviklet og det oppfordres også til å bruke disse. Integrert plantevern (IPV) som nå brukes i mange land og defineres som en bærekraftig strategi for å håndtere planteskadegjørere ved å følge åtte prinsipper (ifølge EU regelverket som Norge implementerte i 2018) på en måte som reduserer risiko for økonomi, miljø og helse. Disse åtte prinsippene inkluderer blant annet bruken av naturlige fiender og manipuleringen av deres trofiske samspill med skadegjørere for å beskytte plantene (biologisk kontroll).

De to viktigste bladlusartene som opptrer som skadedyr på høsthvete og andre kornarter i Europa er kornbladlusa, *Sitobion avenae,* og havrebladlusa, *Rhopalosiphum padi.* Begge artene har mange naturlige fiender og blant disse hører insektpatogene sopp i underrekke Entomophthoromycotina. I denne gruppen er *Pandora neoaphidis* og *Entomophthora planchoniana* blant de mest lovede artene for biologisk kontroll. For alle vert-patogensamspill er det tre hovedgrupper av faktorer som er viktige drivere for en epidemisk utvikling: vertpopulasjonen, patogen populasjonen og miljøet. Dette kalles sykdomstriangelet. Målet med denne PhD oppgaven var å identifisere viktige drivere i sykdomstriangelet og som påvirker (1) hvor vellykket disse insektpatogene soppene kan infisere bladlus i korn og (2) soppens evne til å spre seg i bladluspopulasjonene.

I denne oppgaven gjennomgår jeg først hvilke faktorer som er drivere for bladlusenes (*S. avenae* og *R. padi)* og mottakelighet for eller resistens mot de insektpatogene soppene (*P. neoaphidis* og *E. planchoniana*). Følgende faktorer ser ut til å være de viktigste for en vellykket soppinfeksjon av bladlusene: Blaldusartenes adferd og valg av økologisk nisje, bladlusas morf, bladlusarten soppisolatet er isolert fra og om endosymbiotiske bakterier er tilstede i bladlusa. Videre setter jeg opp en hypotese om at disse bladluspatogene soppene følger sine verter gjennom hele deres livssyklus og derfor også følger bladlusenes romlige utbredelse.

Den hypotesen tester jeg i en studie av havrebladlus *(R. padi)*. Havrebladlusa overvintrer som egg på hegg (*Prunus padus)*. I studiet fant vi soppdrepte bladlus med overvintrende soppstrukturer i det samme mikrohabitatet som vi fant egg av havrebladlus. Nyttesopp tilhørende *Zoophthora* sp. overvintret som hvilesporer mens soppen *Entomophthora*

planchoniana overvintret som modifiserte hyfelegemer. Det var ingen signifikant negativ sammenheng mellom antall overvintrende havrebladlusegg og soppdrepte havrebladlus per heggkvist. Antall havrebladlusegg og soppdrepte havrebladlus varierte betydelig mellom år og geografisk lokalitet. Jeg diskuterer den potensielle rollen hegg kan ha som reservoar for sopp som dreper bladlus i korn.

I et laboratorieforsøk studerer jeg mulig smitte av tre *P. neoaphidis* isolater (fra en og samme kornbladlus populasjon i Norge) mellom kornbladlus og havrebladlus. Videre studerer jeg effekten av disse soppisolatene på bladlusenes dødelighet og fertilitet ved tre ulike temperaturer som er relevant for norske forhold. Våre resultater viser at smitte fra en bladlusart til en annen er mulig men at den antagelig er asymmetrisk. Dette vil si at *P. neoaphidis* fra kornbladlus dreper flere kornbladlus enn havrebladlus og at den også dreper kornbladlusa raskere. Det ble funnet en signifikant variasjon i virulens og sub-letal effekt knyttet til bladlusas fertilitet mellom de tre soppisolatene. Videre fant vi at høyere temperaturer ga høyere dødelighet hos soppinfiserte bladlus men vi fant ingen konsistent effekt av temperatur på tid brukt til å drepe bladlusa eller effekt på fertilitet. Disse resultatene er viktige for å kunne forstå og modellere den epidemiologiske utviklingen av *P. neoaphidis* i ulike bladlusarter i korn.

Til slutt i denne PhD oppgaven har jeg brukt modellering for å undersøke epizootiologien til *P. neoaphidis* som smitter kornbladlus i høsthvete. En mekanistisk tre-trofisk modell ble bygget og denne inkluderer en høy kornbladluspopulasjon for å sørge for at den ligger over en potensiell terskelverdi for vertstetthet. Elleve parameter var knyttet opp mot nyttesoppens biologi og til klimatiske faktorer og disse fikk variere på en slik måte at vi kunne identifisere hvilke som kunne være viktigst for en god epidemisk utvikling av nyttesoppen i bladluspopulasjonen og hvilke som dermed muliggjorde biologisk kontroll. Tre parameters ble identifisert som vesentlige: (1) soppens spredningsevne (2) terskelverdi for fuktighet som skal til for at soppen skal sporulere og (3) værforhold (temperatur og fuktighet). "Levetid" for soppdrepte bladlus (hvor lenge de kan fungere som smittekilde) var veldig viktig for den epidemiske utviklingen av soppen i denne modellen. Andel soppinfiserte bladlus som koloniserte høsthvete var det parameteret som i størst grad førte til mindre avlingstap som følge av økt biologisk kontroll.

List of publications

I. Eilenberg J., Saussure S., Ben Fekih I., Bruun Jensen A., Klingen I. (**2019**). Factors driving susceptibility and resistance in aphids that share specialist fungal pathogens. Current Opinion in Insect Science, 33, 91-98

II. Saussure S., Bruun Jensen A., Davey M. L., Folkedal Schjøll A., Westrum K., Klingen I. Entomophthoromycotan fungi overwinter with their host the bird-cherry oat aphid *Rhopalosiphum padi* on its winter host *Prunus padus*. (**manuscript**)

III. Saussure S., Westrum K., Roer Hjelkrem A.-G., Klingen I. (**2019**). Effect of three isolates of *Pandora neoaphidis* from a single population of *Sitobion avenae* on mortality, speed of kill and fecundity of *S. avenae* and *Rhopalosiphum padi* at different temperatures. Fungal Ecology, 41, 1-12.

IV. Saussure S., Roer Hjelkrem A.-G., Klingen I., Meadow R., Holst N. The population dynamic of *Sitobion avena*e in presence of its fungal pathogen *Pandora neoaphidis* in winter wheat. (**manuscript**)

Foreword

The thesis presented here has been conducted in the department Invertebrate Pests and Weeds in Forestry, Agriculture and Horticulture at the Norwegian Institute for Bioeconomy Research (NIBIO). The work was financed by the Research Council of Norway through the project SMARTCROP (project number: 244526). This project aimed at promoting and innovating in Integrated Pest Management (IPM) by combining biology, social sciences and technological approaches to elaborate new knowledge, strategies and tools for use in IPM. This thesis also received financial support by two personal grants from the European COST Action FA1405. This COST Action promoted collaboration between institutes to develop models on tri-trophic interactions occurring in agroecosystems. I used this funding to visit and work in close collaboration with Aarhus University in Denmark.

Susceptibility: Lack of ability to resist some extraneous agent (such as a pathogen or drug).

Resistance: The inherent ability of an organism to resist harmful influences (such as disease, toxic agents, or infection).

Virulence: The disease producing power of an organism *i.e.* the degree of pathogenicity within a group or species.

Prevalence: The total number of cases of a particular disease at a given time, in a given population.

Conspecific host: host belonging to the same host species as the inoculum source

Heterospecific host: host belonging to a different species than the inoculum source

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Box 1: Some important definitions of terms related to epidemiology as used in this work

1. Agronomical and ecological context

1.1. Agroecosystems and plant protection

An ecosystem is a biological system composed of species interacting with each other and with their physical environment. A trophic network emerges from the ecosystem, with at least three levels: (i) the producers: plants, (ii) the primary consumers: herbivores and plant pathogens, and (iii) the secondary consumers: predators, parasites/parasitoids and entomopathogens. An agroecosystem is particular in the sense that usually only one producer is interesting for famers: the crop. All primary consumers feeding on it are considered pests and all competing species with the crop are called weeds. Further, all organisms consuming pests or weeds are called natural enemies and provide an ecosystem service called biological control. The trophic interactions between primary consumers, competing species and secondary consumers greatly influence the yield. Current estimations of total global potential yield losses due to pests and weeds can reach up to 40-80 % of yield (*e.g.* Lake and Wade, 2009; Oerke, 2006). Further, climate change may increase insect pest pressure and crop losses as for example shown for cereals production (Deutsch *et al.*, 2018; Lesk *et al.*, 2016). Efficient plant protection strategies are required and should be adapted to the changing environmental conditions.

Since the beginning of agriculture (10,000-16,000 years ago), farmers have modified the environment and interactions within the agroecosystem to favour the crop (Gray *et al.*, 2009). At the beginning of plant protection (from 2.500 BC and onwards), pesticides were simple inorganic (*e.g.* sulfur, arsenic) and organic molecules (*e.g.* olive oil). Then extraction of plant insecticidal compounds increased in the 16th century (Thacker, 2002). Industrial production of pesticides started in 1865 and synthetic pesticides were discovered in 1939 with the infamous dichlorodiphenyltrichloroethane (DTT). Production of pesticides continued growing in the 1950's and 1960's and the first detrimental consequences became evident at that time (Casida, 2012; Gray *et al.*, 2009) and are still being addressed. One of the challenges arising with increased use of pesticides is pesticide resistance, when pests and weeds become less sensitive to previously effective compounds, rendering the pesticide of little use to control a certain pest (Gould *et al.*, 2018). Further, some pesticides can reduce the population of the biological control agents and may disrupt biological control (*e.g.* Klingen and Westrum,

2007). Finally, increased use of pesticides may also put at risk human health and the environment, with for example pesticide residues in the food chain (Gonzalez-Rodriguez *et al.*, 2011) and decrease in biodiversity (*e.g.* Beketov *et al.*, 2013). Consequently, nowadays, national and international regulations have prohibited certain compounds (*e.g.* ban of neonicotinoid to protect bees, European regulation No. 485/2013) and require ecotoxicological tests before approving new products (European regulation No. 1107/2009). Despite this, agronomists still use substantial quantities of pesticides with an estimation of four million tons of pesticides per year worldwide (Gavrilescu, 2005). At the same time, alternative plant protection strategies have been developed and promoted to reduce pesticide use while conferring efficient plant protection. One approach is called Integrated Pest Management, which incorporates biological control.

1.2. Integrated Pest Management and biological control

Integrated Pest Management (IPM) is a conceptual framework that includes advice for farmers on how to build plant protection strategies and to help them decide if, when and how to apply control measures for pests (insects and diseases) and weeds (Barzman *et al.*, 2015). IPM uses a holistic approach by integrating all pest and weed risks based on the specific agricultural context of the crop. Further, decisions for pest control is based on economic thresholds, which are estimations of the maximal pest population a crop can tolerate before a significant yield loss occurs. IPM is defined by Endure, the European network for plant protection, as "a sustainable approach to manage pests by combining biological, cultural and chemical tools in a way that minimizes economic, environmental and health risks." (www.endure-network.eu). This concept has been developed first by entomologists as "a wise combination of biological and chemical control of pests" (Stern *et al.*, 1959). Over the years, IPM has been recognised as an interdisciplinary and flexible approach, which constantly evolves to integrate new knowledge and technological tools (Barzman *et al.*, 2015; Gray *et al.*, 2009). Barzman *et al.* (2015) defined eight principles of IPM: (i) prevention and suppression, (ii) monitoring, (iii) decision based on monitoring and thresholds, (iv) non-chemical methods, (v) pesticide selection, (vi) reduced pesticide use, (vii) anti-resistance strategies, (viii) evaluation. The EU has given a legislative framework to IPM and promotes it through the EU-Directive 2009/218/EC. Norway also implemented this directive in 2015.

As mentioned earlier, natural enemies of pests provide an ecosystem service, which results in the regulation of pest populations, called biological control. Biological control is the use of living organisms to control pest populations. Eilenberg *et al.* (2001) listed four strategies of biological control including conservation biological control, which is defined as “modification of the environment or existing practices to protect and enhance specific natural enemies or other organisms to reduce the effect of pests”. Consequently, biological control and especially conservation biological control fulfil IPM requirements. Indeed, by manipulating the environment around the crop, farmers could protect their crop in a sustainable manner and decrease the cost of plant protection. Several groups of natural enemies have been considered for use as biological control agents such as parasitoid wasps and predators (*e.g.* Ramsden *et al.*, 2015). The use of insect pathogens (virus, bacteria, fungi) as biological control agents for IPM has been recently reviewed by Lacey *et al.* (2015) and are a promising alternative to pesticides in some cases. Among pathogens, entomopathogenic fungi are potential biological agents, with great control potential as they can significantly reduce the pest population given optimal environmental conditions (Lacey *et al.*, 2015; Pell *et al.*, 2001). This is particularly true in the case of aphids feeding on cereals (Ben Fekih *et al.*, 2015; Li and Sheng, 2007; Barta and Cagáň, 2006). To be able to use biological control efficiently and avoid unintentional reduction of the natural population, further studies are required to understand the biology and manipulate environment and trophic interactions of aphids, their natural enemies and the wheat crop.

2. Aphids and entomopathogenic fungi in cereals: a case study

2.1. Pest aphids in cereals

Aphids (Hemiptera: Aphididae) in cereals have become a recurrent problem in Western Europe. Two main species are known to attack cereals in this region: the English grain aphid *Sitobion avenae* and the bird cherry oat aphid *Rhopalosiphum padi* (Blackman and Eastop, 2007). Their high reproduction and dispersion capacities enable them to exploit ephemeral habitats such as cereal fields (Fereres *et al.*, 2017; Winder *et al.*, 2013; Dedryver *et al.*, 2010). Aphids damage cereals first through direct consumption of plant nutrients (*i.e.* by sucking phloem), and indirectly by disruption of photosynthesis (*i.e.*

honeydew production and mould development on green leaves), and finally by transmitting plant viruses (Rabbinge *et al.*, 1981; Wratten, 1975).

Aphids overwinter as diapausing eggs on their winter host plant. *Sitobion avenae* overwinters on grasses or cereal stubble; while *R. padi* overwinters on the bird cherry tree *Prunus padus* (Rosales: Rosaceae) (Blackman and Eastop, 2007; Halkett *et al.*, 2004; Rispe *et al.*, 1999). In spring, overwintering eggs of both *S. avenae* and *R. padi* hatch and a spring generation feeds and reproduces parthenogenetically on the winter host (Fig. 1). Both species produce 2-3 generations before producing winged females, which migrate from the winter host to grasses and cereals (Hansen, 2006). During summer, aphids keep reproducing parthenogenetically with many generations. Winged and apterous (*i.e.* without wings) females are produced depending on environmental conditions. Under high density in colonies and with decrease in plant nutritional quality, more winged females are produced for dispersion (*e.g.* Duffy *et al.*, 2017). Cereal development greatly influences survival and reproduction capacity of aphids (Dean, 1974). For instance, *S. avenae* reproduction capacity is multiplied by 1.6 between flowering and milk development of cereals due to the plant allocating nutrients to grain formation through the phloem (Watt, 1974). However, when cereals ripen, they become unsuitable for aphid development, hence their survival rate decreases, and winged females are produced for dispersion (Duffy *et al.*, 2017; Plantegenest *et al.*, 2001). In autumn, specific females, the sexuparae, produce males and egg-laying females, which migrate back to their respective winter host (Leather, 1992). After mating, egg-laying females deposit overwintering eggs either at the basis of Poaceae stems for *S. avenae* or in the axil of *P. padus* buds for *R. padi* (Leather, 1993; Leather, 1981). Under mild winter conditions, both species can keep reproducing parthenogenetically and can also overwinter as parthenogenetic viviparous females (Dedryver *et al.*, 2010). However, these latter forms experience high mortality rates under -10 ^{o}C; while diapausing eggs can survive down to -40 ^{o}C (Dedryver *et al.*, 2010; Leather, 1992; Sömme, 1969). Therefore, in Northern Europe, only sexual reproduction and overwintering as eggs are favoured for both species.

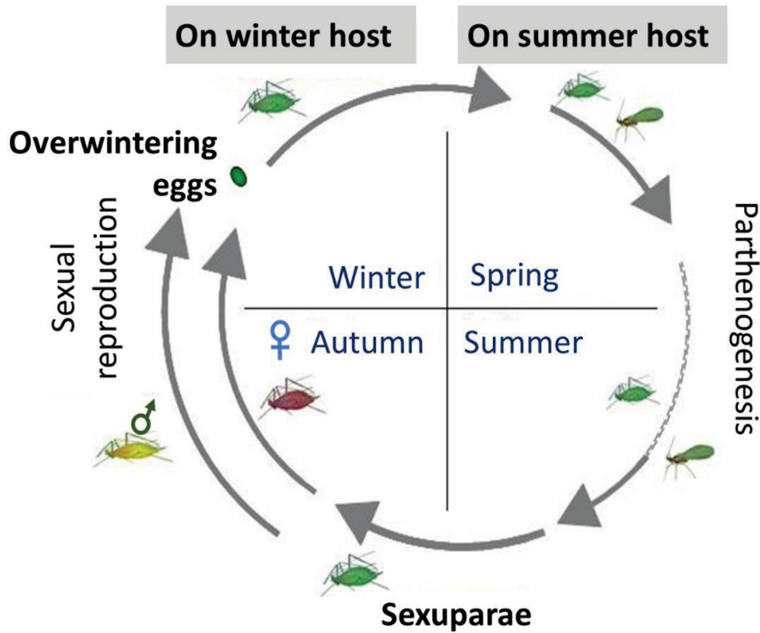

Figure 1: Generic biological cycle of *Sitobion avenae* and *Rhopalosiphum padi*. Overwintering eggs, located on the aphid's winter host, hatch at the beginning of spring. Aphids start reproducing parthenogenetically and building up populations. During the middle of spring, winged females are produced. They disperse and colonise summer hosts: Poaceae plants including cereals. Aphids continue reproducing parthenogenetically and produce winged or apterous females depending on environmental conditions. In autumn, aphids produce sexuparae females that produce males and egg-laying females, which migrate to their respective winter host. After mating, females lay overwintering eggs on sheltered locations. Adapted from Encyclop'Aphid, INRA.

Within a cereal field, aphid colonisation and dispersion drive their population dynamic during the whole growing season (Winder *et al.*, 2013; Fievet *et al.*, 2007). Both processes are continuous (Winder *et al.*, 2014; Dedryver *et al.*, 2010; Vialatte *et al.*, 2007) and modulated by climatic conditions (Harrington *et al.*, 2007) and population density (Bommarco *et al.*, 2007). At the beginning of field colonisation, aphid distribution is random, and several genotypes start establishing colonies. However, a rapid selection of genotypes adapted to the crop occurs (Fievet *et al.*, 2007) leading to the predominance of only some genotypes in one field (Haack *et al.*, 2000). These selected genotypes tend to disperse within the field and their distribution becomes more homogenous (Winder *et al.*, 2014; Fievet *et al.*, 2007). For instance, Dedryver *et al.* (2009) estimated that in a wheat field during a week, 20-60% of *S. avenae* colonies disappeared. At the same time, each day 20-35% of aphids are estimated to fall to the ground, disperse by walking and colonise

(winged or apterous) depend on the cereal growth stage. Their implications for aphid population dynamics have been studied since the 1970s (Dean, 1974; Carter *et al.*, 1992). Many studies have measured, estimated or predicted aphid colonisation (Ciss *et al.*, 2014; Hansen, 2006), population dynamics on cereals (Honek *et al.*, 2018; Duffy *et al.*, 2017; Plantegenest *et al.,* 2001), damage on crop yield quantity (Rossing, 1991; Entwistle and Dixon, 1987; Wratten, 1975) and quality (Lee *et al.*, 1981; Wratten, 1975). These studies can be used in the framework of IPM as monitoring and predicting tools. For instance, based on the knowledge acquired, threshold values of aphid density have been estimated depending on cereal growth stage and yield expectations for both *S. avenae* and *R. padi.* A density higher than these thresholds would cause significant yield loss (Klingen *et al.*, 2008; Larsson, 2005; Oakley and Walters, 1994).

Aphids are major pests in cereals. However, outbreaks occur periodically and only some years in Europe (Barbec *et al.*, 2014; Dedryver *et al.*, 2010; Larsson, 2005; Hansen, 2000), which ultimately leads to significant variation in yield loss over the years. Climatic conditions (Barbec *et al.*, 2014; Gilabert *et al.*, 2009) and natural enemies (Bonsall, 2004; Dwyer *et al.*, 2004) are commonly identified as potential drivers of oscillations in insect populations. Nevertheless, these threshold values established for aphid density in cereals (see above) do not consider biological control by natural enemies. For example, biological control of the cotton aphid in the United States is efficiently implemented in plant protection strategies. Indeed, farmers withhold insecticide spraying when an entomopathogenic fungus is predicted to efficiently decrease the pest population (Hollingsworth *et al.*, 1995). Aphids in cereals have many natural enemies. Predators such as ladybirds and parasitoid wasps are the most studied for biological control. Predictive models have been built to estimate the biological control they confer (*e.g.* Maisonhaute *et al.*, 2018; Leblanc and Brodeur, 2018). However, aphids are also attacked by entomopathogenic fungi as mentioned earlier. Entomopathogenic fungi have been identified as crucial for regulation of insect populations (*e.g.* Wang and Wang, 2017) and have long been investigated for biological control purposes (Lacey *et al.*, 2015; Pell *et al.*, 2010). Potential use of these in IPM require a good understanding of the ecology and factors promoting fungal spread and establishment in host populations, communities and landscapes.

2.2. Entomopathogenic fungi infecting aphids

The most important fungi infecting aphids in cereals belong to the sub-phylum Entomophthoromycotina and more specifically to five genera: *Pandora*, *Entomophthora*, *Zoophthora*, *Conidiobolus*, *Neozygites* (Hajek and Meyling, 2018; Humber, 2012; Barta and Cagáň, 2006). Among them, the two most important species attacking cereal aphids are *Pandora neoaphidis* and *Entomophthora planchoniana* (Barta and Cagáň, 2006; Pell *et al.*, 2001). These ubiquitous fungi are pathogenic to more than 70 and 30 aphid species for *P. neoaphidis* and *E. planchoniana* respectively (Barta and Cagáň, 2006; Pell *et al.*, 2001). Further, they do not infect other natural enemies such as ladybirds (Baverstok *et al.*, 2009). They are, therefore, good candidates for biological control. However due to difficulties in mass-production, Entomophthoromycotina have mostly been considered for conservation biological control purposes (Lacey *et al.*, 2015; Pell *et al.*, 2010).

As any fungi, they are highly sensitive to environmental humidity for development and dispersion (Steinkraus, 2006). A fungus-killed aphid is called a cadaver and needs optimal conditions to sporulate, among which optimum temperature or humidity (Shah *et al.*, 2002; Xu and Feng, 2002). Therefore, there can be a time-delay between aphid death and fungus sporulation. For example, *P. neoaphidis* requires 3 hours at 20°C and 95% relative humidity to sporulate from *S. avenae* cadavers (Ardisson *et al.*, 1997) (Fig. 2A). During sporulation, Entomophthoromycotina produce tens of thousands of infective units, called conidia, which are actively projected in the environment (Pell *et al.*, 2001). Hemmati et al (2001a) showed that conidia of *P. neoaphidis* are projected high enough to leave the leaf boundary layer and are able to become airborne. Further, Steinkraus *et al.* (1993) found that 76% of *N. fresenii* conidia projected from a sporulating cotton aphid cadaver become airborne; while 24% land on the substrate in the aphid vicinity (Fig. 2B). These airborne conidia can form conidia clouds as found above cereal fields in summer by Hemmati *et al.*, (2001b). In this study, the maximum concentration of conidia in the air reached 1,373 conidia m^{-3}. These clouds could come from infections occurring in flower strips close to the field and infect aphids in cereals (Baverstock, 2012; Ekesi *et al.*, 2005). If conidia don't come in contact with a susceptible host, they produce and actively project secondary or tertiary conidia, which are also infective (Sierotzi *et al.*, 2000; Eilenberg *et al.*, 1995). Moreover, Entomophthoromycotina transmit horizontally only, meaning that they infect their host only by contact (Vega *et al.*, 2012). This characteristic is crucial for biological

control of aphids because aphids are sap-sucking insects and, therefore, less exposed to ingestible pathogens. If conidia land on a suitable host, they germinate and penetrate through the aphid cuticle (Vega *et al.*, 2012) (Fig. 2C). Inside the host, the fungus multiplies and develops first as protoplasts (*i.e.* without cell walls) then as hyphal bodies (Barta and Cagáň, 2006). Hyphal bodies invade the whole host body and the host is killed. Conidiophores (the structure producing conidia) are produced and break through the cuticle only under optimal conditions (Kalkar, 2005).

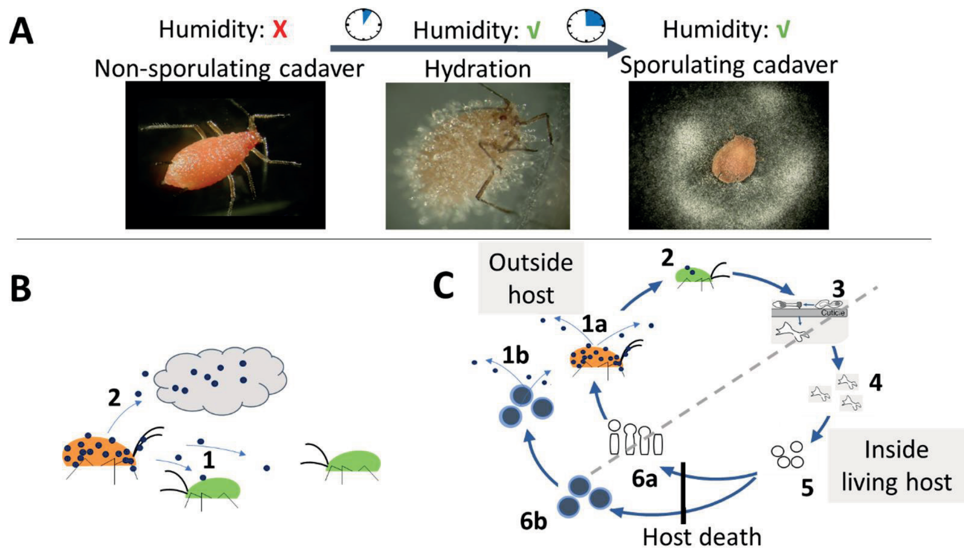

Figure 2: Important characteristics of Entomophthoromycotina. **A)** Influence of environmental humidity on the sporulation process. Once aphids are killed by the fungus, it becomes a non-sporulating cadaver. All fungal structures are still inside the dead body of the host. When environmental conditions are optimum, in particular humidity is high enough, the fungus hydrates for few hours and sporulation occurs. **B)** During sporulation, conidia are actively projected from the cadaver. (**1**) Some spores land in the aphid vicinity; (**2**) while others become airborne and passively disperse in the airstream. **C)** Generic biological cycle. (**1a**) A sporulating cadaver projects spores in the environment. (**2**) Some may land on the cuticle of a suitable host. (**3**) Spores germinate and penetrate inside the host body. Once inside the host, (**4**) the fungus multiplies first as protoplasts, then (**5**) as hyphal bodies. The host death occurs prior to sporulation. (**6a**) Conidiophores (*i.e.* structure producing conidia) develop first inside the host and if conditions are optimal grow outside the host's dead body. (**6b**) Under certain circumstances, hyphal bodies conjugate and produce overwintering structures such as resting spores. (**1b**) These structures can germinate and produce infective spores after breaking their dormancy under the proper conditions. Cycle adapted from Jon Zawislak.

Under certain conditions, hyphal bodies developing inside the host conjugate and produce overwintering structures. Entomophthoromycotinan fungi overwinter as many different structures (Hajek *et al.*, 2018; Eilenberg *et al.*, 2013; Nielsen *et al.*, 2003). Specifically, *Pandora neoaphidis* and *E. planchoniana* can overwinter as long-lived and dormant resting spores (Scorsetti *et al.*, 2012; Keller, 1991a, 1991b) (Fig. 2C) or as modified hyphal bodies for *E. planchoniana* (Keller, 1987). Triggers of overwintering structures' formation and germination are difficult to study and are mostly unknown for many species (Hajek *et al.*, 2018). However, Hajek *et al.* (2018) listed several drivers of resting spore formation such as change in host morph, food quality, daylength, decrease in temperature. Overwintering structures are not infective and usually stay inside the host's dead body (Hajek *et al.*, 2018). However, they germinate and produce infective spores, the germ conidia, which are also projected in the environment (Humber, 2012; Keller, 1987). Many resting spores do not sporulate after their first winter and may survive for many years (Hajek *et al.*, 2018; Pell *et al.*, 2001). They can, therefore form pathogen reservoirs. Among usual reservoirs of fungi, we can find the soil or some trees (Hajek *et al.*, 2018; Nielsen *et al.*, 2003; Baverstock *et al.*, 2008; Keller, 1987). Finding reservoirs can be difficult, nevertheless Hajek *et al.* (2018) speculate that overwintering fungi should be located close to their host's overwintering site. Indeed, the first spring fungal infections due to overwintering structures initiate the annual fungus life cycle. These first infections are called primary infections and initiate secondary cycling and disease transmission (Hajek and Shapiro-Ilan, 2017). Identifying and quantifying such reservoirs would be a first step to include them in biological control measures and IPM.

Because Entomophthoromycota are biotrophic, the host is killed only prior to sporulation (Fig. 2C). Consequently, there is a time-delay between the host getting infected and dying because of the fungus. This time-delay is called lethal time and usually ranges from 3-5 days at 18-20 ^{o}C for *P. neoaphidis* for example (*e.g.* Nielsen *et al.*, 2001). During this time, an infected aphid can disperse, reproduce, and damage the crop. Further, a fungal infection does not always result in host death and fungal sporulation; fungal infection may fail resulting in host resistance and fungus death (Milner, 1982). Consequently, we define a fungus as virulent (Box 1) if it expresses a high capacity to kill aphids and if it kills them fast. The infection output is called disease expression and the transmission of the pathogen between an infectious host (*i.e.* sporulating cadaver) and a susceptible host (Box1) is called disease transmission. When disease transmission is efficient inside a host

population, the fungus can spread and create an epizootic, which results in the host population crashing (Hajek and Meyling, 2017; Steinkraus, 2006; Pell *et al.*, 2001). An epizootic is an epidemic in animal populations and is defined as "an unusual large number of cases of disease in host population" (Fuxa and Tanada, 1987). Otherwise, the fungus is in an enzootic state. This definition adds a spatio-temporal dynamic to the process but is vague and context dependent (what is a usual number of disease cases?). However, in practice, epizootics are characterised by a high fungal prevalence (Box 1) and the crash of the host population. Epizootic is a fast and ephemeral event characterised by a rapid increase in prevalence (Fig 3) associated with an important mortality in a host population. After a peak, prevalence decreases quickly due to the mortality of susceptible hosts and the survival of resistant (Box 1) individuals. From a biological control point of view, we want to facilitate disease transmission inside the host population by for instance manipulating the environment to initiate epizootic.

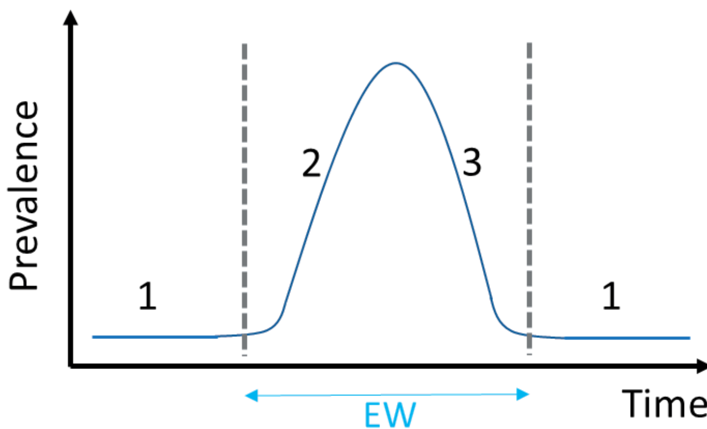

Figure 3: Epizootic dynamic. When the fungus prevalence is low in a host population (**1**), the fungus is in enzootic. Under optimal conditions, the fungal prevalence increases quickly and abruptly, the fungus is in epizootic (**2**). After a peak, the prevalence decreases quickly due to the death of the susceptible population and resistant host selection (**3**). The combination of steps 2 and 3 is called an epizootic wave (EW). After an epizootic wave, the fungus is again enzootic inside its host population. Adapted from Shapiro-Ilan *et al.* (2012).

Epizootiology of insect diseases is "the science of causes and forms of the mass phenomena of disease at all levels of intensity in a host population" (Fuxa and Tanada,

1987). Many factors influence the different steps occurring inside the host body during disease expression and outside the host body during disease transmission. It is of critical importance to understand their implication on the development of Entomophthoromycotinan infection and spread for biological control purposes.

3. Epizootiology and biological control of aphids in cereals

3.1. The specific case of one pest, one pathogen

There are three groups of factors influencing disease expression and transmission: host population, pathogen population and environment (Antonovics, 2017; Fuxa and Tanada, 1987). Together, they are called the disease triangle (Antonovics, 2017) (Fig. 4A). All factors interplay and influence both organisms host and pathogen but also their interactions. Altogether, they determine the infection output *i.e.* host resistance or host susceptibility (*e.g.* Thomas *et al.*, 2003). First, let us consider the disease expression that starts once conidia land on a susceptible host. Cereal aphid host populations are not homogenous, as seen earlier with colonisation. Indeed, distribution and genotype frequencies vary over the growing season. Further, different aphid genotypes of one species can express different levels of susceptibility to Entomophthoromycotina from resistant (always survive the fungal infection) to highly susceptible (Parker *et al.*, 2014; Milner, 1982). Moreover, aphids may harbour facultative endosymbiotic bacteria that increase their resistance as shown for the pea aphid *Acyrthosiphon pisum* (Heyworth and Ferrari, 2016; Łukasik *et al.*, 2013). Second, variability in the pathogen population is also important for the disease expression. For example, different isolates of one fungal species can express different virulence to an aphid host (Barta and Cagáň, 2009; Sierotzi *et al.*, 2000; Rohel *et al.*, 1997). The struggle between host and fungus during the infection results in both organisms developing adaptations and counter-adaptations resembling an arms race (Boomsma *et al.*, 2014; Roy *et al.*, 2006). Finally, many environmental factors shaped the disease expression and the struggle between host and pathogen. For example, temperature influences the host susceptibility (Stacey *et al.*, 2003) and the virulence of Entomophthoromycotina, either the aphid mortality (Blandford *et al.*, 2003; Stacey *et al.*, 2003) or the lethal time (Nielsen *et al.*, 2001; Shah *et al.*, 2002). However, temperature can have a complex and non-linear influence on the host-pathogen interaction, especially during an entire growing season. Few studies consider the interaction effect of

temperature and fungal isolates, for example. Investigating the interaction of seasonal temperatures on aphid-Entomophthoromycotina interactions would enable us to increase knowledge and conceptualise seasonal biological control.

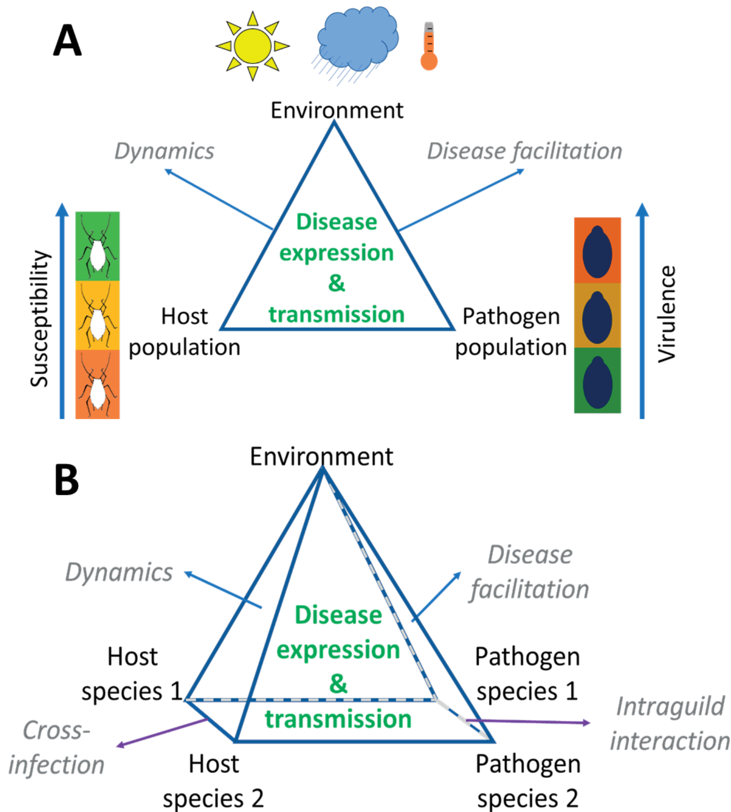

Figure 4: Factors influencing disease expression and transmission **A)** In a system with only one host and one pathogen. The host population is heterogenoeous and different genotypes can express different susceptibility to the pathogen. On the other hand, the pathogen population is also heterogenous, with for instance different genotypes expressing different virulence. The interaction between host and environment shapes the dynamics and dispersion of the host population. While the interaction between pathogen and environment influences the disease spread and in the case of bioloigcal control the disease facilitation. **B)** In a system composed of two host and two pathogen species the same processes occur (dynamics and disease facilitation). In addition, cross-infection between host species is possible and intra-guild interactions between pathogens are likely. Fig. 4A inspired from Antonovics (2017).

Once the fungus has successfully infected its host and killed it, sporulation begins, and disease transmission starts. Entomophthoromycotina transmission results from the combination of the following steps: (1) departure from an infectious host (sporulating cadaver), (2) dispersion per se (*e.g.* airborne conidia) and survival, (3) contact with a susceptible host, and finally (4) infection of a new host (conidia germination and penetration inside a susceptible host) (McCallum *et al.*, 2017). The same three factors (host, pathogen and environment) influence these four steps (Fig. 4A). For example, fungal sporulation capacity varies for different fungal isolates as shown for *P. neoaphidis* (Sierotzi *et al.*, 2000). Second, the host morph (winged or apterous) influences its susceptibility; for instance, Dromph *et al.* (2002) found that winged *S. avenae* were more susceptible to *P. neoaphidis* than apterous adults. Many abiotic factors such as temperature, humidity or UV can favour or hinder conidia production, longevity and dispersion (*e.g.* Vega *et al.*, 2012). For instance, temperature influences the temporal pattern of conidia discharge of *P. neoaphidis* (Olsen *et al.*, 2019).

Because it is impossible to track each conidium released in the environment during sporulation, directly quantifying the transmission efficiency or dispersion capacity of fungi is quite challenging (Antonovics, 2017; Lello and Fenton, 2017; Anderson and May, 1980). Therefore, experiments usually aim at quantifying the consequences of disease transmission and estimate its value afterwards (*e.g.* Ekesi *et al.*, 2005; Ardisson *et al.*, 1997). For example, Ardisson *et al.* (1997) estimated the transmission efficiency for *P. neoaphidis* based on a mesocosm experiment with a fungus density of one sporulating cadaver per 10 susceptible *S. avenae* kept at 18°C and 95% relative humidity. Under these conditions, *P. neoaphidis* transmission efficiency was 0.1728 individuals per day.

Modelling tools can be very useful in this case. By simplifying reality, modelling enables us to grasp difficult concepts and predict long-term consequences that could not be possible otherwise. In epizootiology, Brown and Nordin (1982) were the first to model host-pathogen interactions with *Zoophthora phytonomi* infecting the alfalfa weevil *Hypera postica*. They considered lethal time and humidity effect on fungal development and sporulation. However, populations were considered homogenous. Carruthers *et al.* (1986) later modelled the infection of the onion maggot *Delia antiqua* by *Entomophthora muscae* and introduced heterogeneity in populations and lethal time. Later, Hajek *et al.* (1993) modelled the population dynamic of *Entomophaga maimaiga* infecting the gypsy

moth *Lymandria dispar*. They considered different disease transmission efficiency depending on the host stage (or instar). In this model, the number of conidia were directly integrated and linked to the disease transmission among hosts. The latter study showed that variability in climatic conditions resulted in different disease dynamics in the host population. In all these models, the disease transmission was modelled as a linear function of the host number or density. Hajek *et al.* (1993) identified a threshold host density under which the pathogen cannot disperse in the host population (Fig 5A-C).

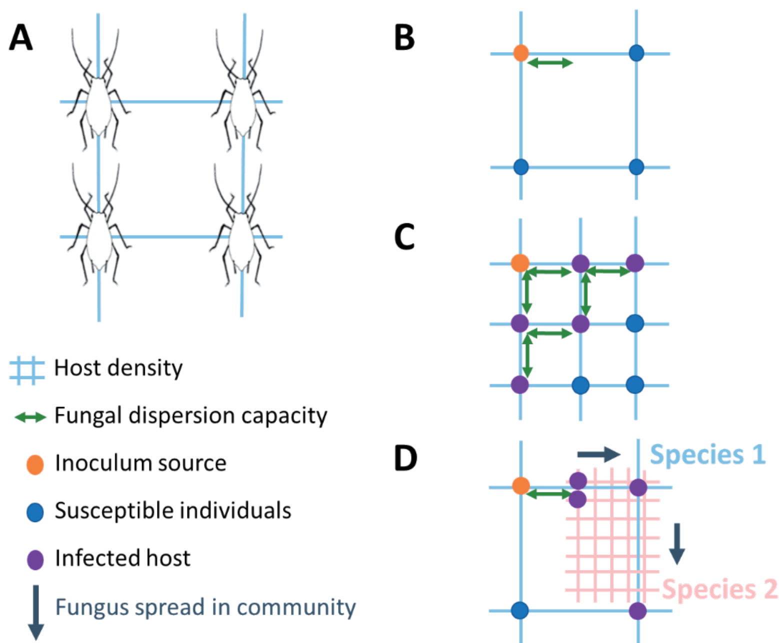

Figure 5: Fungus dispersion capacity and host population density. **A)** Let us consider a uniform distribution of host population with the same distance separating two individuals. In the following, hosts are represented by points. **B)** If the distance between two hosts is superior to the fungus dispersion capacity, it cannot disperse in the host population. **C)** If the distance between two hosts is inferior or equal to the fungus dispersion capacity, it can spread in the host population. **D)** In the configuration of two susceptible host populations, both population densities combine and may enable fungal dispersion in the whole community.

However, the host density threshold seems to be specific to each host-pathogen system (Shapiro-Ilan *et al.*, 2012). Further, there is still a debate among disease biologists about how to model disease transmission either as a linear or non-linear function, which probably depends on the system studied (McCallum *et al.*, 2017; McCallum *et al.*, 2001).

Ardisson *et al.* (1997) investigated this matter for *P. neoaphidis* infecting *S. avenae*. Unfortunately, they could not conclude on whether a linear or non-linear function was the most adapted to model the disease transmission.

Finally, we have seen earlier that cereal growth greatly influenced aphid populations (see Section 2.1.), which influence disease expression and transmission. Moreover, the plant host may directly or indirectly modify interactions between aphids and entomopathogenic fungi by modifying micro-climate or nutritional quality of aphids for fungi, for example (Cory and Ericsson, 2009). To my knowledge, no tri-trophic model has been built on the cereal - aphid - Entomophthoromycotinan fungus system. Tri-trophic models would enable us to estimate the importance of the crop development on the epizootic probability and investigate the key factors enabling epizootics in field conditions.

3.2. The case of several pest and pathogen species

In the case of aphids in cereals, *S. avenae* and *R. padi* interact with two fungal species, *P. neoaphidis* and *E. planchoniana*. This increasing number of players changes the dynamics of the system and the disease triangle becomes a disease square pyramid (Fig. 3B). Two processes emerge: first, fungi can compete for host resources, which we call intraguild competition. For example, both fungal species could co-infect the same populations and even sporulate from the same host individual (Saussure S. pers. obs.). However, different fungi have slightly different niches, for instance *E. planchoniana* prefers dry and moderately humid habitats (Barta and Cagáň, 2006; Keller, 1987) and *Zoophthora* sp. prefers woody habitats (Barta, 2009). No information on specific niche characteristic of *P. neoaphidis* is present in the literature, to my knowledge. Nevertheless, synergy between two fungal species could also occur and result in an increased biological control. On the other hand, cross-infection of one fungus between two host species is possible (Ben Fekih *et al.*, 2019; Shah *et al.*, 2004). Consequently, the disease transmission dynamic is changed by the combined effect of different host densities. For instance, *R. padi* density may be under the host density threshold prohibiting fungal dispersion (Fig. 5B). However, if *S. avenae* density is high enough and enables the fungus to spread, *R. padi* can still get infected and take part in a potential epizootic wave (Fig. 5D). Holt (1977) called this process "apparent competition". It can be an essential concept for biological control. However, different aphid species express different susceptibility to fungi (e.g. Shah *et al.*,

2004). For example, Ben Fekih *et al.* (2019) showed that *P. neoaphidis* collected from *S. avenae* is more virulent to its conspecific host (Box 1) (*S. avenae*) than to its heterospecific host (Box 1) (*R. padi*). However, no isolates from *R. padi* were tested to identify if *R. padi* is generally less susceptible than *S. avenae* or if *P. neoaphidis* is more virulent to its conspecific host. The possible asymmetry in disease transmission inside different host populations may result in different dynamics, especially under different temperatures. Conducting further studies on this matter would enable us to consider biological control as one process in cereals through the whole growing season.

4. Objectives

As opposed to most epidemiological research, for biological control of aphids in cereals we do not want to prevent diseases spreading in the host community. On the contrary, we aim at facilitating disease transmission in the host community and target pest populations. Therefore, we need to understand abiotic and biotic factors influencing the biology, ecology, and spatial distribution of cereal aphids and Entomophthoromycotina. Section 3 underline the impact of the three pillars shaping disease expression and transmission: host population, pathogen population and the environment. In this thesis, I focused on three IPM principles: (1) prevention and suppression of pests, (2) monitoring pest pressure and natural enemy prevalence, (3) estimation of biological control realised and potentially providing a basis for a Decision Support System.

The following research questions were the basis of this thesis:

1. Which factors drive cereal aphid resistance and susceptibility to fungi from the sub-phylum Entomophthoromycotina?
2. Do Entomophthoromycotina overwinter with their host *R. padi* on *P. padus*?
    - If so, which fungal species are present, at which prevalence and what are their overwintering strategies?
    - Can we identify a new reservoir for Entomophthoromycotina?
3. Is cross-infection of *P. neoaphidis* possible between two cereal aphid pests, *S. avenae* and *R. padi*?
    - Is the cross-infection asymmetrical between the two aphid species leading to one species being more susceptible to *P. neoaphidis* than the other?
4. How does the interaction between fungal isolates, host aphid species and temperature influence the fungus virulence and sub-lethal effect on host fecundity?
5. In a tri-trophic model between winter wheat, *S. avenae* and *P. neoaphidis*, which parameters are the most important for the pest and natural enemy population dynamics and for the potential biological control?
    - Do the most important parameters vary with the trophic level studied *i.e.* aphid and fungus population dynamics and biological control?

There have been many recent studies focusing on resistance and susceptibility of aphids infected with Entomophthoromycotina. The objective of **Paper I** was to answer Question 1 by reviewing biotic and abiotic factors influencing resistance and susceptibility of the two cereal aphid species, *S. avenae* and *R. padi*. Both host species are infected by many fungi from Entomophthoromycotina such as *P. neoaphidis* and *E. planchoniana* (Barta and Cagáň, 2006; Pell *et al.*, 2001). This two-host-two-pathogen system, depicted in Fig. 3B, is an excellent model to study host behaviour and environmental conditions favouring or hindering disease transmission among host communities.

Question 2 is linked to a critical step in fungal disease transmission: the primary infections initiated by fungal overwintering structures leading to the beginning of fungal spread in their host community. Winter is a critical season for Entomophthoromycotina since their hosts are usually sparse and inactive during this period of year. Therefore, pathogens have developed several overwintering strategies (Hajek *et al.*, 2018; Eilenberg *et al.*, 2013; Nielsen *et al.*, 2003; Keller, 1987). However, it has proved complicated to find overwintering sites and forms of these fungi and only few have been discovered as yet (Hajek *et al.*, 2018). For instance, *P. neoaphidis* resting spores have only recently been discovered (Scorsetti *et al.*, 2012). Consequently, the focus of **Paper II** was to identify overwintering sites and potential reservoirs of Entomophthoromycotina. Identifying such reservoirs may improve the understanding of fungus population dynamics and facilitate better estimations of their importance for biological control.

Questions 3 and 4 focused on cross-infection potential during the season by considering the three pillars of the disease triangle (Fig. 3A). The influence of the variability in the host community is investigated with two aphid species co-occurring in cereals field. The variability in the pathogen population is studied with three isolates of *P. neoaphidis* collected from one population on the same date. Finally, the influence of the environment on the host-pathogen interaction is considered with three temperatures characterising different periods of the growing season of cereals. The focus of **Paper III** was to understand these abiotic and biotic factors influencing the disease expression under realistic conditions to shed light on the mechanisms occurring in the field. This knowledge could be used in the building of host-pathogen models to understand the system dynamics over a whole season.

Question 5 focused on the modelling of host-pathogen dynamics over a whole growing season to grasp the complexity of the interactions between host, pathogen and environment. Therefore, in **Paper IV**, we developed a mechanistic tri-trophic model to simulate the daily interactions between *Triticum aestivum* (winter wheat), *S. avenae* (English grain aphid), *P. neoaphidis* (natural enemy) under prevalent weather conditions. The model gave four outputs: the aphid and fungus densities over the growing season, and the biological control realised by the fungus measured by the decrease in the pest population, and the decrease in yield loss due to the presence of the fungus. We aimed at identifying key factors enabling or prohibiting an epizootic and efficient biological control of the aphid among twelve parameters describing various aspects of weather conditions, crop development, aphid development and aphid-fungus interactions.

5. Results and Discussion

5.1. Factors driving susceptibility and resistance in cereal aphids

In **Paper I**, the heterogeneity of the host community and the factors influencing the susceptibility and resistance of aphids are reviewed with the two hosts *S. avenae* and *R. padi* and two fungal pathogens *P. neoaphidis* and *E. planchoniana* as model species. Risk avoidance was identified as the first line of defence. Aphid behaviour, such as their niches on cereal plants, may help them escape fungal diseases. For example, *R. padi* feeds on the lower part of cereals close to the soil surface, while *S. avenae* feeds on the upper part. Consequently, *R. padi* may be protected by cereal leaves from sporulating cadavers showering conidia from the upper part of the plant, while *S. avenae* may be more exposed on cereal heads *e.g.* to airborne conidia. The second line of defence is the host cuticle as a barrier to conidia germination and penetration. Some of its chemical compounds can inhibit conidial germination and its physical hardness can limit penetration. Once inside the host, immune response is triggered; however, *S. avenae* does not seem to have many genes involved in the response to fungal infection. In addition, aphids may harbour protective facultative symbiotic bacteria. In *Acyrthosiphon pisum*, five species were identified as conferring resistance to fungal infection of *P. neoaphidis* or decreasing the fungal sporulation capacity when the host is killed. More studies are needed on both *S. avenae* and *R. padi* to determine if such endosymbiotic bacteria are pertinent protectors of cereal aphids. Further, conspecific versus heterospecific hosts may be among the predominant factors influencing disease expression. However, our review found that there may be no host-driven divergence of fungal genotypes for some species, like *E. planchoniana*. For this fungus, genotypes collected from different aphid host species are not expected to vary greatly in virulence. Finally, our review found that both *P. neoaphidis* and *E. planchoniana* infect cereal aphids during their entire life cycle whether they feed on their winter or summer host plant. Moreover, the higher susceptibility of winged compared to apterous aphids to Entomophthoromycotina may enable the fungi to follow their host's spatial distribution.

5.2. Entomophthoromycotina overwinter with *Rhopalosiphum padi*

In **Paper II**, we investigated the possibility of Entomophthoromycotina overwintering with *R. padi* on *P. padus*. A total of 10 branches of 17 tree locations have been monitored over three years and 879 fungus-killed cadavers were analysed. These cadavers were filled with fungal overwintering structures of two fungal species. First, an unidentified species of *Zoophthora* was dominant and overwintered as resting spores. Further, *E. planchoniana* overwintered as modified hyphal bodies. The latter species is a common pathogen of cereal aphids (*e.g.* Barta and Cagáň, 2006) and has been observed overwintering as modified hyphal bodies by Keller (1987) on the sapling sycamore aphid *Depranisiphum acerinum*. However, this is the first report of *E. planchoniana* overwintering as modified hyphal bodies in *R. padi* cadavers on *P. padus.* The discovery of *Zoophthora* sp. being the dominant species (87% of our samples) was unexpected. Indeed, in Europe no fungus in the genus *Zoophthora* has been recorded on aphids feeding on cereals. On only a few occasions, have some species of *Zoophthora* been recorded infecting *R. padi* feeding on *P. padus* (Barta and Cagáň, 2006; Nielsen *et al.*, 2001). Consequently, *Zoophthora* has not been considered as a potential biological control agent in the latter studies. However, it seems that in our study *Zoophthora* participated significantly to reduce overwintering populations of *R. padi*. Interestingly, *Zoophthora* is a genus that Barta (2009) reported as preferring a woody habitat. Therefore, we hypothesised that *R. padi* encountered *Zoophthora* sp. while migrating to or feeding on *P. padus*. This could be due to cross-infection and apparent competition between *R. padi* and other aphid species feeding on different trees in the vicinity of *P. padus.* Therefore, even though not prevalent in aphid populations feeding on cereals, *Zoophthora* could still be an interesting biocontrol agent. This possibility needs to be studied in more detail.

We also found 3,599 overwintering eggs of *R. padi* on branches of *P. padus*. Both, eggs and cadavers, were found at sheltered locations on branches *i.e.* in bud axils. Keller (1987) also found cadavers of *D. acerinum* containing fungal overwintering structures located on the same microlocation as overwintering eggs. Thus, newly hatched nymphs have a high chance of being exposed to the fungi. However, a significant negative correlation was found between number of eggs and cadavers per branch (Fig. 6). This might be explained by the fact that 70.6% of the sample we observed were killed by Entomophthoromycota

during the nymphal stage, which might lead to a decrease of aphid reproduction and egg-laying.

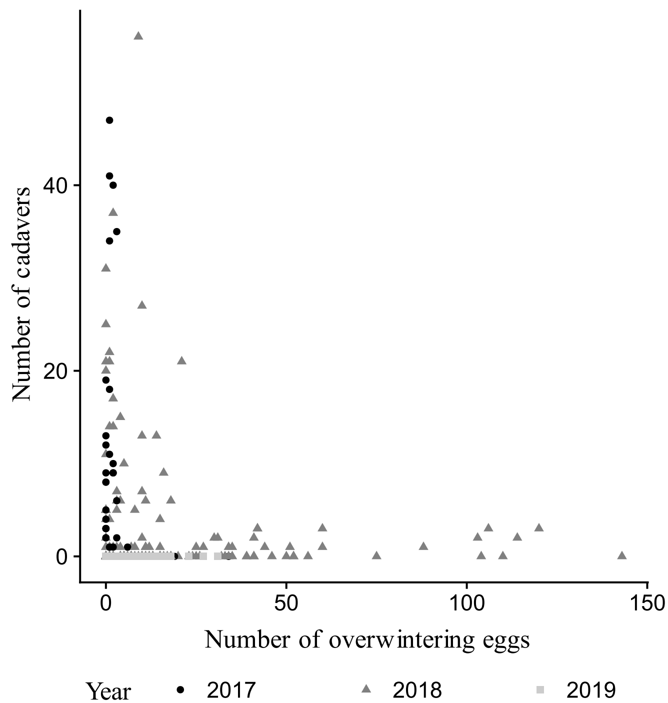

Figure 6: Correlation between numbers of *Rhopalosiphum padi* eggs and overwintering fungus-killed *R. padi* cadavers per bird cherry (*Prunus Padus*) branch in 2017, 2018 and 2019. Ten branches were collected and examined from 17 *P. padus* tree locations. Both live and dead eggs are included.

The negative correlation between eggs and cadavers may result in a low risk for *R. padi* to encounter Entomophthoromycota in spring on *P. padus*. Further, *R. padi* spring generations induce galls on unfurling leaves that they feed on (Leather and Dixon, 1981). They could, therefore, be efficiently protected from airborne conidia or sporulating cadavers. Nevertheless, the dominant fungal species overwintering on *P. padus* with *R. padi* was *Zoophthora* sp. and it overwintered as long-lived and dormant resting spores. In spite of the negative correlation between *R. padi* eggs and cadavers containing overwintering fungus structures, *P. padus* may act as a fungal reservoir.

A significant annual variability was found in fungal overwintering populations on *P. padus*, which could be explained by variability in the host population susceptibility, or in the fungus population virulence (see above). Further, as we found two species of

Entomophthoromycotina (*Zoophthora* sp. and *E. planchoniana*) overwintering in *P. padus*, intraguild competition may occur and result in annual variability among the overwintering pathogen populations. Finally, climatic conditions during the previous season, may favour or hinder the disease transmission and lead to annual variability (Finlay and Luck, 2011; Steinkraus, 2006). However, the annual variation in overwintering populations associated with resting spore longevity may influence primary infections the following spring, and thus disease transmission. Disease transmission will only be possible when both *R. padi* eggs and overwintering fungus-killed cadavers are present on the same tree. Over several years, if *R. padi* eggs and fungi overwinter at the same location, but during different winters, remaining dormant fungi may still be able to initiate primary infections the following spring. This seems to be the case, as we found a maximum of 222 cadavers on 10 branches of one tree location to contain fungal overwintering structures. Considering that our study covered a small proportion of the total habitat, a high overall prevalence of overwintering structures can be assumed. These findings highlight the need to investigate the long-term role of *P. padus* as a reservoir of Entomophthoromycotina and its potential use for biological control.

5.3. The disease triangle effect on disease expression of *Pandora neoaphidis*

5.3.1. Variability within the host community

In the laboratory study presented in **Paper III**, we showed that *R. padi* was much less susceptible to *P. neoaphidis* isolated from a *S. avenae* population than was *S. avenae*. A total of 38% of *S. avenae* died after infection with the fungus compared to only 7% of *R. padi*. Furthermore, *P. neoaphidis* killed *S. avenae* 30% faster than *R. padi.* Ben Fekih *et al.* (2019) also found that *S. avenae* was more susceptible to *P. neoaphidis* and *E. planchoniana* than *R. padi.* In that study, aphids were allowed to position themselves on a host plant depending on their niche preferences (*R. padi* at the bottom and *S. avenae* in the upper part of the plant). Conversely, in **Paper III** both aphids were contained in one Petri dish without a plant host during the conidia shower. Therefore, it seems that the higher resistance of *R. padi* to Entomophthoromycotina may have a behavioural and an immunological component. Shah *et al.* (2004) also showed that *R. padi* was among the least susceptible aphid species to 20 isolates of *P. neoaphidis*. However, in no studies were the tested Entomophthoromycotina isolates collected from *R. padi*. There is consequently,

a confounding effect between host species susceptibility and conspecific versus heterospecific effect. This matter requires further studies in order to untangle the two possibilities. For instance, isolates of Entomophthoromycotina collected from *R. padi* should be tested against *R. padi* and *S. avenae*.

5.3.2. Variability within the fungus population

We showed in **Paper III** that the variability in virulence between fungal isolates, measured by aphid mortality, depended on the host species. Indeed, the three tested isolates expressed similar virulence to *R. padi* but different virulence to *S. avenae*. Barta and Cagáň (2009) previously showed differences in virulence between isolates of *P. neoaphidis*. Furthermore, the lethal time (*i.e.* time needed for the fungus to kill its host) varied between the three isolates of *P. neoaphidis* for *S. avenae* and *R. padi*. However, the differences in lethal time between isolates did not depend on the host species. Even so, the magnitude of differences between isolates was twice as great when infecting *R. padi* compared to *S. avenae*. Finally, the sub-lethal effect of infection on host fecundity varied with fungal isolates, aphid species and infection output. Isolates from one *P. neoaphidis* metapopulation have been shown to express different germination rates, sporulation capacities (*in vitro* culture), conidial sizes and fungal biomass production (in liquid media, $g.l^{-1}$) (Barta and Cagáň, 2009; Sierotzki *et al.*, 2000), which could explain the differences observed and reported in **Paper III**. This may be crucial for disease transmission. Chen and Feng (2006) infected the peach-potato aphid *Myzus persicae* with *P. neoaphidis.* They showed that infected winged *M. persicae* could disperse, establish on cereal plants, and initiate colonies. In those colonies, they observed secondary infections in 80% of cases within two weeks of establishment. However, a high variability in the disease transmission was found and depended on the lifespan of the infected adults once established on a new plant. Therefore, the heterogeneity within one fungal population may lead to different probabilities for the fungus to spread into its host populations. This may be critical at the beginning of the season when aphid distribution within the field is random and hinders disease transmission.

5.3.3. Variability of environmental conditions

In **Paper III**, three temperatures relevant for the Norwegian climate (7.5, 14.0 and 18.0 ˚C) have been used to investigate the influence of temperature on host-pathogen interactions. Aphid mortality for infections with *P. neoaphidis* increased with increasing temperatures. This was consistent with the literature showing that the optimal temperature for vegetative growth, lethal time and host mortality of European *P. neoaphidis* ranges around 15-25 ˚C (Barta and Cagáň, 2006; Stacey *et al.*, 2003; Schmitz *et al.*, 1993). However, we did not find that temperature differently influenced the three isolates tested. Morales-Vidal *et al.* (2013) and Filotas *et al.* (2006) found that temperature differently influenced fungal isolates of *Z. radicans* and *Furia gastropachae* infecting the diamondback moth (*Plutella xylostella*) and the forest tent caterpillar moth (*Malacosoma disstria*) respectively. However, the isolates used in both studies were collected from different geographical locations. Therefore, we hypothesised that temperature did not affect our *P. neoaphidis* isolates differently since they were collected from the same geographical origin.

Secondly, we did not find any temperature effect on the lethal time of *P. neoaphidis* expressed in degree-days (DD) when pooling the three tested fungal isolates. This is in contrast to studies showing a longer lethal time at lower temperature (*e.g.* Nielsen *et al.*, 2001). However, in these studies lethal time was expressed in days. Investigating temperature effect on a process expressed in DD enable us to differentiate between the temperature influence on ectotherm species (the higher the temperature, the faster the biological process) and the temperature influence on the process itself. For instance, if a fungus needs 40 DD to kill *S. avenae*, it will take 2 days at 20 ºC and 4 days at 10 ºC. If the fungus requires 60 DD at 20 ºC and 40 DD at 10 ºC, we proved a temperature effect on the host-pathogen interaction itself. We argue that the significant temperature effect on lethal time found in the literature (see above) does not enable us to identify a temperature effect on the lethal time itself. When temperature effect was studied on each fungal isolate separately, we found a significant influence of temperature on lethal time. Thus, different *P. neoaphidis* isolates kill *S. avenae* faster under different temperatures (Fig. 7). As shown by Bonsall (2004), such differences in lethal time may have dramatic consequences for the epizootic development of the fungus in a host population. These results suggest that temperature modifies the disease transmission of different isolates in different ways.

Therefore, we can speculate that different isolates will be selected in different seasons and a possible shift in fungal population may occur during the whole season.

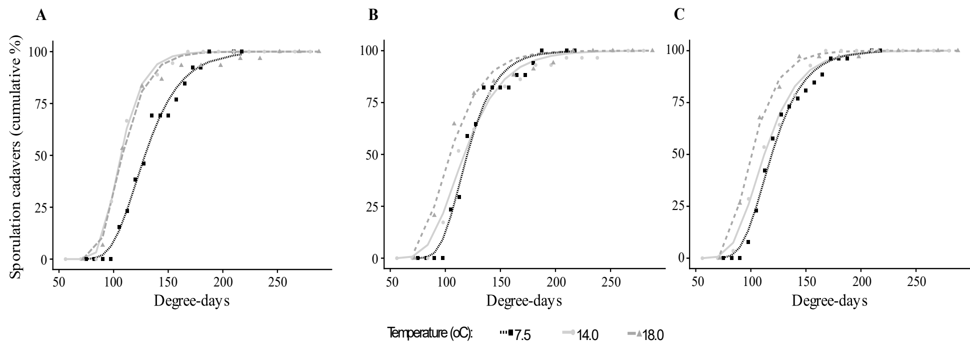

Figure 7: Effect of temperature per degree-day on cumulative sporulation percentage of three *Pandora neoaphidis* isolates (A) NCRI 459/15, (B) NCRI 460/15, and (C) NCRI 461/15 from fungus killed *Sitobion avenae*. The lines represent the fitted models and the points represent the observed values.

We also found that the sub-lethal effect of *P. neoaphidis* on the fecundity of *S. avenae* depended on the temperature. The highest decrease in fecundity of inoculated *S. avenae* that survived the fungus occurred at different temperatures depending on the fungal isolate used in infection. To our knowledge, no study investigated the interaction between different Entomophthoromycotina isolates and temperature. However, several studies showed that different genotypes of *A. pisum* expressed different susceptibility to a given *P. neoaphidis* isolate depending on temperature (Baverstock *et al.*, 2006; Stacey *et al.*, 2003; Blandford *et al.*, 2003). The influence of temperature on the disease expression may greatly impact the probability for Entomophthoromycotina to disperse and create an epizootic.

5.4. Modelling the population dynamics

In **Paper IV**, we built a mechanistic tri-trophic model simulating the population dynamic of *S. avenae* feeding on winter wheat in the presence of *P. neoaphidis*. We investigated twelve important parameters of the fungus' ecology and climatic conditions to identify the most important ones for aphid and fungus population dynamics and biological control. In parallel, we fixed the number of aphids migrating to winter wheat to enable a high host population. This enable us to overcome any potential host threshold density (Fig. 5B and C) and allow the fungus to initiate epizootics if the conditions are optimal. We identified

three parameters as crucial for the whole system dynamic: the fungus transmission efficiency, the humidity threshold that triggers fungal sporulation, and the weather (temperature and humidity). These parameters are linked to each other through the influence of environmental humidity on the fungus. Transmission efficiency has been recognised as a key process in host-pathogen interactions (McCallum *et al.*, 2017; Steinkraus, 2006; McCallum *et al.*, 2001). An active debate exists on how to model it correctly (e.g. McCallum *et al.*, 2001). The difficulty in finding a consensus might be due to (1) specificity of the studied host-pathogen system (e.g. Elder *et al.*, 2008; Reeson *et al.*, 2000), and (2) the composite nature of the parameter (McCallum *et al.*, 2017). Transmission efficiency combines the probability of a host to come in contact with a pathogen and the probability of this contact to initiate an infection (McCallum *et al.*, 2017; Reeson *et al.*, 2000). It is therefore difficult to attribute its variation to environmental conditions or to biological characteristics. In **Paper IV**, transmission efficiency sums up fungus sporulation capacity and transmission within and between colonies (Baverstock, 2012; Steinkraus *et al.*, 2006; Ekesi *et al.*, 2005). Due to crucial influence of environmental humidity on fungal sporulation, germination, conidia and fungus-killed cadaver longevity (Filotas and Hajek, 2004; Xu and Feng, 2002; Furlong and Pell, 1997), we modelled the transmission efficiency at zero under sub-optimal conditions and at its maximal value when conditions are optimal. Conditions in our model, are suboptimal when humidity is below the humidity threshold. This threshold varies with fungus species and ranges between 80 and 100% among Entomophthoromycotan fungi (reviewed by Sawyer *et al.*, 1997). We showed that the higher the threshold, the lower the fungus population and the biological control it confers. However, we did not consider the microclimate at the leaf boundary layer, where fungi and aphids live. Many studies investigated the influence of environmental humidity on disease transmission with a rough index such as rainfall, relative humidity, leaf wetness, free water or soil moisture content (Furlong and Pell, 1997; Sawyer *et al.*, 1997; Wilding, 1969). We also used a rough measurement of environmental humidity for practical purposes and parsimony in model complexity (Fargues *et al.*, 2003). However, all these factors capture the influence of the humidity on the fungal development. We encourage the collection of further experimental data and studies on how to model transmission efficiency for Entomophthoromycotan infections. This will enable us to directly integrate the influence of abiotic (*e.g.* humidity) and biotic (*e.g.* fungal virulence or host susceptibility) factors on the fungal transmission efficiency

and could help us to better understand this crucial process (McCallum *et al.*, 2017; Steinkraus, 2006).

The most important parameters for the fungus population dynamics were linked to the longevity of the infective units in the environment. We did not directly model conidia, but rather cadaver units based on their sporulation capacity (Hemmati *et al.*, 2001a). Fungus-killed cadaver longevity and the increase of energy consumption due to sporulation (decreasing cadaver longevity) are two crucial steps according to our mechanistic model. Fungus-killed cadavers can undergo several cycles of hydration and dehydration as shown in Sawyer *et al.* (1997) with an Entomophthoromycotan fungus infecting grasshoppers. As sporulation is energy demanding (conidiophores development and active projection of conidia), the fungus will age faster when sporulating. Brobyn *et al.* (1985) estimated conidia longevity on crop leaves as up to 14 days, and laboratory studies of *P. neoaphidis* sporulating cadaver estimate that cadavers can sporulate continuously for 2-3 days at 20 °C (Bonner *et al.*, 2003; Ardisson *et al.*, 1997). However, to our knowledge no studies estimated for how long cereal aphid cadavers can sporulate and survive under field conditions. However, Thomas *et al.* (1995) showed that disease transmission among grasshopper population varies through time, partially depending on the sporulation pattern. Conducting experiments similar to those of Sawyer *et al.* (1997) on the species studied in our system could be useful to estimate longevity of cadavers depending on weather conditions, saprophytic or dislodging of cadavers.

Interestingly, the most important parameter for biological control expressed as yield loss was the proportion of infected aphids colonising winter wheat (**Paper IV**). Therefore, it would be interesting to manipulate the environment to increase this proportion at the beginning of the field colonisation and not only during the building-up of aphid populations during summer.

6. Conclusion and perspectives for biological control

Entomophthoromycotina are interesting potential biological control agents. They are specialists on aphids and do not harm biodiversity. They horizontally infect aphids by contact and do not require to be ingested. They are ubiquitous and infect many aphid species, which they follow during their entire lifecycle. Consequently, many sources of inoculum and reservoirs can be located in different habitats such as soil (**Paper I**) or trees (**Paper II**). The results presented in **Paper IV** suggests that they may help in improving cereal yield and significantly decrease host populations under certain circumstances. Thus, manipulating inoculum sources of these fungi may be useful for biological control.

To sum up, there are several inoculum sources of Entomophthoromycotina infecting aphids in cereals. First, overwintering fungal structures of Entomophthoromycotina are expected to be located on the same site as overwintering eggs of *S. avenae* and *R. padi* on grasses and on the bird cherry tree, respectively (Fig. 8A). In spring, overwintering eggs hatch and some fungal overwintering structures germinate, leading to primary infection and the beginning of disease transmission in host populations (Fig 8B). In middle of spring, both *S. avenae* and *R. padi* migrate from their winter host to cereal fields. Infected winged individuals disperse with the fungi (Fig 8C). During summer, there are several ways for Entomophthoromycotinan fungi to disperse within a cereal field (Fig. 8D). First, infected winged individuals may enable the transmission of Entomophthoromycotina. Second, airborne conidia clouds could passively disperse above cereal fields in summer. These clouds could come from (1) overwintering structures sporulating in the surrounding of the field (even though no literature on the subject was found), from (2) infections occurring in flower strips close to the field, or (3) from infections occurring within the pest populations in the field. Third, the soil is a potential reservoir for Entomophthoromycotina like *P. neoaphidis*. As aphids drop regularly from cereals, they can get in contact with these fungi. Finally, because Entomophthoromycotina infecting aphids are only pathogenic to aphids, other natural enemies may act as passive vectors and disperse fungal conidia from one infected colony to a susceptible one. The importance of each route of transmission probably depends on year, community context and environmental conditions. Quantifying the contribution of each transmission route constitutes a major challenge. It is however, interesting in the context of conservation biological control to investigate more on this matter.

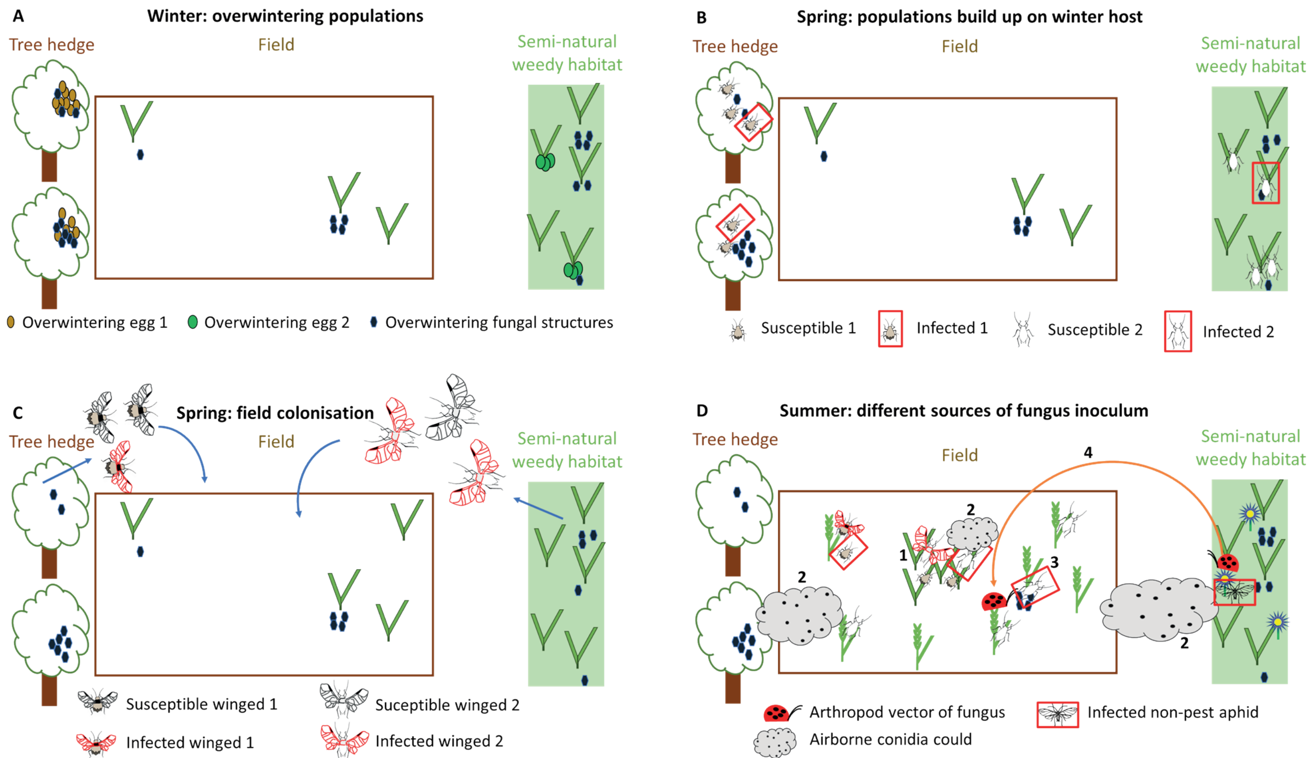

Figure 8: Different sources of Entomophthoromycotina inoculum in pest aphid populations in cereal. **A)** In winter, overwintering fungi and aphids are on the aphid winter host. Species 1 is Rhopalosiphum padi on Prunus padus and species 2 is Sitobion avenea on grasses. **B)** Beginning in spring, new generations of aphids develop and get in contact with germinating overwintering fungal structures. Primary infection occurs and disease transmission starts. **C)** In the middle of spring, aphids leave their winter host to colonise cereal fields. Infected winged individuals disperse with the fungus. **D)** During summer, (1) cross-infection occurs between aphid species. (2) Conidia clouds can passively disperse from sporulating cadavers within or outside the field or directly from germinating overwintering fungal structures. (3) Aphids dropping to the ground get in contact with overwintering fungal structures in the soil and get infected. Finally, (4) because aphid-killing Entomophthoraceae are not pathogenic to other natural enemies, the latter can vector them from colony to colony.

In order to facilitate the disease transmission and epizootics for the purpose of conservation biological control, farmers need to manipulate the environment. This may be easier in greenhouses for example where abiotic factors (temperature, humidity, light, *etc.*) are controlled. Under field conditions, these factors usually cannot be manipulated but other suggestions may be possible. This is the major drawback of Entomophthoromycotina. The results in **Paper IV** showed that the influence of weather on these fungi seems crucial and affects the probability of an efficient biological control. Therefore, it must be acknowledged and accepted that some years, these fungi will not be efficient to control cereal aphids. This is especially true, since the mass production of conidia of Entomophthoromycotina is not yet possible. Consequently, inundation biological control (the massive release of fungi in the environment to control pest population) cannot yet be performed with this group of fungi.

In our model (**Paper IV**), we showed that the proportion of infected aphids colonising winter wheat is the most influential parameter for the biological control effect on the potential yield loss. Therefore, it would be interesting to manipulate the environment to increase this proportion. As explained in Section 3, cross-infection of a fungal disease between two host species in a community is theoretically possible. However, an asymmetrical cross-infection of *E. planchoniana* and *P. neoaphidis* on *S. avenae* and *R. padi* can occur (**Paper I**, **III**). More specifically, the difference in lethal time between the two host species (**Paper III**) may have dramatic consequences for the fungus population dynamics and the disease transmission within the aphid community. It would be interesting for further studies, to investigate the difference in lethal time for a larger number of aphid species within a cereal field. Additional species that could be investigated are the pest *Metopolophium dirhodum*, or aphid species found in semi-natural habitats such as *Aphis fabae* or *Myzus persicae*. This would enable the identification of transmission routes with low resistance to fungal spread and the optimization of biological control. For instance, if *P. neoaphidis* kills *A. fabae* faster than *M. persicae*, it could be beneficial to seed host plants of *A. fabae* in flower strips. This could promote an epizootic in pest populations by increasing the likelihood of a high amount of airborne conidia above the cereal field (Fig 8D). The selection of semi-natural habitats harbouring highly susceptible alternative hosts could enable an increase of fungal inoculum in the field (Fig. 8).

Paper II demonstrated the potential role of the bird cherry tree *P. padus* as a fungal reservoir (Fig. 8A). However, we also identified a significant annual and geographical heterogeneity of the fungal overwintering populations on *P. padus*. This spatio-temporal variability may lead to different fungal inoculum levels in the surrounding aphid community. Therefore, further studies are needed to link these overwintering structures to primary infection cycles in spring and an increase of biological control. Further *P. padus* is also the winter host plant of *R. padi* and, therefore, its role may be versatile depending on the year. However, alternative aphid species overwinter on different shrubs and trees. It would be interesting to investigate the potential reservoir roles of their winter host plants, and if possible, manipulate the landscape around cereal fields to increase the presence of such potential reservoirs.

In conclusion, this thesis work provided several novel and important findings on diverse aspects of the disease triangle on disease expression and transmission that could be useful against cereal aphids in Norway. Nevertheless, conclusions and ecological concepts used in this thesis are highly relevant for other host-pathogen systems.

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Papers I-IV

Paper I

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Factors driving susceptibility and resistance in aphids that share specialist fungal pathogens

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Pandora neoaphidis and *Entomophthora planchoniana* are widespread and important specialist fungal pathogens of aphids in cereals (*Sitobion avenae* and *Rhopalosiphum padi*). The two aphid species share these pathogens and we compare factors influencing susceptibility and resistance. Among factors that may influence susceptibility and resistance are aphid behavior, conspecific versus heterospecific host, aphid morph, life cycle, and presence of protective endosymbionts. It seems that the conspecific host is more susceptible (less resistant) than the heterospecific host, and alates are more susceptible than apterae. We conceptualize the findings in a diagram showing possible transmission in field situations and we pinpoint where there are knowledge gaps.

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Introduction

Species in the fungal subphylum Entomophthoromycotina (Zoopagomycota) play an important role in regulating host populations [1,2]. To understand the epizootic development of these fungi and their transmission within host species and between host species, we need to understand the abiotic and biotic factors affecting host susceptibility and resistance. Susceptibility to a disease can in general be defined as '*lack of ability to resist some extraneous agent (such as a pathogen or drug)*' [3] and resistance to a disease can in general be defined as '*the inherent ability of an organism to resist harmful influences (such as disease, toxic agents, or infection)*' [4]. Thereby, the two terms are somehow linked; a high susceptibility means a low resistance.

In this review, we focus on a host–pathogen system in aphids in cereals that includes two aphid pest species (the grain aphid etc. *Sitobion avenae* and the bird cherry etc. oat aphid *Rhopalosiphum padi*). Two specialist fungal pathogens are commonly reported on these two aphid species, namely *Pandora neoaphidis* (Syn. *Erynia neoaphidis*) and *Entomophthora planchoniana* (Tables 1 and 2). Since *P. neoaphidis* and *E. planchoniana* are shared between these two aphid species, this provides an excellent model system to understand which aphid species or morphs are most susceptible or resistant and which behaviors and environmental conditions favor or disfavor infections among these aphids. Information from other aphid-fungal systems is included when appropriate. Both fungal species have a global distribution and are found on many aphid species (Table 1). Indeed, several other entomophthoralean fungi occur on aphids in cereals but our review will focus on *P. neoaphidis* and *E. planchoniana*.

Epizootiological principles and life cycles of fungal pathogens in aphids in cereals

Epizootiology of insect diseases is the science of causes and forms of the mass phenomena of disease at all levels of intensity in a host population [5]. The primary factors that are involved in the cause, initiation, and development of epizootics of infectious diseases in insects are the pathogen population with its variable virulence and efficient means of transmission and the susceptibility of the host population to the pathogen [6]. We use the terms 'pathogenicity' and 'virulence' as defined by Refs. [7,8••]: pathogenicity is a qualitative character describing if a microorganism causes disease, while virulence is a quantitative expression of the power of a pathogen toward a specific host, for example, an aphid.

Host–pathogen interaction can be depicted as a struggle between competing species that develop adaptations and counter-adaptations against each other, resembling an arms race [7,9]. Some pathogens are able to colonize new hosts for example, if these hosts represent a resource similar to the original host and/or if host populations are genetically diverse allowing parts of the new host population to be susceptible at a given point in time [10••].

Table 1

Observational field studies on occurrence of fungal pathogens in a host–pathogen system consisting of aphids in cereals (*Sitobion avenae* and *Rhopalosiphum padi*) and entomopathogenic fungi (*Pandora neoaphidis* and *Entomophthora planchoniana*)

Observational field study	Aphid species studied	Fungus species found	Results	References
Occurrence in infected aphids on cereal crops	*S. avenae* and *R. padi*	*P. neoaphidis* and *E. planchoniana*	*P. neoaphidis* or *E. planchoniana* have a wide distribution and can develop epizootics	[54–60]
Occurrence in infected aphids in winter wheat fields with weeds	*S. avenae*	*P. neoaphidis* and *E. planchoniana*	Higher fungal prevalence in weedy plots than in herbicide treated plots	[61]
Occurrence in infected aphids on overwintering site, *Prunus padus*	*R. padi*	*P. neoaphidis* and *E. planchoniana*	Infections in *R. padi* in autumn much more common than in spring	[27]
Occurrence in soil in spring	*S. avenae*	*P. neoaphidis*	Inoculum present in soil samples, infective to *S. avenae* crawling on soil	[28]
Occurrence in infected aphids trapped from air	*S. avenae* and *R. padi*	*P. neoaphidis* and *E. planchoniana*	*P. neoaphidis* more common than *E. planchoniana*	[62]

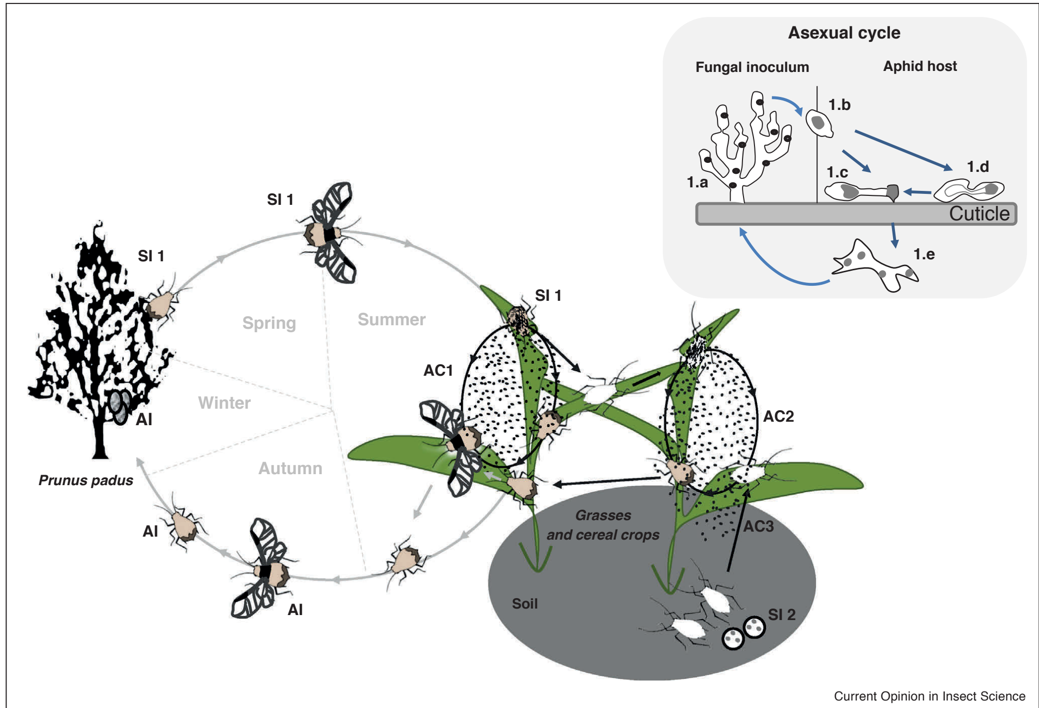
This might, in particular for obligate pathogens, result in a host driven divergence of genotypes, although this divergence may be specific to the host pathogen systems. In the case of the genus *Entomophthora*, a study [11] documented that the aphid pathogen *E. planchoniana* was much less prone to such divergence than the dipteran pathogen *Entomophthora muscae*. In other words, *E. planchoniana* from different host aphid species are rather similar with respect to genotype and we hypothesize that in a cereal field, *P. neoaphidis* and *E. planchoniana* are pathogenic to both aphid species (Table 1) and that transmission between hosts in cereals may take place (Table 2). The question is, whether one aphid species or one morph is in general more susceptible than the

Table 2

Experimental laboratory studies on factors influencing susceptibility in a host–pathogen system consisting of aphids in cereals (*Sitobion avenae* and *Rhopalosiphum padi*) and entomopathogenic fungi (*Pandora neoaphidis* and *Entomophthora planchoniana*)

Experimental laboratory study	Aphid species studied	Fungus species tested	Results	References
Aphid species	*S. avenae* *R. padi*	*P. neoaphidis*, several isolates	*S. avenae* more susceptible than *R. padi* but none of the isolates tested where conspecific with *R. padi*	[63]
Conspecific versus heterospecific hosts	*S. avenae* (conspecific) and *R. padi* (heterospecific)	*P. neoaphidis* and *E. planchoniana*	Conspecific host *S. avenae* more susceptible than heterospecific host *R. padi*. Fecundity was negatively affected in inoculated (but surviving) *S. avenae*	[37•,53]
Two heterospecific hosts	*R. padi* (heterospecific) and *Myzus persi-cae* (heterospecific)	*P. neoaphidis* (from *S. avenae*)	Heterospecific host *M. persicae* more susceptible than heterospecific host *R. padi*	[64]
Aphid morph	*S. avenae*	*P. neoaphidis*	Alate aphids more susceptible than apterous aphids	[40]
Aphid clone	*S. avenae*	*P. neoaphidis*	No effect of clones in susceptibility in laboratory experiments and in field data	[40,41]
Temperature	*S. avenae*	*P. neoaphidis*	Lethal time decreased with increasing incubation temperature, although not consistently. Increased temperature gave higher mortality but did not consistently affect lethal time or fecundity	[37•,65]
Fungal isolates	*S. avenae* *R. padi*	*P. neoaphidis* three isolates	No fungal isolate related difference in numbers of sporulating cadavers of *R. padi*. For *S. avenae,* a difference was found	[37•]

Figure 1



Life cycle of the insect pathogenic fungi from Entomophthorales (*Pandora neoaphidis* and *Entomophthora planchoniana*) in bird cherry-oat aphid *Rhopalosiphum padi* (with dark posterior) and grain aphid *Sitobion avenae* (without dark posterior). Dioecious holocyclic *R. padi* alternates between the winter host bird cherry (*Prunus padus*) and the summer hosts cereals and grasses.
Primary spring inoculum (**SI 1**) of *P. neoaphidis* or *E. planchoniana* will be brought via infected alates of *R. padi* upon migrating to the summer hosts. On the summer hosts, infected *R. padi* die and produce infective conidia, which are actively discharged. These conidia can infect alate or apterous *R. padi* and several asexual infection cycles (**AC1**) can occur during the cropping season. During autumn, *R padi* alates migrate to the winter host and infected individuals bring autumn inoculum (**AI**) to the winter host. If transmission of fungal disease from conspecific host *R. padi* to heterospecific host grain aphid *Sitobion avenae* or vice versa takes place in nature, such transmission may result in asexual infection cycles (**AC2**) in the heterospecific host and eventually back to conspecific host. Another source of spring inoculum (**SI 2**) is infective conidia produced by overwintering stages (most probably resting spores) in soil. This may in particular apply to *S. avenae*, which remains on grasses during autumn and winter and may become infected (**AC3**) during spring and initiate asexual infection cycles in the conspecific host *S. avenae.* We hypothesize that transmission to the heterospecific host *R. padi* can occur (**AC 3**), but probably with less success than transmission to the conspecific host (Table 2). Insert upper right shows infection process. Conidiophores (1.a) emerge through cuticle from killed host and primary conidia are forcibly discharged (1.b). Once the conidia land on the cuticle of a suitable aphid host, each will produce a germ tube that can penetrate the cuticle (1.c). Under unfavorable conditions, primary conidia may instead produce secondary conidia (1.d). Inside the aphid host, the fungus will develop as hyphal bodies (1.e), invade the host tissues, kill the host and finally produce conidiophores.

other, and whether the conspecific host (belonging to the same species as the inoculum source) is more susceptible than the heterospecific host (belonging to a different species than the inoculum source).

In northern Europe, the two main aphid species in cereals are the grain aphid *S. avenae* and the bird cherry-oat aphid *R. padi* from the family Aphididae (Hemiptera). They are serious pest insects in cereals and are two of the 14 aphid species considered the most important worldwide and share host plants in the Poaceae (grass) family, which includes crops like wheat [12]. In aphids, overwintering can be achieved by: diapausing eggs produced by mated sexual females in autumn, and/or persistent parthenogenetic viviparous females. When winters are cold, the first strategy is favored because sexual eggs are very cold-resistant [13]. *S. avenae* remains on grasses and overwinters as eggs or parthenogenetic viviparous females

on the stems, whereas *R. padi* migrates to its winter host bird cherry (*Prunus padus)* where it overwinters as eggs on the branches [12,14,15].

The anholocyclic life cycles of these two species of aphids during summer in cereal crops are similar but their niches differ. During the cropping season, *S. avenae* at first colonizes the underside of the leaves of cereal plants and later upper parts of the plant, for example the ears [16,17]. *Rhopalosiphum padi* shows another pattern: first, they position themselves on the cereal plant, but close to the soil surface. Later they colonize the more parts of the plant and position themselves mainly on the underside of the leaves [16,17]. Aphid populations in cereals are clonal during summer in Northern Europe, and some genotypes can be predominant throughout a growing season or year [18]. However, during only a week 20–60% of *S. avenae* colonies in a population may disappear and can be replaced by new colonies originating from airborne immigrants landing in the field [19]. Hence, the genetic structure of an *S. avenae* population may vary significantly throughout a growing season or between years and this may influence aphid resistance to fungal pathogens.

Pandora neoaphidis and *E. planchoniana* have been known as aphid pathogens since the 19th century [20]. They infect their aphid host by conidia (asexual spores) landing on and penetrating the aphid cuticle (Figure 1) initially developing inside the host as protoplasts/hyphal bodies [21]. Once the host is killed, the fungus breaks through the cuticle and produces primary conidia on conidiophores [22]. Primary conidia are actively discharged if conditions are favorable and initiate another infection cycle if they land on the integument of a suitable host, or they produce secondary conidia, which are also infective [23–25]. Thick walled resting spores [26] are produced in the dead host for winter survival. In autumn, infected alate *R. padi* migrate to the winter host, bird cherry, and may bring with them the pathogen to their overwintering site (Table 1), where conidia or resting spores are produced [27] (Figure 1). When *R. padi* eggs hatch in early spring, aphid nymphs may then become infected by overwintering fungal inoculum present on the bird cherry (S. Saussure., pers. obs.) and then probably transport it to the field via infected alates. For *S. avenae*, winter survival takes place in the cereal stubble or on grasses, and infected aphids will probably remain there and eventually drop to the soil surface. Inoculum of *P. neoaphidis* is therefore present on the soil surface in spring and can infect aphids exposed to soil and litter [28] (Figure 1 and Table 1). In Ref. [28], the authors provide a review on studies of winter survival of *P. neoaphidis*. Fungal inoculum may also be transported over long distances by infected alates (Table 1) or possibly also as air-borne conidia, and infect new individuals [29].

Host insect resistance against fungal pathogens

Insects have a complex hierarchy of defenses or resistance mechanisms that pathogens must overcome before a successful infection may occur. The main behavioral and physical barriers to infection are behavioral avoidance of the pathogen [30], morphological barriers to infection (cuticle, digestive system and tracheal system), or physiological responses to infection (distinguishing self from non-self or altered self, humoral responses, cellular responses, melanizaton, intracellular defenses) [31]. Aphids are not social insects and cannot perform social immunity against specialist fungi from Entomophthoromycotina like ants can do [32•]. However, aphid behavior may still be important. The different positions of *R. padi* and *S. avenae* on plants may be ecological traits leading to different susceptibilities. Differences in position on the plant are significant for insect pathogenic fungi, affecting their ability to target the host cuticle by their infection propagules. The more hidden the host, the lower the chances of spores landing on the cuticle and the lower the risk of infection. In arthropods, both attraction and avoidance of conspecifics infected with specialist entomopathogenic fungi have been noted in insect species [9,32•] and mite species [33]. The pea aphid, *Acyrthosiphon pisum*, seems to be indifferent to infected aphids and colonize new plants without regard to the presence of cadavers infected by *P. neoaphidis* [34]. An interesting behavioral resistance has been shown for milkweed aphid, *A. asclepiadis* [35]. Here, it is not the aphid itself having a behavior supporting resistance, instead ants (*Formica podzolica*) in the field quickly removed fungal killed aphids and in that way significantly lowered the possibilities of disease transmission among the aphids.

As almost all insect pathogenic fungi use the cuticle as their point of entry, the cuticle forms the first physical barrier for the pathogen to overcome. The resistance mechanisms in the cuticle may include both chemical compounds that inhibit germination of fungal propagule and/or hardness of the insect cuticle that inhibits penetration [31]. One study [36] aimed to discover fungal secretomes from field collected *S. avenae* and the authors discovered several fungal gene products involved with host cuticle penetration. The aphids themselves had, however, few genes involved in response to pathogen invasion. Even when the aphids received a high conidial dose, the host response by *S. avenae* was weak and probably of little significance in resistance.

A recent study [37•] found that *S. avenae* fecundity was reduced for aphids that were inoculated with but not killed by *P. neoaphidis*. This may be because some aphids escaped from becoming lethally infected, for example due to resistance responses. The loss in fecundity of infected but yet surviving *S. avenae* can be interpreted as energy losses of the host due to the immune response fighting the infection.

Aphids are polyphenic (multiple, discrete phenotypes that arise from a single genotype), and can exhibit different forms, or morphs, during the course of their seasonal life cycle. Among these, they produce apterae (lacking wings) or alate (with wings) adults in response to different conditions. Alates are probably subjected to higher disease pressure than apterae, because of their larger range of activity. Further, the energy cost of producing wings can be high and the energy allocated to resistance to pathogens may be lower. The high susceptibility of alate aphids is an advantage for the fungus, since migrating aphids may disperse the pathogen (Tables 1 and 2). The suggested advantage in fungal dispersal via alates finds further support in a study [38], which documented that infection of *A. pisum* with *P. neoaphidis* resulted in the production of a higher proportion of alates. In a study on *A. pisum* [39•] the authors showed that alates were more susceptible than apterae to infections by *P. neoaphidis*, fitting with the hypothesis of energy limitation. Similarly, in the case of *S. avenae* and *P. neoaphidis*, a bioassay study [40] showed that alates were more susceptible. Because of the migrating alates, cereal fields can contain several clonal lineages of aphids with potential differences in susceptibility, although results so far (Table 2) do not suggest major differences. Results from a two-year study in a winter wheat field in Denmark suggests that neither *P. neoaphidis* nor *E. planchoniana* affected the clonal distribution of *S. avenae* [41].

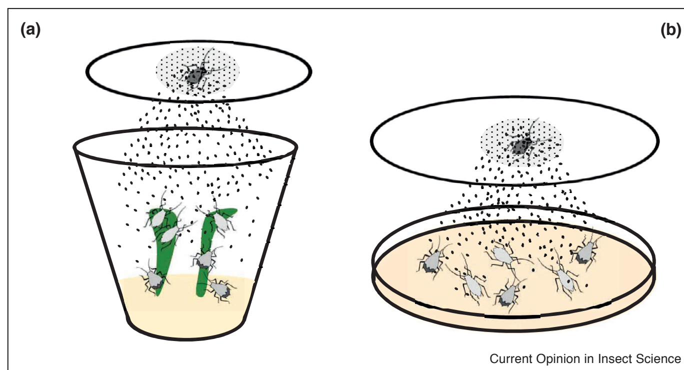
Role of symbionts

Aphids harbor many bacterial species, including endosymbionts [42,43•], and we may speculate if they assist in protecting aphids from fungal infections. The obligate symbiont *Buchnera aphidicola* was reported from aphids, including *S. avenae* and *R. padi* [43•,44]. A few facultative endosymbionts have been reported from *S. avenae*, namely *Hamiltonella defensa*, *Regiella insecticola* and *Serratia symbiotica* [43•,45–47] while no facultative endosymbionts to our knowledge are reported from *R. padi*. Five species of facultative endosymbionts (*R. insecticola*, *Spiroplasma*, *Rickettsia*, *Rickettsiella* and X-type) have been identified as conferring significant protection to *A. pisum* against *P. neoaphidis* [48,49]. They can reduce the mortality of *A. pisum* when infected and decrease *P. neoaphidis* sporulation from the killed aphids [48]. In a study discriminating between intrinsic (genetic) resistance and extrinsic (symbiont conferred) resistance to *P. neoaphidis* across host, it was shown that *A. pisum* biotypes with a higher probability of carrying protective endosymbionts also have a higher intrinsic resistance [50••]. Authors therefore did not find evidence that aphid carrying protective endosymbionts lose (or 'chose to outsource') their own genetic resistance.

Methodological challenges to compare aphid susceptibility

Pandora neoaphidis and *E. planchoniana* conidia can be produced from dead aphid cadavers, but due to their sticky nature [22,24] they cannot be mixed with water to obtain a predefined concentration. Therefore, infection bioassays to study pathogenicity and virulence have to use methods mirroring their biology by allowing one or more dead infectious aphid cadaver(s) to discharge infective conidia to a cohort of aphids [51,52]. Since a predefined conidia concentration cannot be obtained, various

Figure 2



Bioassay procedure to document pathogenicity and virulence of entomophthoralean fungi to grain aphid *Sitobion avenae* and bird cherry-oat aphid *Rhopalosiphum padi*. Infective conidia are discharged from one or more aphid cadavers (conspecific or heterospecific host) onto exposed aphids.

(a) Aphids are allowed to position themselves on their host plant. Aphids which prefer to position themselves in lower parts of plants (*R. padi*, with dark posterior) may to some extent benefit from this position in comparison with aphids that normally position themselves with more exposure to fungal conidia (*S. avenae*, without dark posterior).

(b) Aphids do not have access to their host plant during exposure, so host behavioral effects are avoided.

methods to count conidia on cover slips before and after exposure or during exposure have been applied [52]. In studies comparing virulence toward two aphid species, the challenge is to apply the same dose to each aphid species. Such bioassays may in addition take aphid host behavior into account. An example of a bio-assay, where *S. avenae* and *R. padi* are allowed to position themselves on a host plant according to their biological preferences is depicted in Figure 2a; *R. padi* will position themselves lower than *S. avenae*. Results suggest that a conspecific host *S. avenae* is more susceptible than the heterospecific host *R. padi* [53] (Table 2). In that study, aphid behavior is taken into account and therefore results can more readily be extrapolated to real field conditions. Another set up is to leave out the position behavior of aphids by placing *S. avenae* and *R. padi* in the same Petri dish without a host plant during exposure (Figure 2b), and in that case both aphid species will receive the same dose. A study using this design [37•] proved that also is this case the conspecific host was more susceptible than the heterospecific host. It seems therefore that the different susceptibilities between the two aphid species may include an immune component and a behavior component. Further studies testing the susceptibility of conspecific versus heterospecific aphid hosts may elucidate more in depth the importance of the different components.

Conclusion

In this cereal host–pathogen system, aphid behavior, aphid morph and fungal isolate are important factors governing host susceptibility and resistance. Hosts' immune responses seem weak in *S. avenae* and of limited importance, but further studies are needed to confirm if this also is the case in *R. padi*. When comparing susceptibility of fungal isolates from the conspecific host *S. avenae*, *S. avenae* was more susceptible than the heterospecific host *R. padi* but fungi conspecific to *R. padi* and heterospecific to *S. avenae* are still left to be tested. Alate *S. avenae* and *R. padi* seem to be more susceptible to *P. neoaphidis* than apterae fitting with the hypothesis of energy limitation (cost spend in nymphal stages on the development of wings versus costs spend to ensure a high level of immune response) that we see for resistant but less fecund *S. avenae*. Symbionts may play an important role in aphid resistance to fungal pathogens and other natural enemies, but comparative studies on the influence of symbionts on the resistance to specialist fungal pathogens in *S. avenae* and *R. padi* are lacking.

Conflict of interest statement

Nothing declared.

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- •• of outstanding interest

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Paper II

Entomophthoromycotan fungi overwinter with their host the bird-cherry oat aphid *Rhopalosiphum padi* on its winter host *Prunus padus*

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Abstract

In Scandinavia, the bird cherry-oat aphid *Rhopalosiphum padi* overwinter as eggs on *Prunus padus*. Branches of *P. padus* were collected at the end of February / beginning of March from 17 locations in Norway over a three-year period. A total of 879 overwintering fungus-killed cadavers were observed. Out of 3 599 overwintering *R. padi* eggs observed, 59.5% were dead. We found a significant negative correlation between eggs and cadavers per branch. Cadavers were infected with either *Zoophthora* sp. overwintering as resting spores, or *Entomophthora planchoniana* overwintering as modified hyphal bodies. Numbers of eggs and cadavers varied greatly between years and among tree locations. Fungus-killed *R. padi* cadavers were found close to bud axils, where overwintering *R. padi* eggs are also usually observed. This is the first report of *E. planchoniana* overwintering in *R. padi* cadavers as hyphal bodies. We discuss whether *P. padus* may act as an inoculum reservoir for fungi infecting aphids in cereals in spring.

Keywords: overwintering strategy; *Zoophthora*; *Entomophthora planchoniana*; hyphal bodies; resting spores

1. Introduction

Winter in temperate climates represents a bottleneck for many species (*e.g.* Leather, 1992). Both pests and their natural enemies have developed strategies to survive the harsh winter conditions and to re-establish new populations the following spring. Some pests overwinter in specific sites or on specific hosts. For example, the bird-cherry oat aphid *Rhopalosiphum padi* (Hemiptera: Aphidoidea) and the black bean aphid *Aphis fabae* (Hemiptera: Aphidoidea) have only one woody winter plant host (Leather, 1983).

Rhopalosiphum padi has a complex life cycle. 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 winter on the bird cherry *Prunus padus* (Rosales: Rosaceae) (Leather, 1992). In Scandinavia, only *R. padi* is reported to occur on *P. padus* (Ossiannilsson, 1964). At the beginning of autumn, aphids produce alate gynoparae and males that migrate to their winter host, the bird cherry *P. padus* (Leather, 1992). They choose *P. padus* 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 are able to mate and produce overwintering eggs containing cryoprotectants that confer cold-resistance to -40 °C (Sömme, 1969). Gynoparae females seem to randomly locate themselves within a tree (Leather, 1981a), although their choice of egg-laying site is non-random (Leather, 1981b). After mating, oviparae females prefer to lay overwintering eggs in sheltered locations close to *P. padus* buds (Leather, 1992). In high population density situations, eggs are laid in cracks of branches as well (Kurpaa, 1989).

Oviparae females compete for the best egg-laying sites: sheltered locations in the bud axils. 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 is estimated, increasing to 6.5% per week in early spring due to the increased activity of natural enemies. Total egg survival is estimated around 30% (Leather, 1980; 1983). At the beginning of spring, eggs hatch and *R. padi* establishes new colonies on unfurling *P. padus* leaves. New fundatrices produce 2-3 wingless generations on *P. padus*, which then produce alates that migrate to cereals or other grass plants (Hansen 2006). Scandinavian studies have shown a correlation between the number of overwintering eggs on *P. padus* at the end of winter and *R. padi*

population size the following summer (e.g. Leather, 1983). In Norway, *P. padus* trees have been monitored for *R. padi* eggs for more than 20 years with the aim of estimating spring *R. padi* pressure for use in a decision support system named VIPS (https://www.vips-landbruk.no/).

Entomopathogenic fungi in the phylum Entomophthoromycota are an important group of natural enemies of *R. padi.* Infection rates can reach up to 46 % of observed individuals (Barta and Cagáň, 2004; 2007; Hatting *et al.,* 2000; Chen and Feng, 2004) and several species have been documented to infect *R. padi*, namely: *Pandora neoaphidis*, *Entomophthora planchoniana*, *Conidiobolus obscurus*, *Neozygytes fresnii*, *Zoophthora aphidis*, *Z. radicans*, and *Z. occidentalis* (Nielsen and Steenberg, 2004; Barta and Cagáň, 2004; Barta 2009; Manferino *et al.,* 2014). Entomopathogenic fungi in the phylum Entomophthoromycota, are mainly biotrophic with a close relationship to their hosts (*e.g.* Pell *et al.,* 2010). They employ several overwintering strategies: 1) forming resting spores in the host or in the ambient environment (*e.g.* Klingen *et al.,* 2008; Duarte *et al.,* 2013; Hajek *et al.,* 2018), 2) forming loricoconidia (thick-walled conidia) in soil (Nielsen *et al.,* 2003), 3) forming modified hyphae in soil or in cadavers (Keller, 1987), 4) forming semi latent hyphal bodies in their live, hibernating hosts (Klingen *et al.,* 2008), and 5) persisting as a slowly developing infection in hibernating hosts that may also be transmitted between individuals in *e.g.* a cluster of flies (Eilenberg *et al.,* 2013). Resting spore formation is triggered 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 might be induced by cues from the host aphid (Nielsen *et al.,* 2003; Hajek *et al.,* 2018). Previous studies of fungus-killed *R. padi* on *P. padus* found cadavers with conidia mainly in autumn and very few in spring; and have not reported the presence of resting spores or overwintering hyphal bodies (Nielsen and Steenberg, 2004; Barta and Cagáň, 2004). However, Hajek et al. (2018) emphasize that overwintering forms of Entomophthoromycotan fungi must be co-located with areas of host activity in spring. As such, Entomophthoromycotan pathogens of *R. padi* would be expected to overwinter in the vicinity of *P. padus* trees.

The aim of this study was, therefore, to answer the following research questions: 1) Do fungi in Entomophthoromycota overwinter in *R. padi* on *P. padus*? 2) If so, what is their overwintering strategy (formation of resting spores, hyphal bodies *etc.*)? 3) Which fungal

species are present in the overwintering aphid population, and how abundant are they? 4) Does the frequency and abundance of *R. padi* eggs and fungus-killed cadavers vary between years or tree locations?

2. Materials and Methods

2.1. Field sampling

For each sampling, ten branches of *P. padus* located at the border of cereal fields in Norway were collected at the end of February or beginning of March (Fig. 1). A total of 17 locations were sampled, of which 13 were monitored over a period of three years (2017-2019), 2 locations for two years (2017-2018), and 2 were sampled only in 2019 (Table 1). Branches were sampled from only 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 enumeration to avoid hatching of eggs. Over a period of three years, 450 branches were examined for overwintering *R. padi* eggs and for fungus-killed overwintering *R. padi* cadavers as described below.

2.2. *Rhopalosiphum padi* overwintering eggs and cadavers counting

In the laboratory, *R. padi* eggs and fungus-killed *R. padi* cadavers were counted under a stereomicroscope (0.71-1.25 X) and processed for further morphological and molecular identification of aphid and fungal species as described below. Open, empty and flat *R. padi* eggs were recorded as dead, while full and shiny black eggs were recorded as live.

2.3. Aphid and fungal species identification

2.3.1. Morphological identification

Ninety-two fungus-killed overwintering *R. padi* cadavers were cut in two. One part was used for morphological observation and the other for molecular identification. Morphological observations were conducted by mounting the cadaver in lactic acid cotton blue (0.075% cotton blue in 50% lactic acid) and fungal structures were observed and measured using a compound microscope (200-400X). Aphid species and morph (adult or nymph) were identified according to Blackman and Eastop (2007).

Figure 1: Bird cherry (*Prunus padus)* branch collection sites (red points) for *Rhopalosipum padi* overwintering eggs and fungus-killed *R. padi* cadavers.

2.3.2. Molecular identification

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

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 volume 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 ^{o}C, followed by 6 cycles with denaturation for 30 sec at 94 ^{o}C, annealing for 30 sec at 45 ^{o}C, extension for 1 min at 72 ^{o}C, followed by 35 cycles with denaturation for 30 sec at 94 ^{o}C, annealing for 1 min at 51 ^{o}C, extension for 1 min at 72 ^{o}C and a final extension for 10 min at 72 ^{o}C. The products were kept at 12 ^{o}C until further analysis.

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 ^{o}C, followed by 35 cycles with denaturation for 30 sec at 98 ^{o}C, annealing for 30 sec at 55 ^{o}C, extension for 30 sec at 72 ^{o}C and a final extension for 10 min at 72 ^{o}C.

PCR amplification was verified by gel electrophoresis (Agarose from Sigma, A9539, 1 %, 90 Voltage for 40 min duration) with intercalant (Ethidiumbromid from Vwr, E406-5mL),

and successfully amplified products were purified and sanger-sequenced in the forward and reverse directions by Eurofins Genomics (Germany).

For each cadaver, consensus sequences for the fungal LSU and insect COI regions 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% percent identity to an aphid reference sequence was required for positive identification to species level. 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 x 106 generations each were made under a GTR+I+G model, with trees sampled every 1000th 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.

2.4. Statistical analysis

We excluded tree location and year without any aphid or fungus overwintering forms 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 was overdispersed, we used negative binomial regression to test the correlation between number of eggs and number of overwintering fungus-killed *R. padi* cadavers and the influence of year. We compared the different years to each other with estimated marginal means (post hoc analysis, R package "emmeans", Lenth (2017)).

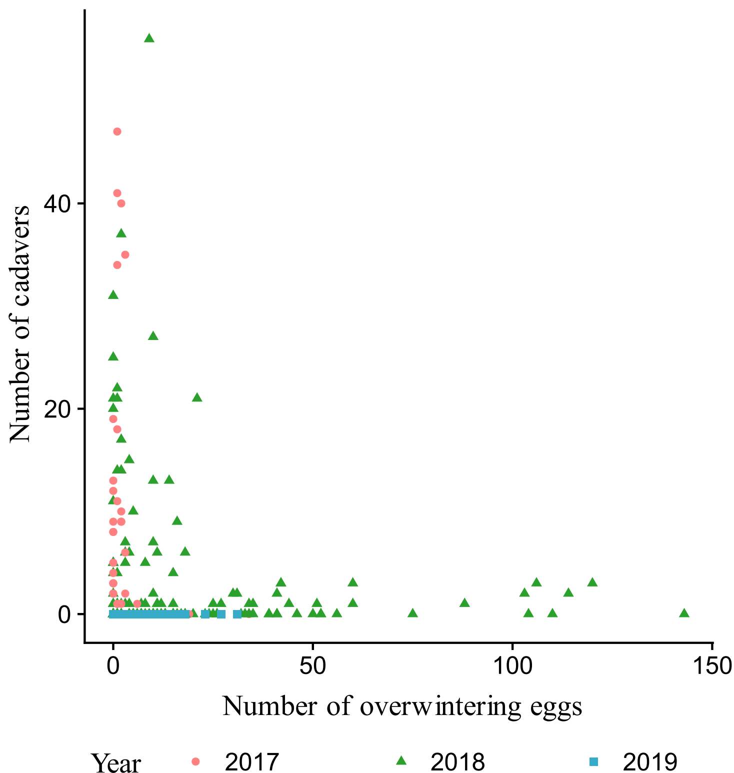


Figure 2: Correlation between numbers of *Rhopalosiphum padi* eggs and overwintering fungus-killed *R. padi* cadavers per bird cherry (*Prunus Padus*) branch in 2017, 2018 and 2019. Ten branches were collected and examined from 17 *P. padus* tree locations. Both live and dead eggs are included.

3. Results

3.1. Overwintering eggs and fungus-killed cadavers per branch

We observed a total of 879 cadavers and 3 599 overwintering *R. padi* eggs, of which 59.5% were dead. 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 only 0 to 10% had only fungal cadavers. The 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. 2). Year significantly influenced the number of overwintering eggs ($F = 74.042$, $df = 2$, $p < 0.001$). More precisely, 2018 was significantly different from 2017 and 2019 ($p < 0.001$ for both comparison), 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. 2).

3.2. Variability between *Prunus padus* tree locations

We observed a high variability between *P. padus* tree locations and years in average number of *R. padi* eggs per branch, and average number of overwintering fungus-killed *R. padi* cadavers per branch (Table 1). No fungus-killed *R. padi* cadavers were found on any 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 of 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.3A), 2) one tree consistently had predominantly overwintering fungus-killed *R. padi* cadavers (Fig. 3B), 3) five trees had predominantly overwintering fungus-killed cadavers one year and predominantly overwintering eggs the following year (Fig 3C), 4) in 2018 only, two trees had a significant mix of overwintering cadavers and eggs simultaneously (Fig. 3D).

Table *1*: Average number (± SD) of *Rhopalosiphum padi* overwintering eggs 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). Black bold: average > 1. Red bold: average > 10.

	2017		*2018*		*2019*	
Tree	*Eggs*	*Cadavers*	*Eggs*	*Cadavers*	*Eggs*	*Cadavers*
Apelsvoll	**5.1** ± 4.0	0 ± 0	**31.6** ± 28.8	0.6 ± 0.8	0.2 ± 0.6	0 ± 0
Auli	**4.0** ± 3.5	0 ± 0	**1.1** ± 2.1	**1.1** ± 1.6	0.4 ± 1.0	0 ± 0
Blaker	**4.9** ± 5.4	**1.3** ± 3.8	**12.6** ± 12.1	**5.2** ± 5.4	0.8 ± 1.7	0 ± 0
Brandval	0.9 ± 1.0	**18.9** ± 13.3	**9.8** ± 5.7	**15.3** ± 16.3	**2.4** ± 2.4	0 ± 0
Buskerud	0 ± 0	0 ± 0	0.1 ± 0.3	**3** ± 3.5	**2.2** ± 2.9	0 ± 0
Grønnesby	-	-	-	-	0.5 ± 0.8	0 ± 0
Kirkenær	**3.6** ± 3.2	0.2 ± 0.4	**11** ± 5.9	0.8 ± 1.9	**8.0** ± 5.1	0 ± 0
Lardal	**1.8** ± 1.1	**13.2** ± 16.4	**9.7** ± 4.6	0.6 ± 1.3	**2.9** ± 3.4	0 ± 0
Leirud	0.7 ± 0.9	0 ± 0	**30.5** ± 17.9	0.7 ± 1.1	**18.1** ± 8.9	0 ± 0
Meeggen	0.6 ± 0.7	0 ± 0	0.5 ± 0.8	0 ± 0	0.1 ± 0.3	0 ± 0
Meldal	-	-	**8.6** ± 9.3	0.2 ± 0.6	0.7 ± 1.6	0 ± 0
Stokke	0 ± 0	**2.1** ± 2.5	0.9 ± 0.9	**22.2** ± 7.2	0.6 ± 0.8	0 ± 0
Storøya	**2.0** ± 1.9	0 ± 0	**29.1** ± 21.1	0.3 ± 0.5	**1.1** ± 1.4	0 ± 0
Stjørdal	-	-	-	-	0 ± 0	0 ± 0
Suleng	**9.1** ± 9.0	0 ± 0	**34.3** ± 11.7	0.5 ± 1.0	1.0 ± 1.0	0 ± 0
Vinstra	**5.4** ± 5.8	0 ± 0	**101.3** ± 26.6	1.0 ± 1.3	-	-
Øsaker	1.0 ± 1.2	0 ± 0	**8.9** ± 9.1	0.8 ± 1.9	**1.9** ± 2.1	0 ± 0

– = no collection of branches

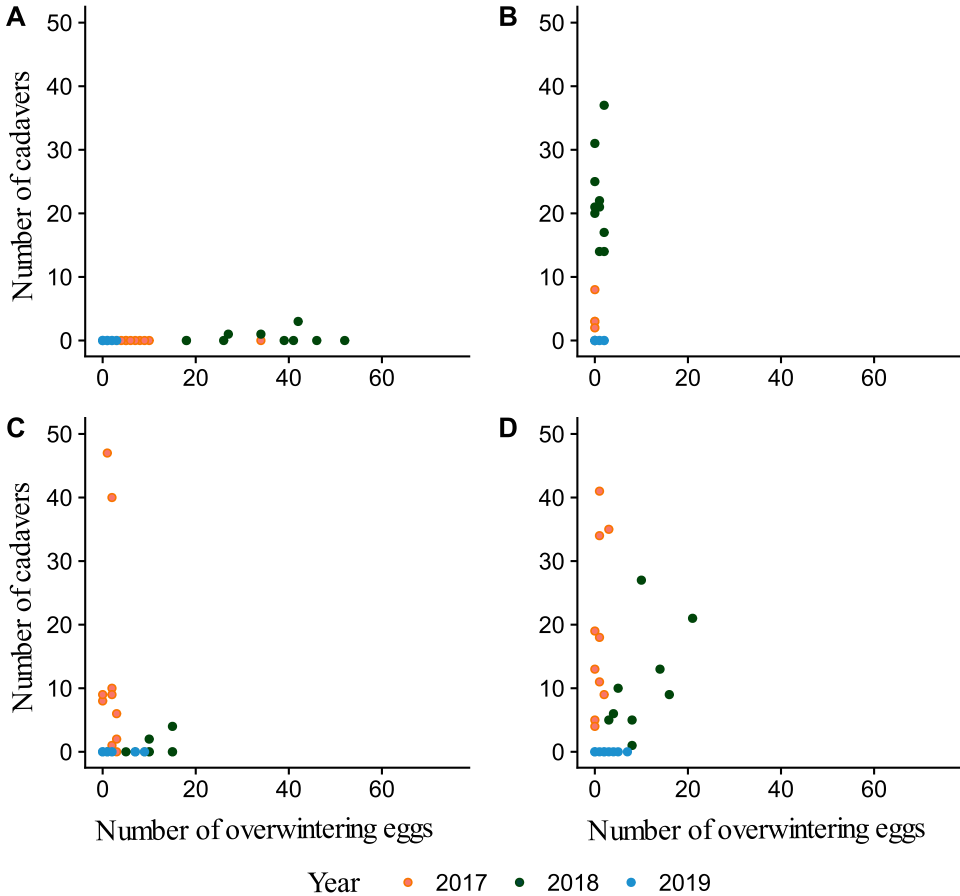


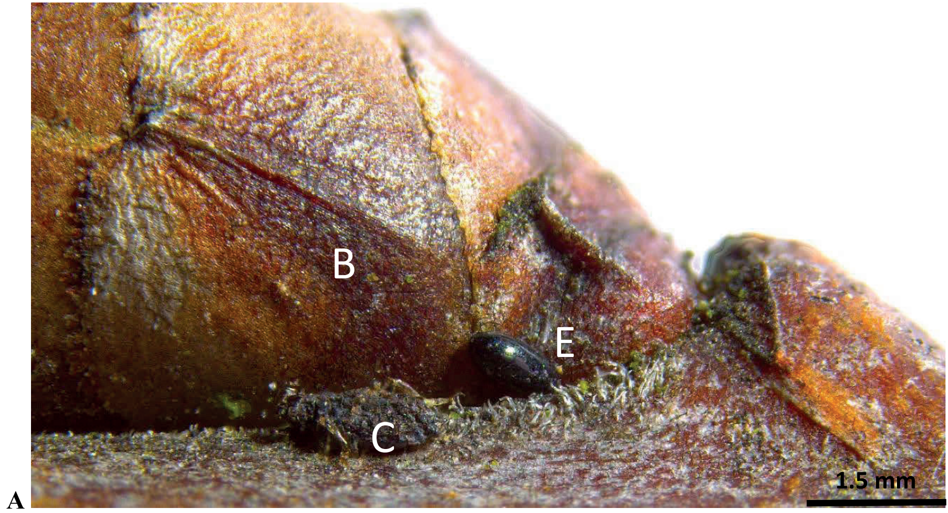
Figure 3: Numbers of *Rhopalosiphum padi* overwintering eggs and fungus-killed cadavers per branch over three years. A) Tree in Suleng with almost only overwintering eggs. Aphids may escape fungi. B) Tree in Stokke with almost only overwintering cadavers. Fungi might not re-infect spring aphid population. C) Tree in Lardal with mostly overwintering cadavers one year and mostly overwintering eggs the following year. Fungi may re-infect spring population 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 can re-infect spring aphid population after their first winter

.

3.3. Description and identification of fungus-killed overwintering *R. padi* cadavers

Fungus-killed *R. padi* cadavers were found close to bud axils, where overwintering *R. padi* eggs are also usually observed (Fig. 4A-C). When the density of cadavers was high, some were also found on the branch between buds (Fig. 4D). Among the 92 fungus-killed cadavers studied, 70.6% were nymphs, 2.2% were adults and 27.2% were not possible to identify to aphid stage.

When observed by stereomicroscope and compound microscope, two cadaver morphotypes were noted. One group of fungus-killed overwintering *R. padi* cadavers were black, dry and hard to break without immersing the body in a liquid (Fig. 5A, B). Legs, antennae and cornicula, when still attached to the body, were usually black but some individuals had brown legs. Many rhizoids 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 that appeared black and "grainy". Resting spores were spherical or slightly pumpkin-shaped and included an epispore (Fig. 6A-C). Resting spores (epispore included) measured 42.62 μm ± 3.25 (mean ± SE) (range: 36.25 – 51.25 μm). The second morphotype included fungus-killed overwintering *R. padi* cadavers that were brown, dry and varied in hardness from very hard to relatively easy to break without immersing the body in a liquid. Legs, antennae and cornicula, when still attached to the body, were brown or yellowish (Fig. 5C). No rhizoids were present (Fig. 5D), and the cadaver was attached to the branch by being intertwined with the branch trichomes. The aphid body (thorax and abdomen) was filled with hyphal bodies that appeared dark-brown or white and "grainy". Ten cadavers were filled with heterogenous hyphal bodies of varying shape and length (Fig. 6D), while one cadaver was filled with homogenous rod shaped hyphal bodies (Fig. 6E). The homogenous rod shaped hyphal bodies had a mean length of 41.69 μm ± 5.07 (mean ± SE) (range: 37.5 - 50 μm) and a mean diameter of 17.99 μm ± 2.98 (mean ± SE) (range: 12.5 – 22.5 μm).



A

B

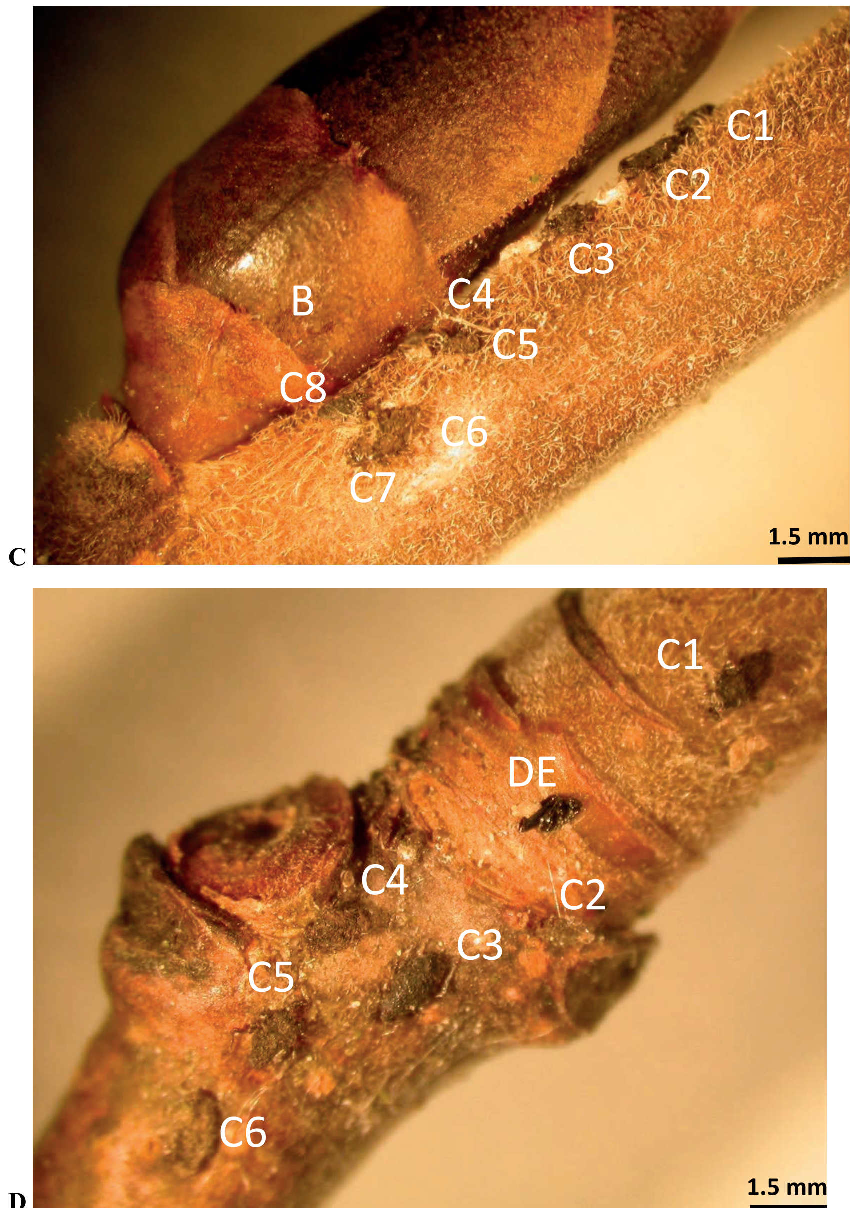


Figure 4: A) Typical microlocation for overwintering fungus-killed *Rhopalosiphum padi* cadavers (C1, C2 and C3) close to bird cherry (*Prunus padus*) bud axil on last annual shoot. One live egg was close to the fungus-killed cadaver. B) Twelve overwintering eggs close to the bud axils. C) Eight fungus-killed *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. Annotations: B: bud; C: cadaver; E: egg; DE: dead egg. Photo: Erling Fløistad and Stéphanie Saussure, NIBIO

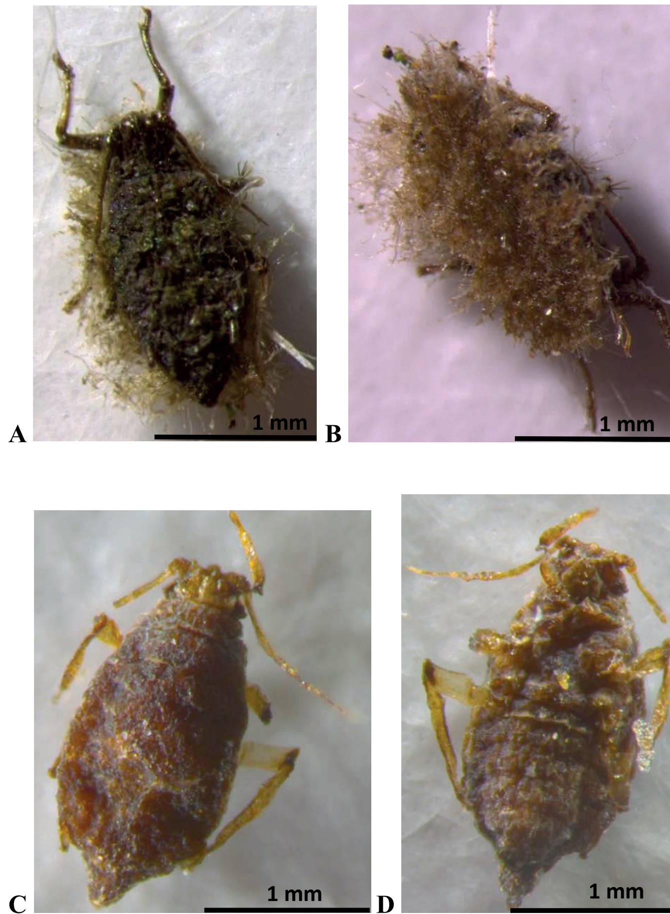


Figure 5: A) and B) Overwintering fungus-killed *Rhopalosiphum padi* cadaver filled with resting spores of an unidentified *Zoophthora* species A) Dorsal face of the cadaver, B) Ventral face showing many rhizoids intertwine with trichomes from the tree, which attached the cadaver to the *Prunus padus* 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 rhizoids. Photo: Stéphanie Saussure, NIBIO

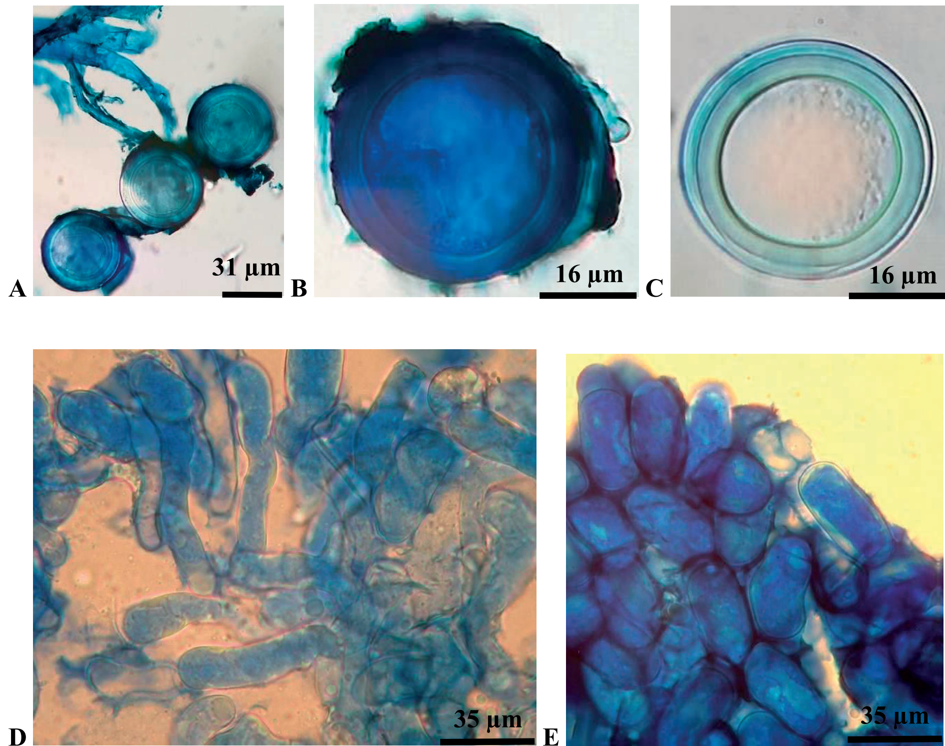


Figure 6: A-C) Pictures of resting spores of an unidentified *Zoophthora* sp. A) Resting spores with epispore and intertwined with rhizoids, B) Resting spore with epispore and C) Smooth and hyaline resting spore without epispore showing two thick walls. D-E) Pictures of overwintering hyphal bodies of *Entomophthora planchoniana*. D) Hyphal bodies of different shapes; E) Rod shaped hyphal bodies. Photo: Karin Westrum and Stéphanie Saussure, NIBIO

Insect COI sequences were successfully generated for 14 of the 92 aphid cadavers investigated, all of which were positively identified as *R. padi* in BLAST searches. The fungal LSU sequences represented two distinct groups with >99% sequence similarity that corresponded to fungus-killed cadaver morphotypes with either resting spores or hyphal bodies in the aphid body (Fig.7). Between-group sequence similarity was 77%. In the phylogenetic analysis, all fungal LSU sequences from the cadavers nested within the Entomophthoraceae, which formed a distinct, highly supported monophyletic clade (96% Bayesian posterior probability (BPP)). Eighty overwintering cadavers belonged to the group of fungus-killed cadavers with resting spores. The fungal LSU sequences from these cadavers formed a distinct clade (100% BPP) that was sister to an unidentified species of *Zoophthora* observed in resting spores infected *Eurois occulta* (Lepodoptera: Noctuidae) (Fig. 7). The genus *Zoophthora* was paraphyletic owing to the exclusion of a well-supported clade (100% BPP) comprised of *Pandora* and *Erynia* species. All these cadavers had the morphology as described for the first group above and in Fig 5 A and B and Fig 6 A to C.

Eleven overwintering cadavers belonged to the group of fungus-killed cadavers containing hyphal bodies. The fungal sequences corresponding to this group formed a strongly supported (100% BPP) monophyletic clade with representative sequences of *E. planchoniana* that was distinct from other *Entomophthora* species, and the fungus is tentatively identified as *E. planchoniana* (Fig. 7). All these cadavers had the morphology as described for the second group above and in Fig 5 C and D and Fig 6 D and E.

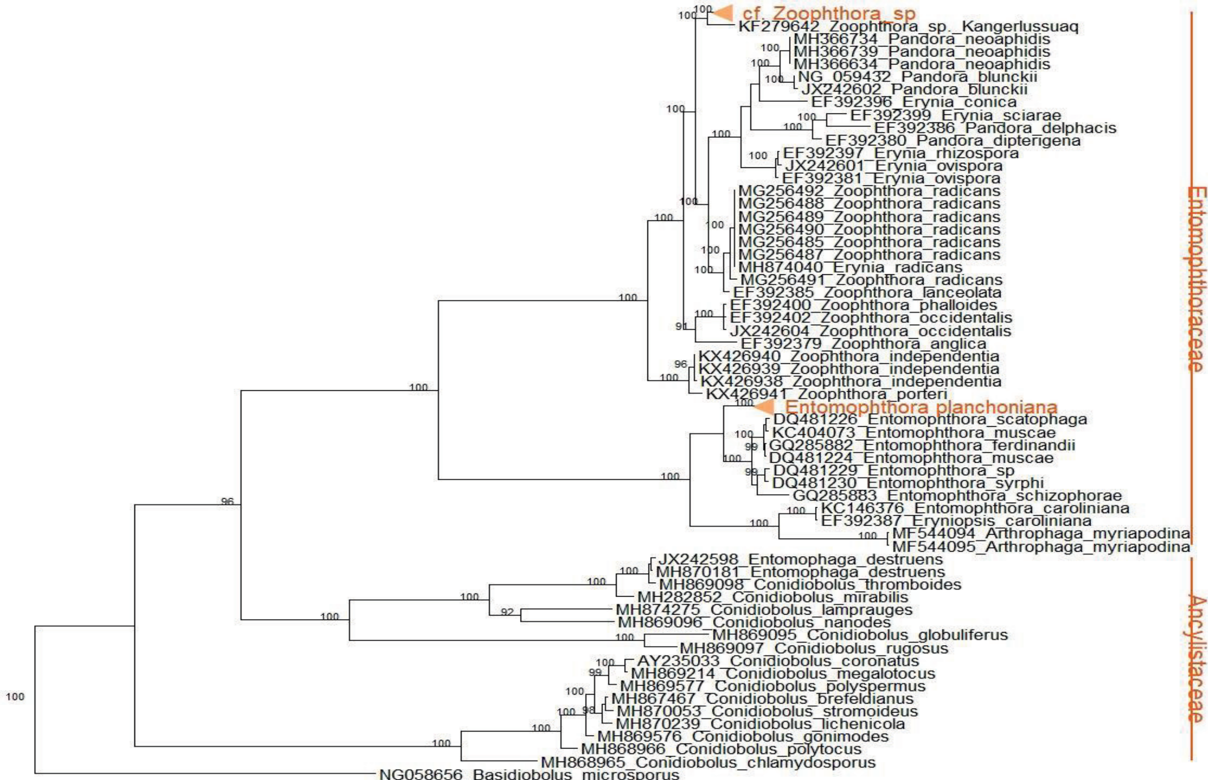


Figure 7: Phylogenetic tree proposed by Bayesian inference for the fungal LSU sequences of two cadaver morphologies (in orange). The fungal LSU sequences from fungus-killed *Rhopalosiphum padi* cadaver with resting spores formed a distinct clade sister to an unidentified species of *Zoophthora* from a resting spore infected *Eurois occulta* (Lepodoptera: Noctuidae). The genus *Zoophthora* was paraphyletic owing to the exclusion of a well-supported clade comprised of *Pandora* and *Erynia* species. 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. Outgroup species are in the Ancylistaceae family.

4. Discussion

This study demonstrates that two fungal species in the Entomophthoromycota overwinter as resting structures in *R. padi* cadavers on *P. padus*. One species exclusively formed resting spores in its host's body and was phylogenetically allied to a clade composed of the genera *Zoophthora* and *Erynia/Pandora*. Multigene phylogenies of the Entomophthoraceae support the existence of *Zoophthora* as a distinct genus separate from an *Erynia/Furia/Pandora* genus complex (Gryganskyi *et al.,* 2013). As our fungal LSU sequences were supported as a group distinct from the *Erynia/Pandora* clade in these analyses, they are provisionally identified as members of the genus *Zoophthora*. 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 observed are morphologically 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 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". However, the lack of a reference 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* have until now not been considered as an important natural enemy of aphids in cereals in Europe. 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 cereal aphids and may cause epizootics (Barta and Cagáň, 2006; Ben Fekih *et al.,* 2015; Hatting *et al.,* 2000). 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* overwinter as modified hyphal bodies inside the oviparae of the sapling sycamore aphid *Drepanosiphum acerinum*. However, our *R. padi* cadavers were filled with hyphal bodies only and it 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, but only the rod shaped were measured and 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 are within the range Keller (1987) reported for *E. planchoniana*.

Our study showed a negative correlation between numbers of overwintering eggs and cadavers per branch and therefore, there may be low infection pressure on spring *R. padi* generations. 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 entomophthoromycotan fungi just prior to migrating to grasses. Moreover, our study showed that aphids typically killed during their nymphal stage (70.6% of our samples). Therefore, we hypothesise that the observed negative correlation may be due to early aphid mortality from fungal infections reducing reproduction and egg laying. 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 microlocation 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 on different locations on the tree depending on whether fungus-killed cadavers have 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 dying on egg-laying sites, which might in turn increase the likelihood of the fungus re-infecting the aphids in spring. By extension, there may be a competition between healthy oviparae females and Entomophthoromycota-infected nymphs for the best microlocations 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)

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 2) fungal isolates with different virulence. A discussion of the two last factors mentioned are 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, *P. padus* may be considered only as an overwintering site for *R. padi.* 2) When only fungi are present, *P. padus* may be considered as an overwintering site for fungi only. 3) When both *R. padi* eggs and fungi are present, fungi may infest *R. padi* the following spring. Over several years, if *R. padi* eggs and fungi 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 favorable conditions, they germinate and produce infective germ conidia (Hajek *et al.,* 2018). Overwintering hyphal bodies are not infective either but produce conidiophores that may produce infective conidia when exposed to favorable 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 fungi in the Entomophthoromycota for summer populations of cereal aphids. However, in our study, some trees harboured many fungal overwintering cadavers (up to 222 cadavers on 10 branches from one tree) and we may, therefore suggest that fungal infected *R. padi* can be an important inoculum reservoir for fungi in the Entomophthoromycota for summer populations of cereal aphids.

5. Conclusion

Overwintering hyphal bodies of *E. planchoniana* and resting spores of an unidentified species within the genus *Zoophthora* were found in *R. padi* on its winter host *P. padus*. Fungus-killed *R. padi* cadavers were attached to bud axils at the same microlocation as overwintering eggs. We found a negative correlation between aphid overwintering eggs and fungus-killed cadavers and a high variation between years and *P. padus* tree location. Some locations hosted only eggs or cadavers, while others hosted a mix of both. Therefore, fungal re-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 an inoculum reservoir even after a one-year delay. We therefore suggest that *P. padus* may act as an inoculum reservoir for these two entomophthoromycotan fungal species.

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Paper III

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Effect of three isolates of *Pandora neoaphidis* from a single population of *Sitobion avenae* on mortality, speed of kill and fecundity of *S. avenae* and *Rhopalosiphum padi* at different temperatures

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We studied the effect of three *Pandora neoaphidis* isolates from one *Sitobion avenae* population, three temperatures, and two aphid species namely *S. avenae and Rhopalosiphum padi* on (i) aphid mortality, (ii) time needed to kill aphids, and (iii) aphid average daily and lifetime fecundity. A total of 38% of *S. avenae* and 7% of *R. padi* died and supported fungus sporulation. *S. avenae* was killed 30% faster than *R. padi*. Average daily fecundity was negatively affected only in *S. avenae* inoculated with, but not killed by, *P. neoaphidis*. Nevertheless, lifetime fecundity of both aphid species inoculated and sporulating with *P. neoaphidis* was halved compared to lifetime fecundity of surviving aphids in the control. Increased temperature resulted in higher mortality rates but did not consistently affect lethal time or fecundity. Results suggest that (i) temperature effects on virulence differ between isolates, even when obtained within the same host population, and (ii) even though an isolate does not kill a host it may reduce its fecundity. Our findings are important for the understanding of *P. neoaphidis* epizootiology and for use in pest-natural enemy modelling.

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1. Introduction

Plant pests (weeds, pathogens, arthropods) and their natural enemies (microorganisms, predators and parasitoids) interact with each other directly and indirectly through the plant. These interactions are affected by abiotic factors such as temperature, pesticides, relative humidity, water, and light (Klingen and Westrum, 2007; Asalf et al., 2012; Caballero-López et al., 2012; Holland et al., 2012; De Castro et al., 2013). An example of non-target effects of plant protection measures are insecticides killing predators and parasitoids, leading to a resurgence of secondary pests (Fernandes et al., 2010). This can also work across pest categories, as observed when fungicides used against plant pathogens also kill beneficial fungi (Klingen and Westrum, 2007), leading to higher levels of pest arthropods and subsequent pesticide use. The effects of natural enemies are sometimes included in decision support systems. In USA, farmers withhold insecticide application for the cotton aphid during epizootics of the aphid-killing fungus *Neozygites fresenii* to enhance control of the aphid population by this beneficial fungus (Hollingsworth et al., 1995). More strategies such as this are needed. However, to build pest-natural enemy models to serve as a basis for such strategies, detailed studies on biotic and abiotic factors affecting these interactions are needed.

Entomopathogenic fungi in the phylum Entomophthoromycota, such as *N. fresenii*, are important natural enemies of foliar pest insects and may cause natural epizootics that can contribute to the control of these pests (Hollingsworth et al., 1995; Pell et al., 2001; Barta and Cagáň, 2006). The major drawback of Entomophthoromycota as biocontrol agents is their primarily biotrophic lifestyle and close association with their insect- or mite-host, which prevents mass-production on artificial media for most species (Jaronski and Jackson, 2012). There are few successful cases of their use in inundation- or inoculation-biological control (Lacey et al., 2001; Shah and Pell, 2003). The use of Entomophthoromycota in

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conservation biological control, as described above for the cotton aphid-killing fungus *N. fresenii,* represents a promising strategy that we would like to develop for *Pandora neoaphidis* (Syn. *Erynia neoaphidis*) (Entomophthoromycota: Entomophthorales) as well.

Pandora neoaphidis is an important fungal pathogen on aphids in temperate agroecosystems (Keller, 1991; Ekesi et al., 2005; Steinkraus, 2006). It has the ability to infect several species of aphid pests on different host plants (Pell et al., 2001; Shah et al., 2004; Barta and Cagáň, 2006; Scorsetti et al., 2007) including the English grain aphid *Sitobion avenae* (Pell et al., 2001; Shah et al., 2004; Chen et al., 2008) and the bird cherry-oat aphid *Rhopalosiphum padi* (Nielsen and Steenberg, 2004; Shah et al., 2004; Chen et al., 2008; Manfrino et al., 2014). In Europe, both *S. avenae* and *R. padi* are important pests in cereals and can co-occur in the same field (Blackman and Eastop, 2007). *P. neoaphidis* penetrates the aphid cuticle, develops inside its host as hyphal bodies, kills the host, breaks through the cuticle, and then produces primary conidia on conidiophores. Primary conidia are then actively projected if conditions (humidity, temperature, light, *etc.*) are favourable. They can then start another infection cycle if they land on the integument of a suitable host. However, if the primary conidia land on unsuitable surfaces (e.g., leaf or soil), secondary conidia may be projected. These are also infective and may infect new aphids or form new infective tertiary or quaternary infective conidia (Shah et al., 1998).

In insect pathology, virulence is defined as “the disease producing power of an organism, *i.e.,* the degree of pathogenicity within a group or species” (Shapiro-Ilan et al., 2005). The virulence of *P. neoaphidis* varies with aphid host species (Shah et al., 2004), the aphid host genotype (Milner, 1982; Stacey et al., 2003; Parker et al., 2017), the geographic origin of the isolate (Shah et al., 2004; Barta and Cagáň, 2009) and even between isolates co-occurring in one aphid metapopulation (Rohel et al., 1997; Sierotzki et al., 2000; Barta and Cagáň, 2009). Because *P. neoaphidis* is a biotrophic fungus, it kills its hosts at the end of the infection process, prior to sporulation. This time between initial host infection and death, *i.e.* lethal time (LT) can dramatically influence the epizootiology of the disease in a host population (Bonsall, 2004). Further, the time between when the infected host dies and the onset of sporulation (becoming infectious) is probably also an important factor in the epidemic development. In Entomophthoromycota sporulation can start at host death, but it can also be delayed if conditions are not suitable. In that case, cadavers may dry and not start sporulating again until a few hours at high humidity triggers the sporulation (Sawyer et al., 1997). Before the infected aphid dies, it may be able to reproduce and contribute to population increase (Schmitz et al., 1993; Baverstock et al., 2006; Chen and Feng, 2006). Consequently, studies of the effect of fungal isolates should also include effect on host fecundity. Lambrechts et al. (2006) highlight the role of both host and pathogen in the expression of various epizootiological traits, including virulence. They emphasise that most epizootiological traits of host-parasite relationships are not host- or parasite-specific but rather the result of complex interactions between the two organisms and, therefore, studying such interactions is encouraged.

Temperature can have complex non-linear effects on host-pathogen interactions such as virulence (e.g. Thomas and Blanford, 2003). Temperature can influence (i) the host mortality caused by a pathogen (Milner and Bourne, 1983; Blanford et al., 2003; Stacey et al., 2003; Eliasova et al., 2004), (ii) the LT (Schmitz et al., 1993; Nielsen et al., 2001; Shah et al., 2002) and (iii) the host's susceptibility (Stacey et al., 2003; Linder et al., 2008; Wojda, 2017; Doremus et al., 2018). Interactive effects between temperature and fungal isolates have also been shown on virulence of Entomophthoromycota; more specifically on the lethal concentration of *Zoophthora radicans* infecting the diamondback moth *Plutella xylostella* (Morales-Vidal et al., 2013) and on the prevalence of *Furia gastropachae* infecting the forest tent caterpillar *Malacosoma disstria* (Filotas et al., 2006). Temperature effects on virulence and sub-lethal effects on the host fecundity may vary between isolates. Exploring the effect of a realistic range of temperatures on several naturally co-occurring isolates would help to reveal the importance of these processes in the disease epizootiology.

In continental Europe (Slovakia) *P. neoaphidis* infects aphids from April to the first frost in mid-November (Barta and Cagáň, 2006). Further, Nielsen et al. (2001) report that a Danish isolate can infect and kill *S. avenae* from 2 to 25 °C. *Pandora neoaphidis* may, therefore, be active from early spring to late summer even at climatic locations similar to Northern Europe (e.g. Agrometeorology Ås, Norway, 2000–2016). Studies on European isolates (from Denmark, Slovakia, UK, and France) suggest that *P. neoaphidis* optimal temperature for vegetative growth, LT and host mortality ranges between 15 and 25 °C (Schmitz et al., 1993; Morgan et al., 1995; Nielsen et al., 2001; Stacey et al., 2003; Barta and Cagáň, 2006). As the virulence of *P. neoaphidis* increases, its LT decreases until the temperature approaches the fungus optimal temperature range (Milner and Bourne, 1983; Schmitz et al., 1993). Although, *P. neoaphidis* infection has been reported to decrease the fecundity of infected aphids compared to uninfected ones for the pea aphid *Acyrthosiphon pisum* (Baverstock et al., 2006; Parker et al., 2017) and the peach-potato aphid *Myzus persicae* (Chen and Feng, 2005, 2006), to our knowledge, no studies have been conducted on the effect of the interaction between temperature and *P. neoaphidis* isolate on aphid fecundity.

The objective of our study was, therefore, to reveal the influence of interactions between three *P. neoaphidis* isolates, two host aphid species (*S. avenae* and *R. padi*), and three temperatures relevant for northern Europe (7.5, 14.0, 18.0 °C) on three fungal virulence traits: (i) aphid mortality *i.e.* the success of the infection, (ii) lethal time (LT), and (iii) decrease of the host fecundity.

2. Materials and methods

2.1. Aphid cultures

Sitobion avenae and *R. padi* cultures were established from a single individual collected on winter wheat (*Triticum aestivum*) in May 2015 in Ås, Norway (59.6607 N, 10.7506 E), and on bird cherry (*Prunus padus*) in 2012 in Toten, Norway (60.5536 N, 10.9309 E), respectively. They were maintained on winter wheat (*T. aestivum* var. Ellvis) at 18 °C, 70% relative humidity and 16:8 h light:darkness. Only 1-3-d-old adult apterae were used in the experiment. The age of the aphids was ensured by controlling nymph production by transferring four apterous adults into a 50 mL plastic vial containing 7.5 mL 1.5% water agar and 6 pieces of winter wheat leaves stuck into the agar. A total of 40 vials were prepared for each aphid species. Adults were left in the vial for 3 d to produce nymphs. In order to avoid the formation of winged individuals among nymphs, each vial was only allowed to contain eight *R. padi* nymphs or five *S. avenae* nymphs. They were maintained until adulthood (10 d for *S. avenae* and 8 d from *R. padi*) at 18 °C, 70% relative humidity and 16:8 h light:darkness. We started adult production of *R. padi* 2 d after *S. avenae* since the two species have different developmental times and we wanted simultaneous adult emergence of both species at the start of the experiment.

2.2. *Pandora neoaphidis* isolates and production of fungal cadavers

The three *P. neoaphidis* isolates (NCRI 459/15, NCRI 460/15 and NCRI 461/15) used in our experiment were collected from three

S. avenae individuals from a spring wheat (*T. aestivum*) field in Ås, Norway (59.6607 N, 10.7506 E) in August 2015. Isolate NCRI 459/15 and NCRI 460/15 were collected 3 m apart from each other and NCRI 461/15 was collected 30 m apart from the two other sites. The three isolates were identified morphologically to *Pandora* spp. according to Keller (1991) and Humber (2012), and to species level as *P. neoaphidis* by the use of molecular methods as described by Thomsen and Jensen (2016). Cadavers of each isolate obtained from the field were individually incubated on a glass slide at 18 °C and high relative humidity (>95%) to trigger sporulation. These spores were used to inoculate new *S. avenae* so that we could establish an *in vivo* culture for each of the three isolates on their original host. This was done by placing 20 apterous *S. avenae* adults from our laboratory culture directly in contact with the spores on the glass slide with a paint brush. The inoculated *S. avenae* were then transferred to a Petri dish (8.6 cm diameter) with wet filter paper and 15–20 wheat leaf pieces. The Petri dish was then covered by a lid with 50–70 holes (3 mm diameter) covered with insect net. Aphids were then allowed to reproduce, and the production of winged individuals was not controlled. Petri dishes were kept at 18 °C, 70% relative humidity and 16:8 h light:darkness. They were monitored twice a week in order to clean the cultures and collect suitable cadavers for this experiment. Only non-sporulating cadavers (generally situated on the underside of the lid close to the holes) were collected by the use of a paint brush from the *in vivo* culture. Collected cadavers where then placed on top of dry filter paper in a Petri dish to dry and be stored in the refrigerator at 7 °C for up to 4 months before use in the experiment. Only cadavers of apterous big nymphs and adults were used in the experiment.

2.3. Fungal inoculation and experimental set up

For each isolate, seven dry non-sporulating cadavers were rehydrated for 24 h in a Petri dish (8.6 cm diameter) with 1.5% water agar at room temperature (23–25 °C) under constant light to trigger sporulation. All rehydrated cadavers sporulated well and spores were present on the bottom and in the lid of the Petri dishes. One inoculation replicate consisted of transferring 40-50 *S. avenae* and 40-60 *R. padi* individuals into a Petri dish with sporulating cadavers and they were kept there for 3 h. Aphids were walking throughout most of the exposure time. Consequently, both aphid species and all individuals of one inoculation replicate were assumed to be exposed to the same amount of fungal inoculum. Control aphids were treated similarly except that no sporulating cadavers were present in the Petri dishes they were transferred to. After inoculation, aphids were individually transferred using 30 mL plastic vials containing 5 mL 1.5% water agar and a piece of winter wheat leaf stuck into the water agar. To ensure high humidity in the vials and good conditions for infection during the first 24 h of incubation, only four holes (1 mm diameter) were made with a pin in the lid. After 24 h, another four holes were made to reduce humidity and the risk of growth of saprophytic fungi. The experimental units were then placed at 70% relative humidity and 16:8 h light:darkness at three different temperatures: 7.5 ± 1, 14.0 ± 1 or 18.0 ± 1 °C. The temperatures were selected based on average spring and autumn temperatures (6 °C) and the range in average summer temperatures (14–18 °C) in Ås, Norway between 2000 and 2016 (Agrometeorology Norway, 2000–2016). Aphids were monitored daily for fecundity, mortality and fungal sporulation from cadavers. Every day, the newly produced nymphs were removed from the vials. Aphids were categorized as follows: (i) Inoculated dead sporulating aphids, that were inoculated with, died from and sporulated with *P. neoaphidis*; (ii) Inoculated surviving aphids, that were inoculated with *P. neoaphidis* but survived and were still alive at the end of the experiment; and (iii) Inoculated dead non-sporulating aphids, that were inoculated with *P. neoaphidis* and died but without any signs of fungal growth. Inoculated dead non-sporulating aphids from the two first replicates were dissected to look for fungal growth (hyphal bodies, conidia or other fungal structures) under the microscope. No sign of fungal infection was found. Therefore, inoculated dead non-sporulating aphids from replicates 3 to 6 were only observed under binocular microscope without dissection. Again, no sign of fungal infection was found. In the control, aphids were scored as: (i) control surviving aphids, that were alive at the end of the experiment; and (ii) control dead aphids, that died for unknown reasons before the end of the experiment. Aphids that died 1 d and 2 d after inoculation were considered killed during transfer and removed from the dataset. No sporulating cadavers were found in the control. Based on pilot studies, the treated aphids were monitored for at least 180 degree-days (DD), which corresponds to 10 d at 18 °C, 13 d at 14 °C and 24 d at 7.5 °C. If a sporulating cadaver was observed at the end of the initial 180 DD monitoring period, the treatment was observed for three more days to ensure that all potentially inoculated dead sporulating aphids had died and sporulated. We aimed for a total of 70 individuals per treatment (temperature, isolate and aphid species). Therefore, we conducted six replicates of the protocol described above. To optimize the production of 1-3-d-old aphids for the experiment, we conducted the replicates two by two, with one day difference between the paired replicates. Replicates 3 and 4 started 3 weeks after Replicates 1 and 2. Finally, Replicates 5 and 6 started seven weeks after Replicates 3 and 4. Fecundity was recorded only in the first four replicates (corresponding to 572 *S. avenae* and 597 *R. padi*) due to the extensive work load.

2.4. Statistical analysis

R program version 3.4.2 (R Core Team, 2017) and R studio (R Studio Team, 2016) were used for statistical analysis. Because the host species effect was clearly the dominant trend in the data (data not shown), we present the results separately for each aphid species to allow visualization of the interactive effects of temperature and isolate.

2.4.1. Pandora neoaphidis virulence

Generalised Linear Mixed Models (GLMM, random effect: replicate, family: binomial) were used to investigate the effect of fungal isolate, temperature and their first order interaction on: (i) the number of sporulating cadavers produced through the mortality of inoculated dead sporulating aphids, and (ii) the mortality of inoculated dead non-sporulating aphids compared to the mortality of dead aphids in the control for each aphid species. We used the R. package *lme4* (Bates et al., 2015) for this and we compared the different isolates and temperatures with estimated marginal means (*post hoc* analysis, R package *emmeans*, Lenth (2017)).

2.4.2. Lethal time (LT) of Pandora neoaphidis

We modelled the cumulative percentage of sporulating aphid cadavers per DD as a sigmoid Gompertz equation (Batschelet, 1976) (Equation (1)), referred to as the LT distribution in the following.

$$Y_i = \alpha \left(e^{-\beta e^{-k\ DD_i}} \right) \tag{1}$$

Where Y_i is the cumulative percentage of sporulating cadavers at day i; DD_i is the degree-day accumulation at day i; α is the asymptote *i.e.* the maximal number of sporulating aphid cadavers (here fixed at 100%); β is the curve displacement: the higher, the more DD are needed for the first sporulating cadavers to occur. Finally, k is the curve slope or growth rate: the higher the growth rate, the faster the fungus kills all the infected hosts. For a more

flexible fit (Equation (1)), the parameters (displacement: β and growth rate: k) were subdivided according to the different values of the studied factor (two species, three temperatures or three isolates) (Equation (2)).

$$\begin{cases} \beta = \beta 0 + \beta 1 * X1 + \beta 2 * X2 \\ k = k0 + k1 * X1 + k2 * X2 \end{cases} \tag{2}$$

X1 and *X2* are binary variables (*X1* equals 1 for the second value of the tested factor and *X2* equals 1 for the third value). The LT models (Equations (1) and (2)) were fitted to test (i) species effect on LT by pooling all temperatures and isolates together (one parameter per species in Equation 2), (ii) temperature effect on LT by pooling all the isolates together (one parameter per temperature in Equation (2)) (Because there were only four *R. padi* sporulating cadavers at 7.5 °C, we compared only 14.0 and 18.0 °C for this host species.), (iii) isolate effect on LT by pooling all the temperatures together (one parameter per isolate in Equation (2)) and finally, (iv) for *S. avenae*, we tested the temperature effect on the LT distribution of each isolate by fitting one model per isolate with one parameter per temperature in Equation (2). This model was impossible to fit for *R. padi* due to low numbers of sporulating cadavers per isolate and temperature (insufficient replication). The standard LT50 (defined as time needed to reach 50% sporulating cadavers) can be derived from Equation (1). The LT distribution models (Equation (1)) were fitted with nonlinear least-square estimators (R package *minpack.lm*, Elzhov et al. (2016)). The difference between the parameter $\beta 1$ and $\beta 2$ and between $k1$ and $k2$ were tested with the Delta method (*post hoc* analysis, R package *car*, Fox and Weisberg (2011)).

2.4.3. Aphid fecundity

We ran GLMMs (random effect: replicate, family: Poisson) to investigate the explanatory power of *P. neoaphidis* inoculation (by comparing all inoculated aphids to the aphids in the control), temperature and their first order interaction on aphid daily fecundity and lifetime fecundity of (i) inoculated dead sporulating aphids compared to surviving aphids in the control, and (ii) inoculated surviving aphids compared to surviving aphids in the control. Ongoing infection processes could have been hidden by the fact that mortality of inoculated dead non-sporulating *R. padi* occurred quickly and before mortality of inoculated dead sporulating *R. padi*. To investigate this possibility, we also studied the effect of the factors listed above on the fecundity of inoculated dead non-sporulating *R. padi* compared to dead *R. padi* in the control. If there was a significant effect of *P. neoaphidis* inoculation (all isolates pooled together versus the control), we further studied the effect of each of the three fungal isolates compared to the control. Results are shown for the pooled data in the case of no effect, and for individual isolates where an inoculation effect was detected. Because fecundity depends on the longevity of the aphids, log-transformed longevity was included as a co-variable in all GLMM in order to study the average daily fecundity. For both average daily fecundity and lifetime fecundity, we compared the different isolates and temperatures to each other with estimated marginal means (*post hoc* analysis, R package *emmeans*, Lenth (2017)).

3. Results

3.1. Effect of *Pandora neoaphidis* isolate and temperature on aphid mortality and fungal sporulation

For all three *P. neoaphidis* isolates tested, significantly more *S. avenae* (38%) than *R. padi* (7%) died and developed into sporulating cadavers (Chi^2 = 123.140, df = 1, $p < 0.001$).

3.1.1. *Sitobion avenae*

Isolate significantly influenced the number of *S. avenae* sporulating cadavers (Chi^2 = 6.779, df = 2, $p = 0.034$) (Fig. 1A). Isolate NCRI 461/15 caused significantly more sporulating cadavers than NCRI 459/15 ($p = 0.030$, *post hoc* comparison), while no significant difference was found between the other isolates ($p > 0.05$). Further, temperature also significantly influenced the number of sporulating cadavers (Chi^2 = 17.895, df = 2, $p < 0.001$) (Fig. 1B). An increase in temperature from 7.5 to 18.0 °C resulted in a significant increase in sporulating *S. avenae* cadavers ($p < 0.001$, *post hoc* comparison). No interaction between temperature and isolate was found (Chi^2 = 3.879, df = 4, $p = 0.423$). Only 6% of the inoculated *S. avenae* (all isolates together) were dead non-sporulating aphids

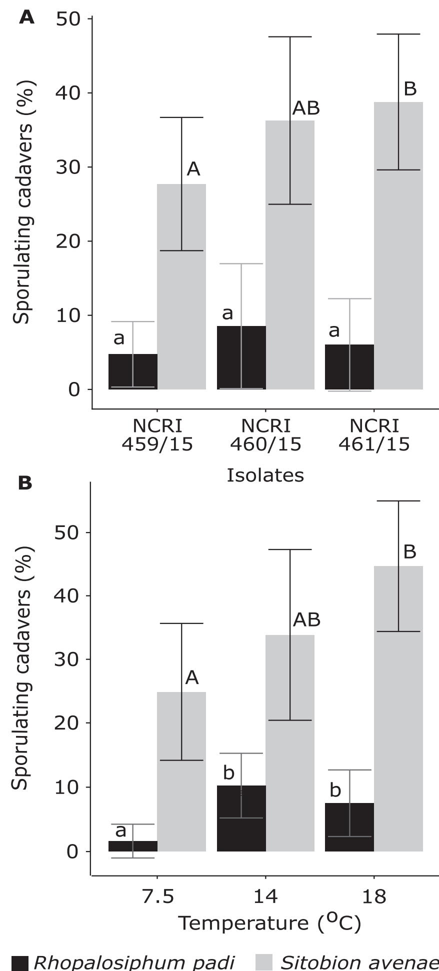

Fig. 1. *Effect of Pandora neoaphidis* isolates (A) and temperature (B) on mean percentage sporulation (±SD) of two inoculated aphid species, *Sitobion avenae* and *Rhopalosiphum padi*. Means with different letters are significantly different based on GLMM and *post hoc* estimated marginal means analysis ($p < 0.05$). Uppercase letters indicate comparisons among *S. avenae* and lowercase letters among *R. padi*. The experiment was repeated six times and a total of 68–75 individuals for each treatment were tested.

(died without fungal growth). In the control, the mortality was 4% and was not significantly different to the mortality of the inoculated dead non-sporulating aphids ($Chi^2 = 0.462$, $df = 1$, $p = 0.497$). Neither the temperature ($Chi^2 = 1.720$, $df = 2$, $p = 0.423$), nor the interaction between the inoculation and the temperature ($Chi^2 = 3.728$, $df = 2$, $p = 0.155$) significantly influenced the mortality of inoculated dead non-sporulating *S. avenae*.

3.1.2. *Rhopalosiphum padi*

There was no significant difference between *P. neoaphidis* isolates in sporulation of *R. padi* cadavers ($Chi^2 = 1.459$, $df = 2$, $p = 0.482$) (Fig. 1A). However, the temperature significantly influenced it ($Chi^2 = 10.992$, $df = 2$, $p = 0.004$) with significantly higher numbers of sporulating cadavers occurring at 14.0 and 18.0 °C than at 7.5 °C ($p = 0.003$ and $p = 0.025$ respectively) (Fig. 1B). There was no significant interaction between temperature and isolate ($Chi^2 = 7.463$, $df = 4$, $p = 0.113$). Only 19% of the inoculated *R. padi* (all isolates together) were dead non-sporulating aphids (died without any fungal growth). In the control, the mortality was 26% and not significantly different to the mortality of inoculated dead non-sporulating aphids (all isolates together) ($Chi^2 = 3.752$, $df = 1$, $p = 0.053$). However, there was a temperature effect on the inoculated dead non-sporulating *R. padi* mortality ($Chi^2 = 21.471$, $df = 2$, $p < 0.001$) and it was significantly higher at 7.5 °C compared to 14.0 and 18.0 °C ($p < 0.001$, $p < 0.001$, respectively). The temperature effect on the mortality of the inoculated dead non-sporulating *R. padi* was not significantly dependent on the isolate ($Chi^2 = 3.086$, $df = 2$, $p = 0.214$).

3.2. Effect of *Pandora neoaphidis* isolates and temperature on lethal time

Each model describing the LT distribution fitted the data well with an R^2 value exceeding 0.96.

Pandora neoaphidis killed all inoculated dead sporulating *S. avenae* significantly faster ($T = 5.419$, $p < 0.001$) than all inoculated dead sporulating *R. padi,* with an estimated growth rate that was 30% higher for *S. avenae* (LT curve slope k, Equation (1)). However, the time needed for the first sporulating cadavers to occur (curve displacement β, Equation (1)) was not significantly different between the two host species ($T = 0.785$, $p = 0.434$). The estimated LT50 was 116.2 DD for *S. avenae* and 147.7 DD for *R. padi*.

A significant effect of *P. neoaphidis* isolate on LT was detected for both *S. avenae* and *R. padi* (Fig. 2A). Isolate NCRI 460/15 killed both aphid species slower than isolate NCRI 459/15 (parameter k, $T = -3.004$, $p = 0.003$ for *S. avenae* and $T = -5.047$, $p < 0.001$ for *R. padi*, Fig. 2B). However, NCRI 460/15 resulted in a significantly shorter time–to-first-sporulating-cadavers compared to the isolate NCRI 459/15 (parameter β, $T = -2.173$, $p = 0.031$ for *S. avenae* and $T = -2.610$, $p = 0.010$ for *R. padi*, Fig. 2C). The difference in LT between the two isolates was more than twice as big for *R. padi* compared to *S. avenae* (Fig. 2B and C). The estimated decrease in growth rate was 21% for *S. avenae* and 43% for *R. padi.* For *R. padi*, isolate NCRI 460/15 resulted in a significantly shorter time (about 10 times) for the first sporulating cadaver to be observed than for NCRI 461/15 (parameter β, $p = 0.009$, *post hoc* comparison, Fig. 2C). However, isolate NCRI 460/15 killed significantly slower (30%) than NCRI 461/15 (parameter k, Equation (1), $p < 0.001$, *post hoc* comparison, Fig. 2B).

The temperature did not influence LT distribution for any of the aphid species when all the isolates were pooled. Neither how fast the inoculated aphids were killed (the growth rate of the LT distribution: curve slope k), nor the time needed for the first sporulating cadavers to appear (curve displacement β) were significantly influenced ($p > 0.05$) by temperature. When the isolates were studied separately, the time needed for the first sporulating *S. avenae* cadaver to occur (parameter β) did not depend on temperature for any of the isolates ($p < 0.05$). However, temperature significantly influenced how fast *S. avenae* were killed (LT growth rate, parameter k) by each isolate. Isolate NCRI 459/15 (Fig. 3A) killed *S. avenae* significantly faster at 14.0 and 18.0 °C than at 7.5 °C ($T = 3.886$, $p < 0.001$ and $T = 3.138$, $p = 0.003$, respectively) with an estimated increase in k of 60 and 47% respectively. Isolate NCRI 460/15 (Fig. 3B) killed *S. avenae* faster at 7.5 °C ($T = -2.366$, $p = 0.023$) than at 14.0 °C with an estimated increase in the growth rate of 34%. Finally, the isolate NCRI 461/15 (Fig. 3C) killed *S. avenae* faster at 18.0 °C than at 14.0 °C ($p = 0.020$, *post hoc* comparison) with an estimated increase in the growth rate of 30%.

3.3. Effect of *Pandora neoaphidis* isolates and temperature on aphid fecundity

3.3.1. *Sitobion avenae*

The fecundity of 124 inoculated dead sporulating *S. avenae* and 139 surviving *S. avenae* in the control were monitored. Average daily fecundity of inoculated dead sporulating *S. avenae* was not significantly different from average daily fecundity of surviving *S. avenae* in the control ($Chi^2 = 0.051$, $df = 1$, $p = 0.821$ all isolates combined). However, it was influenced by temperature ($Chi^2 = 139.073$, $df = 2$, $p < 0.001$). Fecundity increased with temperature ($p < 0.001$ for all pairwise comparisons). The temperature effect on *S. avenae* fecundity was not influenced by isolate ($Chi^2 = 3.012$, $df = 2$, $p = 0.222$).

Lifetime fecundity of inoculated dead sporulating *S. avenae* was significantly influenced by isolate ($Chi^2 = 37.221$, $df = 3$, $p < 0.001$), and its interaction with temperature ($Chi^2 = 54.759$, $df = 6$, $p < 0.001$). Lifetime fecundity of inoculated dead sporulating *S. avenae* was lower than for surviving aphids in the control ($p < 0.001$ for all pairwise comparisons) with a mean decrease of 51%. At 7.5 °C, lifetime fecundity of sporulating *S. avenae* inoculated with NCRI 460/15 and NCRI 461/15 was lower than lifetime fecundity of surviving *S. avenae* in the control ($p < 0.001$ and $p = 0.002$ respectively), with a mean decrease of 44 and 29% for NCRI 460/15 and NCRI 461/15 respectively (Fig. 4A). At 14 °C, the life time fecundity of sporulating *S. avenae* inoculated with the three isolates was lower than the lifetime fecundity of surviving aphids in the control ($p < 0.001$ for all pairwise comparisons), with a mean decrease of 65, 54 and 51% for NCRI 459/15, NCRI 460/15 and NCRI 461/15 respectively. Furthermore, the lifetime fecundity of dead sporulating *S. avenae* inoculated with NCRI 459/15 was lower than for dead sporulating *S. avenae* inoculated with NCRI 461/15 ($p = 0.047$) with a mean decrease of 29%. We found the same pattern for 18 °C as for 14 °C, and the lifetime fecundity of sporulating *S. avenae* inoculated with the three isolates was lower than the lifetime fecundity of the surviving aphids in the control ($p < 0.001$ for all pairwise comparisons). At 18 °C, the mean decrease in lifetime fecundity was 55, 56 and 64% for NCRI 459/15, NCRI 460/15 and NCRI 461/15 respectively. However, at 18 °C the lifetime fecundity of sporulating aphids inoculated with NCRI 461/15 was lower than for NCRI 461/15 ($p = 0.021$), with a mean decrease of 24%. Finally, the aphid lifetime fecundity was not influenced by the temperature ($Chi^2 = 4.982$, $df = 2$, $p = 0.083$).

Fecundity of 270 inoculated surviving *S. avenae* was recorded. Their average daily fecundity was significantly lower compared to surviving aphids in the control ($Chi^2 = 4.334$, $df = 1$, $p = 0.037$, all isolates combined). This decrease in fecundity was different depending on the isolate ($Chi^2 = 18.672$, $df = 3$, $p < 0.001$). *Post hoc* comparisons showed that average daily fecundity of inoculated surviving *S. avenae* decreased when inoculated with isolate NCRI 460/15 compared to surviving aphids in the control and the

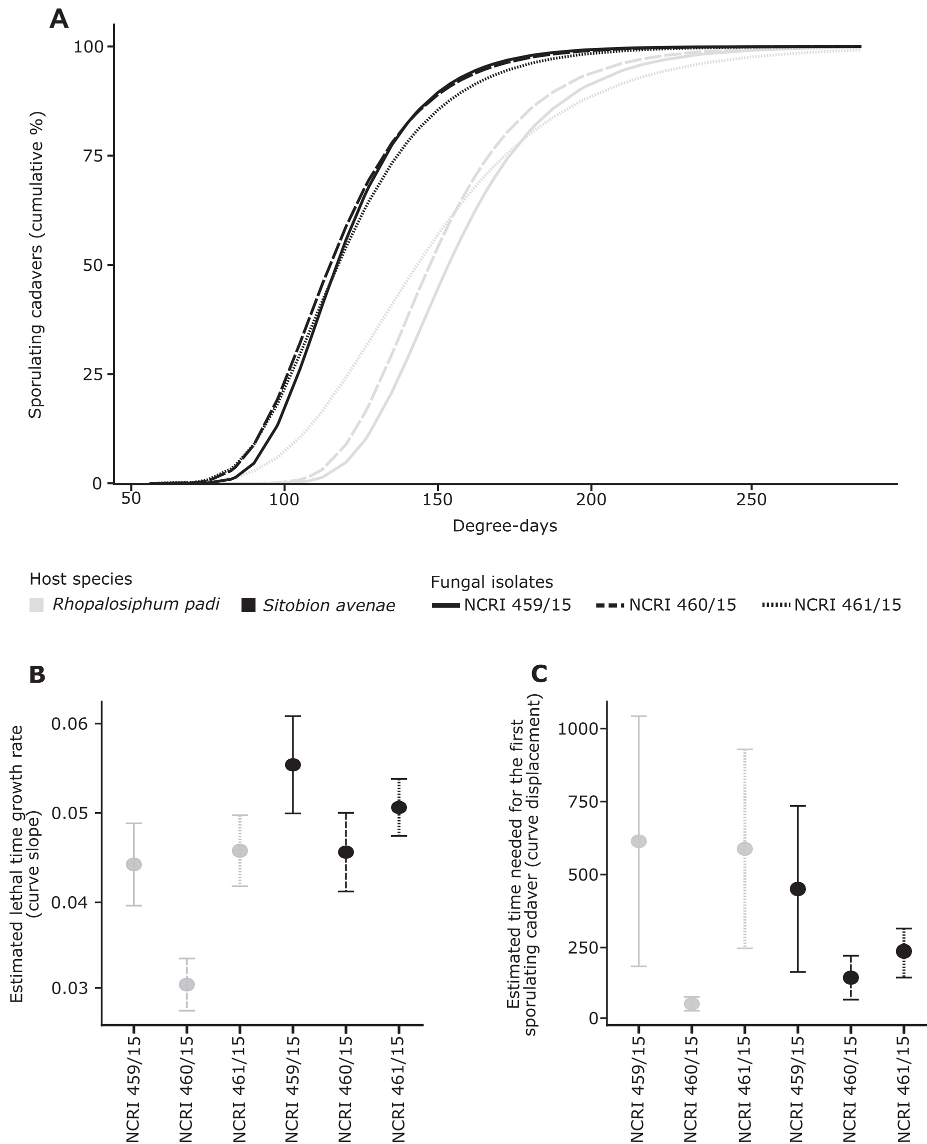

Fig. 2. (A) Fitted lethal time distribution of *Pandora neoaphidis* isolates to *Sitobion avenae* (black lines) and *Rhopalosiphum padi* (grey lines) expressed in cumulative percentage of sporulating cadavers. For each host species, the three *P. neoaphidis* isolates NCRI 459/15, NCRI 460/15 and NCRI 461/15 are represented. Corresponding mean estimates and their 95% confidence interval of (B) the curve slope (growth rate k, Gompertz equation) and (C) the curve displacement (parameter β, Gompertz equation) for each fitted lethal time distribution.

surviving aphids inoculated with NCRI 459/15 ($p = 0.022$ and $p = 0.047$, respectively). Further, temperature influenced average daily fecundity of inoculated surviving *S. avenae* ($Chi^2 = 520.590$, $df = 2$, $p < 0.001$). The higher the temperature the higher the fecundity was observed ($p < 0.001$ for all of the pairwise comparisons). However, the effect of temperature depended on the *P. neoaphidis* isolate aphids were inoculated with ($Chi^2 = 31.042$, $df = 6$, $p < 0.001$) (Fig. 5A). At 7.5 °C, the fecundity of the surviving *S. avenae* inoculated with isolate NCRI 460/15 was lower than the fecundity of surviving *S. avenae* in the control and the surviving aphids inoculated with NCRI 459/15 and 461/15 ($p < 0.001$, $p < 0.001$ and $p = 0.044$ respectively). At 14.0 °C, no significant difference was observed in the average daily fecundity between surviving *S. avenae* in all combinations, inoculated or not. At 18.0 °C, the fecundity of surviving *S. avenae* inoculated with NCRI 461/15 was lower than the fecundity of surviving aphids in the control and surviving aphids inoculated with NCRI 459/15 ($p = 0.004$ and $p = 0.010$ respectively).

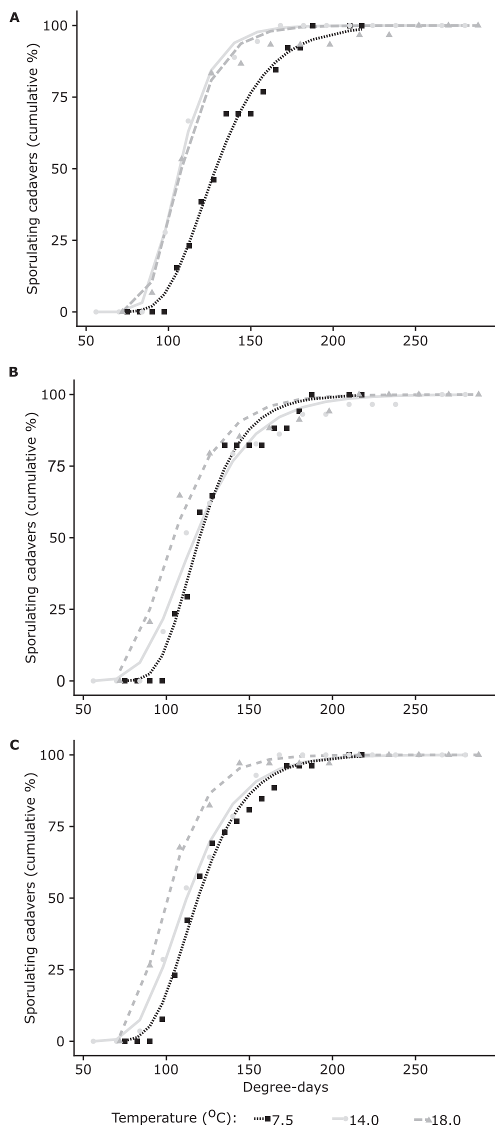

Fig. 3. Effect of temperature per degree-day on cumulative sporulation percentage of three *Pandora neoaphidis* isolates (A) NCRI 459/15, (B) NCRI 460/15, and (C) NCRI 461/15 from fungus killed *Sitobion avenae*. The lines represent the fitted Gompertz equations and the points represent the observed values.

Lifetime fecundity of inoculated surviving *S. avenae* was significantly influenced by isolate ($Chi^2 = 19.005$, $df = 3$, $p < 0.001$), temperature ($Chi^2 = 186.572$, $df = 2$, $p < 0.001$) and their interaction ($Chi^2 = 25.461$, $df = 6$, $p < 0.001$). Lifetime fecundity of surviving *S. avenae* inoculated with NCRI 460/15 was lower than for surviving aphids in the control and for NCRI 459/15 and NCRI 461/15 ($p < 0.001$, $p < 0.001$ and $p = 0.035$ respectively) with a mean decrease in lifetime fecundity of 16, 13 and 8% respectively. At 7.5 °C, the lifetime fecundity of surviving *S. avenae* inoculated with NCRI 460/15 and NCRI 461/15 was lower than the lifetime fecundity of surviving *S. avenae* in the control ($p < 0.001$ and $p = 0.002$ respectively), with a mean decrease of 20 and 5% for NCRI 460/15 and NCRI 461/15 respectively (Fig. 4B). At 14 °C, no significant differences were found between the different treatments ($p > 0.05$). At 18 °C, the lifetime fecundity of surviving *S. avenae* inoculated with NCRI 461/15 was lower than for inoculated *S. avenae* with NCRI 459/15 and for surviving *S. avenae* in the control ($p = 0.020$ and $p = 0.041$, respectively), with a mean decrease in lifetime fecundity of 19 and 12% for respectively.

3.3.2. *Rhopalosiphum padi*

Fecundity of 17 inoculated dead sporulating *R. padi* and 98 surviving *R. padi* in the control was monitored. Average daily fecundity of all the inoculated dead sporulating *R. padi* (all isolates together) was not significantly different from fecundity of the surviving *R. padi* in the control ($Chi^2 = 1.282$, $df = 1$, $p = 0.258$). However, average daily fecundity was influenced by the temperature ($Chi^2 = 210.539$, $df = 2$, $p < 0.001$). The higher the temperature, the higher the fecundity ($p < 0.001$ for all pairwise comparisons).

Lifetime fecundity of inoculated dead sporulating *R. padi* was significantly lower than for surviving aphids in the control ($Chi^2 = 101.540$, $df = 1$, $p < 0.001$), with a mean decrease of 51%. Furthermore, *R. padi* lifetime fecundity was significantly influenced by the temperature ($Chi^2 = 130.050$, $df = 2$, $p < 0.001$) and was lower at 7.5 °C than at 14 °C and 18 °C ($p < 0.001$ for both comparisons). For both analyses, interaction between temperature and the inoculation of *P. neoaphidis* (all isolates together) and the effect of each isolate was not investigated due to low *R. padi* cadaver numbers.

The fecundity of 256 inoculated surviving *R. padi* was monitored. Temperature significantly influenced the fecundity ($Chi^2 = 228.082$, $df = 2$, $p < 0.001$). The higher the temperature the higher the fecundity ($p < 0.001$ for all pairwise comparison). Neither *P. neoaphidis* inoculation ($Chi^2 = 3.403$, $df = 1$, $p = 0.065$), nor interaction between temperature and inoculation ($Chi^2 = 4.477$, $df = 2$, $p = 0.106$) significantly affected average daily fecundity of inoculated surviving *R. padi* compared to surviving *R. padi* in the control (Fig. 5B).

Lifetime fecundity of surviving *R. padi* was also not significantly influenced by either the fungal inoculation ($Chi^2 = 0.092$, $df = 1$, $p = 0.762$) or by the interaction between inoculation and temperature ($Chi^2 = 5.869$, $df = 2$, $p = 0.053$; Fig. 4C). However, it was significantly influenced by temperature ($Chi^2 = 409.352$, $df = 2$, $p < 0.001$). Aphid lifetime fecundity was lower at 7.5 °C than at 14 °C and 18 °C ($p < 0.001$ for both comparisons).

When the first sporulating *R. padi* cadavers occurred, 70% of inoculated dead non-sporulating *R. padi* had died (Fig. 6A). Therefore, the fecundity of 173 inoculated dead non-sporulating *R. padi* was compared to the fecundity of 53 *R. padi* that died in the control in order to reveal a possible ongoing infection processes hidden by the early death of the inoculated dead non-sporulating *R. padi*. Isolate effect on the average daily fecundity of inoculated dead non-sporulating *R. padi* was not significant ($Chi^2 = 0.370$, $df = 3$, $p = 0.946$) when compared to average daily fecundity of dead *R. padi* in the control. However, average daily fecundity of inoculated dead non-sporulating *R. padi* was influenced by temperature ($Chi^2 = 132.343$, $df = 2$, $p < 0.001$) and the interaction between isolates and temperature ($Chi^2 = 41.763$, $df = 6$, $p < 0.001$). There was a similar fecundity at 7.5 °C between inoculated dead non-sporulating and dead *R. padi* in the control ($p > 0.05$) (Fig. 6B). At 14.0 °C, the fecundity of dead non-sporulating *R. padi* inoculated with NCRI 459/15 and NCRI 460/15 was significantly lower than for the control ($p = 0.048$, $p = 0.001$, respectively) and at 18.0 °C, the fecundity of dead non-sporulating *R. padi* inoculated with NCRI

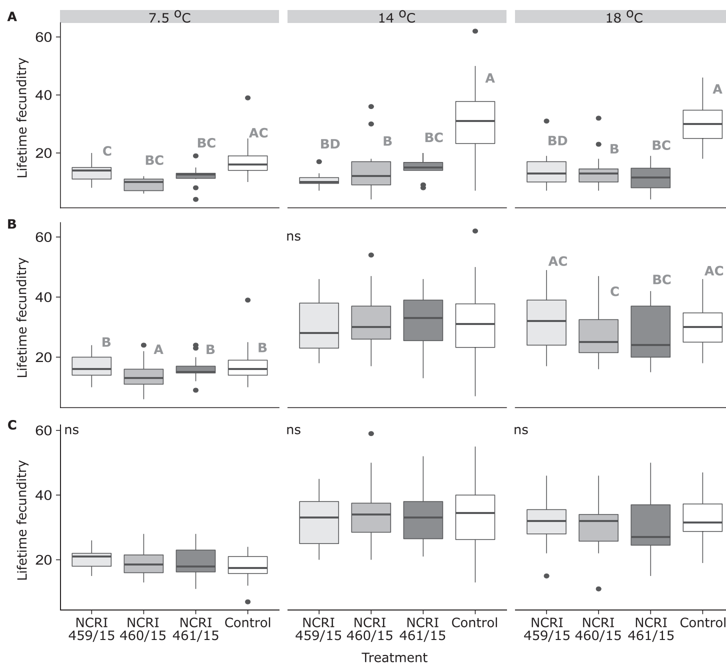

Fig. 4. Interaction effect of temperature and *Pandora neoaphidis* isolates on lifetime fecundity of (A) *Sitobion avenae* that became sporulating cadavers (inoculated dead sporulating) compared to aphids still alive at the end of the experiment in the control (control surviving), (Similar analysis of this interaction could not be investigated for *Rhopalosiphum padi* due to low sporulating cadaver numbers.), (B) *S. avenae* that survived the inoculation (inoculated surviving aphids) compared to aphids still alive at the end of the experiment in the control (control surviving), and (C) *Rhopalosiphum padi* that survived the inoculation (inoculated surviving aphids) compared to aphids still alive at the end of the experiment in the control (control surviving). The boxplots represent the interquartile range (distance between 25 and 75% quantiles), the black line the median and the vertical lines span the largest and smallest value no further than 1.5 x interquartile range. Dots indicate outliers. Results are based on GLMM and uppercase letters indicate comparisons obtained by *post hoc* estimated marginal means analysis ($p < 0.05$). “ns” indicates non-significant difference among all the groups tested.

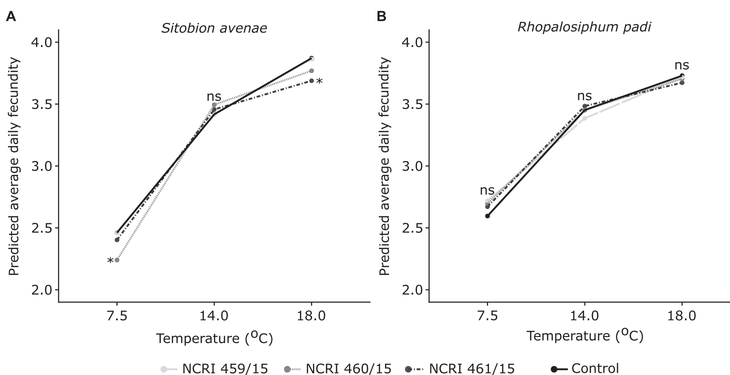

Fig. 5. Interaction effect of temperature and *Pandora neoaphidis* isolates on estimated average daily fecundity of aphids that survived the inoculation and are still alive at the end of the experiment (inoculated surviving aphids). (A) *Sitobion avenae* and (B) *Rhopalosiphum padi*. Estimations are based on GLMM and comparison between estimates based on a *post hoc* estimated marginal means analysis. * indicates a significant difference between the isolates and the control ($p < 0.05$). “ns” indicates a non-significant difference.

460/15 and NCRI 461/15 was lower than for NCRI 459/15 and for the control ($p < 0.001$ for both comparisons). Temperature influenced both the fecundity of inoculated dead non-sporulating *R. padi* and dead *R. padi* in the control ($p < 0.001$). The higher the temperature, the higher the fecundity ($p < 0.001$ for all pairwise comparisons).

4. Discussion

We showed that *P. neoaphidis*, collected from *S. avenae*, can infect and kill both *S. avenae* and *R. padi*. However, *P. neoaphidis* was much less virulent to *R. padi*. Firstly, more *S. avenae* sporulating

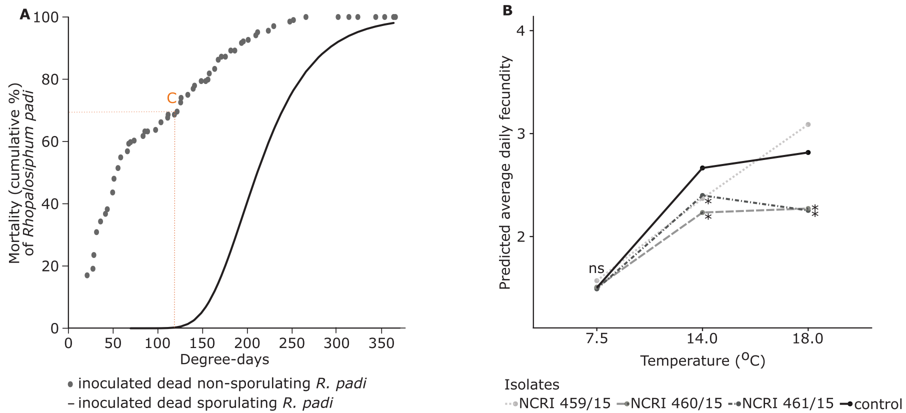

Fig. 6. (A) Cumulative percentage of inoculated *Rhopalosiphum padi* that died without fungal growth (inoculated dead non-sporulating aphids) and estimated cumulative percentage of inoculated dead sporulating *R. padi* (all isolates together) per degree-days. Point C indicates that 70% of the final mortality was achieved in all inoculated *R. padi* when the first sporulating cadavers occurred. (B) Interaction effect of temperature and *Pandora neoaphidis* isolate on estimated average daily fecundity of inoculated dead non-sporulating *R. padi*. Estimations based on GLMM and comparison between estimates based on a *post hoc* estimated marginal means analysis. * indicates a significant difference between the isolates and the control ($p < 0.05$). "ns" indicates a non-significant difference.

cadavers were produced. This is consistent with Shah et al. (2004), who studied aphid susceptibility and median lethal concentration of 20 mostly European *P. neoaphidis* isolates collected from diverse pest and non-pest aphids. They reported *R. padi* to be among the least susceptible aphid species compared to *S. avenae* and five other species (*A. pisum*, the black bean aphid *Aphis fabae,* the rose-grain aphid *Metopolophium dirhodum* and *M. persicae*). Secondly, we showed that *P. neoaphidis* killed *S. avenae* 30% faster (LT growth rate) than *R. padi* and resulted in a lower LT50 for *S. avenae* (116.2 DD) than for *R. padi* (147.7 DD). To our knowledge, no comparison of LT modelled with Gompertz equations has been done among the species we studied. Nielsen et al. (2001) infected *R. padi* and *S. avenae* at 18 °C with a Danish *P. neoaphidis* isolate collected from *R. padi*. This isolate resulted in a lower LT50 for *R. padi* (81 DD (our calculation)) than for *S. avenae* (93.6 DD (our calculation)). This may indicate that *P. neoaphidis* isolates from *R. padi* are more virulent to *R. padi,* but the authors did not conduct any statistical comparisons to confirm this. Thirdly, it is only when comparing inoculated surviving *S. avenae* (inoculated with, but not killed by *P. neoaphidis*) to surviving aphids in the control that we found a small decrease in average daily fecundity. The small decrease in average daily fecundity resulted in a small decrease of the lifetime fecundity of inoculated dead sporulating *S. avenae*, which produced on average 7% less offspring than surviving aphids in the control. A decrease in host average daily fecundity can be interpreted as an effect of the energy the host loses due to the infection, called the immune response cost (*e.g.* Parker et al., 2017). This may indicate that inoculated aphids that did not apparently die from the fungus may have allocated energy to defence responses to the disease rather than using energy to produce progeny. However, since we cannot distinguish aphids surviving the infection from those who escaped it entirely, the immune response cost could be higher than our study indicates. Grell et al. (2011) and Gerardo et al. (2010) suggest that aphids have a reduced immune repertoire and respond only weakly to *P. neoaphidis.* Parker et al. (2017) stabbed *A. pisum* with a needle coated with heat-killed fungal spores and mycelia (non-infectious) of one *P. neoaphidis* isolate collected from *A. pisum* before measuring aphid fecundity. They showed a decreased fecundity in inoculated alate *A. pisum* compared to surviving aphids in the control. As the fungus was non-infectious (heat-killed), the inoculated *A. pisum* in their study could maybe be compared to inoculated surviving *S. avenae* in our study. On the other hand, the complex hierarchy of defence mechanisms to a live and a dead pathogen is probably quite different.

Despite the differences mentioned above, the effect of *P. neoaphidis* was similar for both host species when it came to: (i) the fecundity of inoculated dead sporulating aphids and (ii) the mortality of inoculated dead non-sporulating aphids. Indeed, no effect on the inoculated sporulating aphid average daily fecundity was found for *S. avenae* or *R. padi.* Our results contrast with Baverstock et al. (2006), who calculated the lifetime fecundity over the infection period of inoculated dead sporulating *A. pisum* apterous adults killed by a *P. neoaphidis* isolate, collected from *A. pisum,* at 18 °C. Inoculated dead sporulating *A. pisum* produced 35% less offspring than surviving aphids in the control over the same period of time. However, we showed that for both species there was a significant decrease in lifetime fecundity between inoculated dead sporulating aphids and surviving aphids in the control. Offspring production by inoculated dead sporulating *R. padi* and *S. avenae* was halved compared to surviving aphids in the control (all isolates together). These differences are probably caused by differences in longevity between fungus-killed aphids and surviving aphids in the control since no difference between their average daily fecundity was observed. A similar decrease in lifetime fecundity was shown by Chen and Feng (2006) for inoculated dead sporulating *M. persicae* alates infected with an isolate from unknown origin. The fecundity of inoculated dead sporulating *M. persicae* was reduced by 59% (our calculation) compared to surviving *M. persicae* in the control after 7 d. Also the study by Chen and Feng (2006) suggests that the difference in longevity between inoculated dead sporulating aphids and surviving aphids in the control was the main reason for reduced lifetime fecundity. This decrease in lifetime fecundity could have dramatic importance on the infected aphid capacity to build up colonies and disseminate the disease (e.g. Chen and Feng, 2006). Finally, in our experiment the mortality of inoculated dead non-sporulating aphids was similar to the mortality in the control for both *S. avenae* and *R. padi.* Fungi in the phylum Entomophthoromycota have no or minimal

saprophytic growth since they are considered to be obligate or semi obligate-pathogens. Therefore, our study is consistent with the hypothesis that deadly toxins are probably not produced by *P. neoaphidis* prior to fungal growth in host (*e.g.* Pell et al., 2001).

In our work, *R. padi* exhibits substantial lower susceptibility to *P. neoaphidis* isolates collected from *S. avenae*. However, *R. padi* has been recorded to be infected with *P. neoaphidis* both in cereals (Hatting et al., 2000; Barta and Cagáň, 2006; Chen et al., 2008; Manfrino et al., 2014) and on bird cherry (Barta and Cagáň, 2004; Nielsen and Steenberg, 2004). This suggests a significant variability in *R. padi* susceptibility to *P. neoaphidis*. This may be supported by Parker et al. (2014) who showed that different clones of *A. pisum* vary in susceptibility when infected with a single *P. neoaphidis* isolate. Even though *P. neoaphidis* isolates do not cluster molecularly according to their original host species (Rohel et al., 1997; Tymon et al., 2004; Tymon and Pell, 2005), genetic intra-specific variation among isolates collected from different host species has been identified (Sierotzki et al., 2000; Tymon et al., 2004; Tymon and Pell, 2005; Fournier et al., 2010). This may suggest a variation in virulence between isolates (Tymon and Pell, 2005). We and Shah et al. (2004) did not study isolates originating from *R. padi*. Reyes-Rosas et al. (2012) showed variability in the virulence of isolates collected from the corn aphid *Rhopalosiphum maidis* to the cabbage aphid *Brevicoryne brassicae*. Further, other studies also show both high (e.g. Milner, 1982; Shah et al., 2004) as well as low (e.g. Milner, 1982; Morales-Vidal et al., 2013) virulence of entomopathogenic fungi if tested on other species than the original host. Therefore, *P. neoaphidis* cross-infection between *S. avenae* and *R. padi* could be asymmetrical and needs further investigation to determine if *R. padi* is generally more resistant to *P. neoaphidis* or if it depends on the fungal isolate origin. Information on this would allow estimation of the importance of *R. padi* in the epizootic of the pathogen *P. neoaphidis* in crops with mixed aphid species populations such as cereals.

Regarding the variability of our *P. neoaphidis* isolates, we did not find any difference in numbers of sporulating cadavers between the three *P. neoaphidis* isolates tested for *R. padi*. However, isolate NCRI 461/15 was more virulent to *S. avenae* than isolate NCRI 459/15. Differences in virulence between *P. neoaphidis* isolates have been shown for *A. pisum* by Barta and Cagáň (2009). They reported different median lethal concentration among *P. neoaphidis* isolates collected (i) at the same time in one *S. avenae* population and (ii) at two different dates in one common nettle aphid *Microlophium carnosum* population. Furthermore, our isolates from one fungal population expressed different speeds for killing their aphid hosts, as demonstrated by the LT growth rate. Interestingly, in our study the pattern of LT differences between isolates was consistent between host species, although the magnitude of the difference in *R. padi* was twice that of *S. avenae*. As shown by Bonsall (2004), such differences in LT could have dramatic consequences for the epizootic development of a pathogen in a host population. To our knowledge, no similar study on the effect of temperature to the LT distribution variability among isolates has been conducted on the species studied here. Finally, the suggested immune response cost of inoculated surviving *S. avenae*, shown through a decrease in average daily fecundity and consequently the decrease in lifetime fecundity of inoculated dead sporulating *S. avenae* and *R. padi*, depended on the isolates tested. To our knowledge, no studies have been conducted on the variability of host fecundity among isolates of the same entomophthoromycotan pathogen. However, *P. neoaphidis* isolates collected in one aphid metapopulation have been shown to express different (i) conidia size and fungal biomass production in liquid media (g.l^{-1}) (Sierotzki et al., 2000; Barta and Cagáň, 2009), and (ii) germination rate and sporulation capacity in *in vitro* culture (Sierotzki et al., 2000). These variations could be linked to the variability in virulence that we show in our study. Studying the natural variability of *P. neoaphidis* population in the field in more detail could potentially contribute to estimating its importance in the epizootiology of this fungal species.

Regarding temperature effect on virulence of different *P. neoaphidis* isolates, firstly, we found that *P. neoaphidis* produced more *S. avenae* and *R. padi* sporulating cadavers at 18 and 14 °C, respectively. Temperatures between 18 and 14 °C represent average Norwegian summer temperatures. These results are consistent with other studies that suggest that *P. neoaphidis* is a mesophilic fungus with an optimal temperature around 15–25 °C (Schmitz et al., 1993; Morgan et al., 1995; Stacey et al., 2003; Barta and Cagáň, 2006). Further, our isolates reacted similarly to different temperatures when studying sporulating cadaver production. To our knowledge the effect of the interaction between isolate and temperature on mortality of aphids inoculated with *P. neoaphidis* has not been studied previously. Nevertheless, temperature has been reported to influence differently isolates in the Entomophthoromycota from different geographical origins for *Z. radicans* infecting the diamondback moth *P. xylostella* (Morales-Vidal et al., 2013) and for *Furia gastropachae* infecting the forest tent caterpillar moth *M. disstria* (Filotas et al., 2006). The lack of interaction between temperature and isolate in our study, could be due to the fact that our isolates are from the same geographical origin (same population in one field).

Secondly, when pooling all isolates, no temperature effect on LT was found for any of the aphid species, neither on the LT growth rate (parameter k), nor on time needed for the first sporulating cadavers to occur (parameter β). This is in conflict with other studies that show that the LT50 of *P. neoaphidis* infecting *S. avenae* and *Acyrthosiphon kondoi* decreases when the temperature increases from 2 to 20–25 °C (Milner and Bourne, 1983; Schmitz et al., 1993; Nielsen et al., 2001). However, these authors expressed LT in days and not in DD, and did not use Gompertz equations to model LT distribution. Using DD allows us to focus on the infection process without considering the direct influence that the temperature has on ectotherm species. For instance, if one infection process needs 100 DD to be completed, it should take 10 d at 10 °C or 5 d at 20 °C. If the time in DD changes with temperature then we reveal a temperature effect on the process itself. When our isolates are studied separately, we report a temperature effect on the growth rate of LT with a minimum variation of 30% depending on the temperature. Our *P. neoaphidis* isolates killed *S. avenae* faster under different temperatures. These results suggest that our isolates react differently to temperature.

Finally, we showed that the decrease in aphid average daily fecundity and in their lifetime fecundity depended on the interaction between temperature and isolate, the host species and its health status. The average daily fecundity and lifetime fecundity of inoculated surviving *S. avenae* were slightly reduced at 18 °C when inoculated with NCRI 461/15. At 7.5 °C the average daily fecundity and the lifetime fecundity was reduced only when inoculated with isolate NCRI 460/15. In both cases, the decrease in fecundity occurred at the same temperature as the highest LT growth rate. We, therefore, hypothesize that the immune response cost is higher under more optimal conditions for the fungus to kill its host. Interestingly, when studying *P. neoaphidis* influence on the average daily fecundity of inoculated dead non-sporulating *R. padi*, we also found a significant non-linear temperature effect depending on the isolate. Baverstock et al. (2006), Blanford et al. (2003) and Stacey et al. (2003) found a significant interaction between *A. pisum* clones and temperature on inoculated dead sporulating apterous adult fecundity. Together, these results suggest that *P. neoaphidis* virulence and the host recovery depends on (i) the host and the fungal genotypes as suggested in Lambrechts et al. (2006) and (ii)

their interaction with the temperature as explained in Thomas and Blanford (2003). Consequently, the non-linear effect of temperature on the variability in virulence between isolates could potentially trigger seasonal shifts in the fungal population. It would, therefore, be interesting to study the effect of temperature on *P. neoaphidis* isolates collected from one aphid population but at a different time in the season in order to understand the development and progression of an epizootic.

5. Conclusion

Our study demonstrates three main findings: (i) *P. neoaphidis* collected from one *S. avenae* population infected and killed both *S. avenae* and *R. padi* but it was much less virulent to *R. padi*. Indeed, it produced fewer *R. padi* sporulating cadavers, killed it slower and did not decrease average daily fecundity for either inoculated dead sporulating or inoculated surviving aphids. (ii) *P. neoaphidis* infection caused a decrease in the average daily fecundity of those *S. avenae* that survived the inoculation. This may suggest that *S. avenae* is using energy to combat the infection rather than producing progeny. However, lifetime fecundity of inoculated dead sporulating and inoculated surviving aphids was halved for both host species. (iii) The variability in production of sporulating cadavers between isolates did not depend on temperature but depended on host species. The lowest LT growth rate and decrease in host fecundity occurred at different temperatures according to the isolate and the host species studied. These differences suggest different spread dynamics of the isolates into the two host populations, which can have dramatic consequences for the epidemic development of the pathogen. The non-linear temperature effect on the isolate virulence and sub-lethal effect on the host fecundity emphasises the importance of studying (i) the influence of a realistic range of temperatures on the infection process and (ii) the variability of the isolates present in one fungal population. This information could be useful to understand and model the population dynamics of *P. neoaphidis* and its hosts through the season in order to increase our understanding of its epizootics and its potential use in biological control.

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Paper IV

Paper IV

The population dynamic of *Sitobion avenae* in presence of its fungal pathogen *Pandora neoaphidis* in winter wheat.

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Abstract

Aphids are common pests in cereals, with *Sitobion avenae* being one of the most problematic species in Europe. However, they only have erratic outbreaks, and so many models aim to predict their population development or damage on cereals. Some models estimate the influence of natural enemies (parasitoid and predators) on aphid population dynamics; however fungal pathogen have been mostly forgotten. The most prevalent entomopathogenic fungus attacking *S. avenae* is *Pandora neoaphidis*. Under certain conditions, *P. neoaphidis* can create epizootics that result in collapse of the pest population.

We built a tri-trophic mechanistic model. We modelled host reproduction for both susceptible and infected individuals. We integrated time-delay of the incubation period and for sporulation and we allowed cycles of hydration-rehydration for fungus-killed aphids. However, we did not consider explicitly infective conidia but rather cadaver units based on sporulation capacity. We fixed the aphid inoculum to enable a high host population, in which the fungus could proliferate. This enabled us to overcome any potential host threshold density, if any. Finally, for each simulation, we ran the model twice, with and without the fungus to estimate its biological control.

We aimed to identify, by a sensitivity analysis, the most important parameters of fungus biology and ecology that influence (1) aphid population, (2) fungus population, and the biological control realised by the fungus expressed by (3) a decrease in aphid population and (4) the yield improvement due to this decrease of aphid density.

We showed that the most important parameters depended on the trophic level studied. However, three parameters were always important: the fungus transmission efficiency, the humidity threshold that triggers fungal sporulation and the weather (temperature and humidity). We discuss these results and recommend further studies on some of these parameters.

Keywords: *Erynia neoaphidis*; epizootiology; mechanistic tri-trophic model; host-pathogen interactions; simulation modelling, global sensitivity analysis; Sobol' indices

1 Introduction

Since the 1970's, cereal aphids have become common in Western Europe, with the English grain aphid *Sitobion avenae* being one of the most problematic species (Blackman and Eastop, 2007). Aphids directly damage cereals by sap-sucking and indirectly by (i) transmitting viruses such as the Yellow Dwarf Virus and (ii) hindering photosynthesis by the combination effect of honeydew and fungi reducing the green leaf area (Rabbinge *et al.*, 1981; Wratten, 1975). A high variation in population and outbreaks has been detected between years (Dedryver *et al.,* 2010; Larsson, 2005; Hansen, 2000), with both (i) weather (e.g. Gilabert *et al.,* 2009) and (ii) natural enemies (e.g. Dwyer *et al.,* 2004) commonly identified as potential drivers of oscillations in insect populations. To better understand the system, and in order to optimize the precision of pest management application for crop protection, and to avoid unnecessary effort when there is no risk, a dynamic model would be a useful tool.

In spring, *S. avenae* migrates from its winter host (Poacae) to cereals (*e.g.* Hansen, 2006), colonizes crops and reproduces parthenogenetically with many generations. They have high reproduction and dispersion capacities. Winged and apterous morphs are produced depending on crowding and plant quality (*e.g.* Carter 1982). Before harvest, *S. avenae* emigrates from crops to Poacae. They can either produce sexual morphs and lay overwintering eggs on winter host or continue reproducing parthenogenetically under mild winter conditions (Dedryver *et al.,* 2010).

Aphids have many different natural enemies, including predators, parasitoids and pathogens. The main pathogen group attacking aphids are fungi. Entomophthoraceae is the most important group of fungi causing epizootics. It infects a host with infective spores/conidia that land on a host cuticle. If environmental moisture is high enough, conidia germinate and penetrate inside the host. The fungus multiplies inside its host at a temperature-dependent rate. If the host is susceptible, the infection develops and finally kills the insect. The mummified insect, called a cadaver, sporulates and releases infective conidia in the environment, if moisture levels are high enough. Under certain circumstances, the fungus produces long-lived spores (resting spores), which can enter in pathogen reservoirs. These spores are not infective themselves but may start a new infection after producing infective spores (see full description in Hajek and Meyling, 2018).

In cereals, epizootics occur erratically and locally extinguish aphid populations (*e.g.* Eilenberg *et al.,* 2019). Many models have been published on aphids in cereals, such as *S. avenae*, to understand interactions between aphid population dynamics and (i) weather factors such as temperature, and (ii) its plant host, especially winter wheat (Duffy *et al.,* 2017, Plantegenest *et al.,* 2001; Carter, 1982). Models aim to (1) understand aphid population dynamics and predict outbreaks (Honek *et al.,* 2018; 2016; Duffy *et al.,* 2017; Hansen, 2006;

Carter 1992, Carter and Rabbinge, 1980), (2) estimate their damage on crop yield and quality (Rossing, 1991; Entwistle and Dixon, 1987; Lee *et al.,* 1981) and (3) define agronomic thresholds to treat crops with pesticides only when necessary (Klingen *et al.,* 2008; Larsson, 2005; Oakley and Walters, 1994; George and Gair, 1979; Kieckhefer *et al.,* 1995). However, all of these models only consider aphids and cereals.

In the context of Integrated Pest Management (*e.g.* Barzman *et al.,* 2015), tri-trophic forecasting models that consider the biological control realised by natural enemies will provide additional knowledge to reduce unnecessary treatments. Rabbinge *et al.* (1979) were the first to build a tri-trophic model for wheat, the aphid *S. avenae,* and its natural enemy the hoverfly *Syrphus corollae*. Most tri-trophic models published on cereal aphids focus on aphid predators or parasitoids and some estimate biological control by simulating aphid populations with and without natural enemies and compare pest densities (*e.g.* Maisonhaute *et al*., 2017). However, entomopathogenic fungi in the order Entomophthoraceae have been mostly forgotten, even though they have been used as biocontrol agents in other systems (*e.g.* Hajek and Delalibera, 2010). Brown and Nordin (1982) modelled the population dynamics of *Zoophthora phytonomi* infecting the alfalfa weevil *Hypera postica*. Later, Carruthers *et al.* (1986) modelled the infection of the onion maggot *Delia antiqua* by *Entomphthora muscae*, and Hajek *et al.* (1993) modelled the population dynamics of *Entomophaga maimaiga* infecting the gypsy moth *Lymandria dispar*.

Only two models focused on cereal aphids and Entomophthoraceae (Schmitz *et al*., 1993; Ardisson *et al*., 1997). Schmitz *et al.* (1993) modelled *Pandora neoaphidis* infecting *S. avenae.* They included intermediate stages of host infection to account for delays in the infection cycle. They showed the importance of offspring production by infected hosts, which greatly modifies the disease dynamic. Ardisson *et al.* (1997) continued this work with a model differentiating two stages of cadavers, non-infectious and infectious. They simplified their model by considering environmental conditions to be constant and optimal and by ignoring winged morph production and dispersal. With four differential equations, they proved that oscillations in aphid and fungus populations were possible with epizootics occurring cyclically and separated enzootic periods (low prevalence of the fungus in host population). To our knowledge there is no tri-trophic model including plant host, insect and Entomophthoraceae in the literature, even though host plants may directly or indirectly influence insect-fungal pathogen interactions (see review in Cory and Ericsson, 2010).

We developed a mechanistic tri-trophic model to simulate the daily interactions between *Triticum aestivum* (winter wheat), *S. avenae* (English grain aphid) and *P. neoaphidis* (natural enemy) under prevalent weather conditions. We studied the effect of weather conditions on the tri-trophic system through 40 scenarios defined by historical daily weather records of

ambient temperature and relative humidity. A global sensitivity analysis (Saltelli *et al.,* 2008) was applied to investigate model uncertainty depending on 12 parameters concerning (i) weather (1 parameter, *i.e.* choice of weather file), (ii) crop development (2 parameters), (iii) aphid development (1 parameter) and (iv) host-fungus interactions (8 parameters). Several model response variables were chosen to gauge the importance of the 12 parameters: (i) the aphid and fungus densities, (ii) the reduction in aphid density due to *P. neoaphidis,* (iii) the aphid-induced yield loss. We addressed the following questions: (1) Which of the 12 parameters listed above are the most important for the pest and natural enemy population dynamics and the biological control? (2) Do the most important parameters vary with the output studied? The sensitivity analysis successfully identified the most important parameters common to all outputs and the ones specific to each of them.

2 Material and Methods

2.1 Modelling paradigm

The model follows an object-oriented paradigm in which aphid sub-populations and other model components were represented as objects: software entities that maintain an internal state according to their internal logic receiving inputs and submitting outputs. The model was constructed using the Universal Simulator (Holst 2013, 2019), which provided generic building blocks as well as a framework for coding building blocks specific to this model. Model building blocks were written in C++ and were composed into a hierarchy of interacting objects using the box script language. Simulation outputs were exported to R for visualisation and data analysis. All source code is freely available, together with installation files that will allow anyone to run the model on their own.

The model allows uncertainty in its input parameters. Parameter uncertainty accounts for natural variation in biology and environment, statistical uncertainty in parameter estimates and mechanisms not included in the model. In general, parameter uncertainty was described by the distribution $F_\alpha(x_{min}, x_{max})$ to designate a normal distribution centred around $\mu = (x_{min} + x_{max})/2$ and truncated at $[x_{min}; x_{max})$ to cover only the central $(1 - \alpha)$ part of the normal distribution. F_α will converge toward a uniform distribution as $\alpha \rightarrow 1$. We chose $\alpha = 0.05$ to achieve a central tendency in F_α that matches scientists' intuition about uncertainty. Alternatively, the uniform distribution $U(x_{min}, x_{max})$ was used to choose a random integer value in the interval $[x_{min}; x_{max}]$.

2.2 Model structure

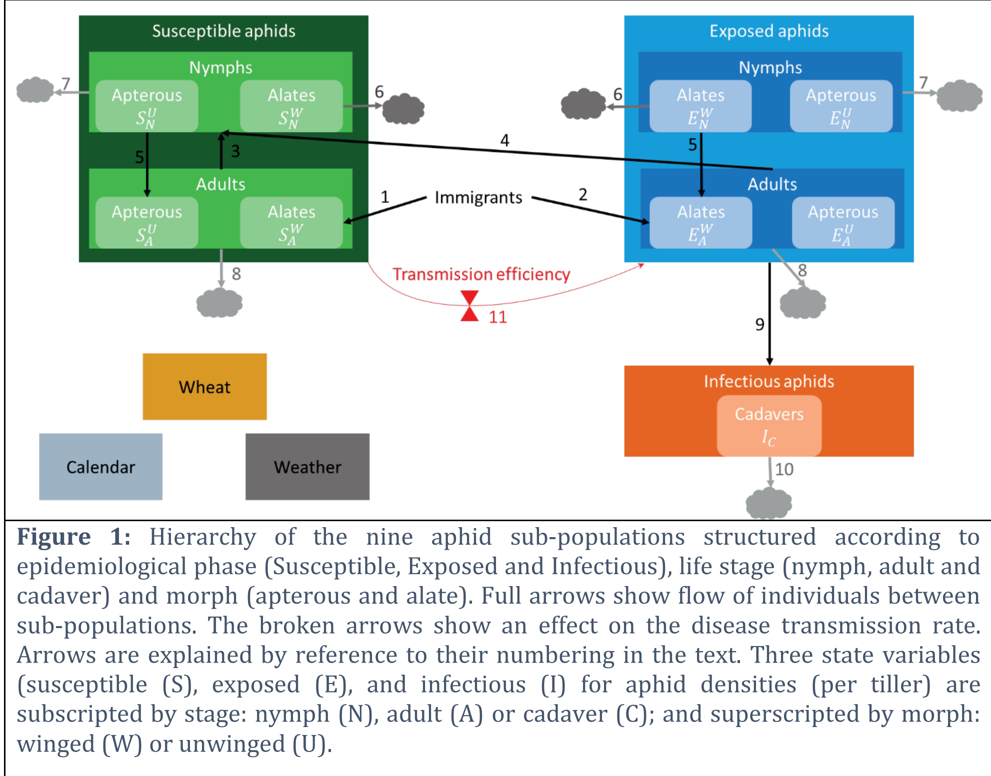

Figure 1: Hierarchy of the nine aphid sub-populations structured according to epidemiological phase (Susceptible, Exposed and Infectious), life stage (nymph, adult and cadaver) and morph (apterous and alate). Full arrows show flow of individuals between sub-populations. The broken arrows show an effect on the disease transmission rate. Arrows are explained by reference to their numbering in the text. Three state variables (susceptible (S), exposed (E), and infectious (I) for aphid densities (per tiller) are subscripted by stage: nymph (N), adult (A) or cadaver (C); and superscripted by morph: winged (W) or unwinged (U).

The most important model building blocks are shown in Fig. 1. Other building blocks provide additional functionality describing fecundity, mortality, morph determination, infection rate, outputs, sensitivity analysis, *etc*. A **calendar** object keeps track of time, which progresses with a time step of 1 day, while **weather** supplies daily weather records, and **wheat** simulates crop development on the Zadoks scale (Zadoks *et al.*, 1974).

Daily fluxes (arrows in Fig. 1) between aphid sub-populations are determined by calendar, ambient temperature and humidity, wheat growth stage and density-dependence. Immigrants provide susceptible (1) and exposed (2) alate adults. Susceptible adults reproduce (3) and give rise to both apteriform and alitiform nymphs (*i.e.* without and with wing buds, respectively). Exposed adults reproduce as well but with a lower reproduction capacity due to the fungal infection (4). Apteriform nymphs develop into apterous adults for both susceptible and exposed aphids (5) whereas alitiform nymphs leave the system when they reach adulthood (6). Nymphs may suffer from mortality (7) while adults die of old age (8). Exposed aphids may turn into cadavers (9), which decay at some rate (10). Susceptible

aphids may become exposed depending on the transmission rate (11). They are removed from the susceptible sub-populations to the corresponding life stage and morph among the exposed sub-populations.

2.3 Aphid development and reproduction

The four sub-populations holding susceptible aphids (susceptible apteriform nymphs, S_N^U; susceptible alatiform nymphs, S_N^W; susceptible apterous adults S_A^U; and susceptible winged adults, S_A^W) and the one with cadavers (I_C) (Fig. 1) were implemented as distributed delays (Manetsch, 1976) which, given an average longevity and a shape parameter (k), produce maturation times following an Erlang distribution, going from a negative exponential at $k = 1$ towards a normal distribution with increasing k. The distributed delay has been used extensively to model physiological development (Gutierrez, 1996). It should be noted that the distributed delay is a deterministic procedure that produces a fixed distribution of maturation times determined by its parameter settings. Maturation time will vary among individuals due to differences in genetics and the experienced microclimate. Earlier modellers have set k to, *e.g.*, 20 (Carruthers *et al.,* 1986) or 30 (Gutierrez *et al.,* 1993; Graf *et al.,* 1990). For all distributed delays, we chose one common $k = U(15,30)$ unless for fecundity (see below).

The attrition parameter was added to the distributed delay model by Vansickle (1977). We set attrition parameter < 1 to account for mortality pertinent to the whole maturation process such as juvenile development. With attrition > 1 we modelled fecundity, in which case 'attrition' is a misnomer at it in effect stands for net reproduction (R_0). For fecundity we set $k = 1$ to obtain a realistic age-dependent fecundity (commonly denoted m_x in life tables).

The four aphid sub-populations holding exposed aphids were implemented as two-dimensional distributed delays, a technique for modelling insect-pathogenic fungi pioneered by Carruthers *et al.* (1986) which includes two orthogonal development processes each following a distributed delay (Larkin *et al.,* 2000).

2.4 Model inputs and outputs

The model was driven by daily average air temperature (T, ºC) and daily maximum relative humidity (H_{max}, %), which were obtained from Agrometeorology Norway (2019). We selected four locations in the cereal production area of southwestern Norway, namely Ramnes (59°25′05″N 10°16′49″E, 116 m a.s.l.), Rygge (59°22′39″N 10°45′01″E, 26 m a.s.l.), Årnes (60°07′20″N 11°28′12″E, 127 m a.s.l.) and Ilseng (60°46′32″N 11°13′38″E, 159 m a.s.l.). We collated 10 years of weather data for each location (2004-2006, 2012-2018). Missing data were interpolated, if there were less than 5 consecutive days without

measurements, or else replaced by corresponding data from the closest weather station. The complete set of weather data allowed us to run model simulations for 40 different scenarios defined by location and year.

We selected four outputs to characterise the outcome of a model simulation: (i) the number of aphid-days (A, d tiller^{-1}), *i.e.* the sum of aphid density over the growing season as calculated by Ruppel (1983); (ii) the number of cadaver-days (C, d tiller^{-1}), *i.e.* the sum of cadaver density over the growing season; and (iii) the yield loss due to aphids (Y_L, %) as a percentage of potential yield expected under conditions (in England) according to Entwistle and Dixon (1987). Each simulation was comprised of two compartments, one with and another without fungus inoculum, allowing us to calculate (iv) the biological control due to the fungus ($\Delta A = A_f - A_0$, d m^{-2}), where A_f and A_0 denote A resulting from the compartment with and without fungus, respectively (*cf.* Maisonhaute *et al.,* 2017). The reduction in yield loss (ΔY_L, % points) due to biological control was calculated similarly as the difference in Y_L between the two compartments.

2.5 Winter wheat sub-model

We developed a phenological model for winter wheat growth stage (GS or G, Zadoks scale) based on three years of Norwegian data. In this model, the crop starts developing in spring after five consecutive days with average air temperature above 5 °C (Korsæth and Rafoss, 2009). Crop development then follows a sigmoid log-logistic function,

$$G = G_0 + \frac{G_{max} - G_0}{1 + \exp\{g(\ln(\tau) - \ln(\tau_{50}))\}} \qquad (1)$$

where G_0 is the crop growth stage reached at the end of winter; G_{max}=99 (secondary dormancy lost) is the final growth stage accounted for in the model; τ is degree-days (°D) above a base temperature of 0 °C; τ_{50} is the inflection point of the sigmoid curve (°D) at $(G_{max} - G_0)/2$; and g is the crop development rate. We fitted this equation to three years data (different wheat varieties and locations) and the three parameters were estimated by non-linear regression. We chose $\tau_{50} = F_\alpha(750, 850)$ °D and $G_0 = F_\alpha(10, 30)$.

2.6 Aphid sub-model

2.6.1 Development

The development rate of *Sitobion avenae* ($\Delta\tau$, °D) was described by a standard degree-day model with a lower threshold for development (T_{min}, °C), only extended with a downward trend between optimum (T_{opt}, °C) and maximum (T_{max}, °C) temperatures,

$$\Delta\tau = \begin{cases} 0 & T < T_{min} \\ (T - T_{min})\Delta t & T_{min} \le T < T_{opt} \\ (T_{opt} - T_{min})\,(T_{max} - T)/(T_{max} - T_{opt})\Delta t & T_{opt} \le T < T_{max} \\ 0 & T \ge T_{max} \end{cases} \qquad (2)$$

where Δt = 1d is the integration time step. We assumed that under Scandinavian conditions, *S. avenae* does not develop under a T_{min} = 3 °C (Hansen, 2006; Dean, 1974) and above a T_{max} = 30 °C (Dean, 1974). In the literature the optimal temperature ranges from 16 to 20 °C (Schmitz *et al.,* 1993; Dean, 1974). We chose T_{opt} = 18 °C.

Based on data from Dean (1974), we estimated that apteriform nymphs spend $L_N^U = 172$ °D to complete their development to adulthood, while alatiform nymphs spend $L_N^W = 195$ °D. Duffy *et al.* (2017) found that apterous adults live on average for 20 days when reared at 10-25 °C. Based on the optimum temperature, we get for apterous adults: $L_A^U = 20$ d·(18 °C–3 °C) = 300 °D.

2.6.2 Aphid immigration

The immigration of winged *S. avenae* is a major factor driving aphid population dynamics during a large part of the season (Jonsson and Sigvald, 2016). Hansen (2006) proposed a migration model for *S. avenae* in winter wheat field based on temperature for Danish conditions. However, trial simulations with his model yielded unrealistic results compared to our field data.

In Norway, *S. avenae* has not been found in winter wheat before stem elongation, GS 31 (unpublished data). When dough formation begins in GS 80, wheat becomes unsuitable for *S. avenae* reproduction (Watt, 1979). Consequently, we modelled *S. avenae* immigration as a constant rate of influx (ΔA_{im}, tiller^{-1} d^{-1}) between GS 31 and GS 80.

The rate of *S. avenae* immigration into cereal fields varies between years and locations. In France, Vialatte *et al.* (2007) measured with a vacuum sampler and found a maximum rate of 15 m^{-2} d^{-1}. In our analysis, we aimed for a pest pressure that could cause a serious outbreak, if not successfully controlled by the fungus *P. neoaphidis*. Hence, we set ΔA_{im}= 0.02 tiller^{-1} d^{-1} since the typical tiller density in Norway is 750 m^{-2} (Einar Strand, pers. comm.). For

simplicity, we considered immigrants as newborn and allocated them the same longevity as apterous adults $L_A^W = L_A^U = 300$ °D.

2.6.3 Aphid reproduction

Sitobion avenae fecundity depends on temperature, wheat GS and morph (apterous or alate). Data from Dean (1974) were used to estimate minimum ($T_{min} = 3$ °C) and maximum ($T_{max} = 30$ °C) temperature for reproduction, while data from Schmitz *et al.* (1993) were used to estimate the optimum temperature ($T_{opt} = 16.1$ °C). We used the same bi-linear equation as for development (eq. 2) to describe temperature-dependent fecundity with an optimum lifetime fecundity R_{opt} = 56.1 reached at T_{opt}.

Data from Watt (1979), which were also used by Carter *et al.* (1982), Plantegenest *et al.* (2001), and Duffy *et al.* (2017), show that *S. avenae* reproduction is enhanced by 60% when the crop is in the growth stage between flowering and milk development (59 ≤ GS ≤ 73); this is due to heightened food quality. Hence in this GS interval, we set R_{opt} = 56.1·1.6 = 89.76. As mentioned above, reproduction stops at GS ≥ 80, *i.e.* R_{opt} = 0. The reproduction of the alate morph is two thirds that of the apterous morph (based on data from Duffy *et al.* (2017). Hence for alates, R_{opt} (computed from temperature and crop GS) was further multiplied by 0.67.

2.6.4 Morph determination

The morph of offspring depends on aphid density and plant food quality. To calculate the proportion of alate offspring ($\alpha \in [0; 1]$), we used the equation of Watt and Dixon (1981), which was also applied by Plantegenest *et al.* (2001) and Duffy *et al.* (2017),

$$\alpha = 0.0260N_{total} + 0.00847G - 0.278 \qquad (3)$$

where $N_{total} = \sum S_i^j + \sum E_i^j$ (tiller^{-1}) is the total density of susceptible (S) and exposed (E) aphids (Fig. 1). Here, i represents the stage (nymph or adult) and j, the morph (apterous or alate). We assumed that alates produced in the field will leave as soon as they develop wings (*cf.* Plantegenest *et al.,* 2001; Duffy *et al.,* 2017). Thus, the only alate adults present in the model are those arriving as immigrants.

2.6.5 Aphid nymph survival

We used the equation of Duffy *et al.* (2017) to take into account the effect of daily average temperature (T, °C) and crop growth stage (G) on nymph survival ($s \in [0; 1]$, d^{-1}),

$$s = \begin{cases} 0.944 - 3.32 \cdot 10^{-10} * \exp(0.726\mathrm{T}) & G < 73 \\ 0.45 & G \geq 73 \end{cases} \qquad (4)$$

This survival rate was used as the attrition parameter in the distributed delay for all nymphs of both morphs (E_N^j, S_N^j) where j denotes morph; see Fig. 1. The fungus causes additional mortality as described below.

2.7 Fungus

2.7.1 The fungal inoculum

Pandora neoaphidis inoculum may enter a *S. avenae* colony from several sources and by several routes (reviewed by Eilenberg *et al.,* 2019). *Sitobion avenae* overwinters as eggs laid at the basis of Poacae plants during cold winters or as adult females still feeding on Poacae under milder conditions (Larsson, 1993). Nymphs newly hatched from overwintering eggs may get in contact with the fungus on the winter host. Later, inoculum can spread with alates invading the field. In wheat fields, aphids are estimated to fall to the ground and climb a straw again at a ratio of 20-35% per day (reviewed in Winder *et al.,* 2013). This promotes the spread of aphids in the field at the risk of picking up soilborne pathogens. Thus Nielsen *et al.* (2003) and Baverstock *et al.* (2008) found that overwintering stages of *P. neoaphidis* (conidia, loricoconidia and hyphal bodies inside cadavers) can remain infective for several months in the soil (depending on environmental conditions).

Conidia are spread by wind and may arrive as inoculum, transported over short or long distances depending on aerodynamic and climatic conditions (Hemmati *et al.,* 2001b; Hajek *et al.,* 1999; Steinkraus *et al.,* 1996). Ekesi *et al.* (2005) showed that conidia of *P. neoaphidis* can disperse passively in the airstream from sporulating aphid cadavers and initiate infections in aphids located within 1 m of the source. Conidia can also be vectored by other natural enemies as they attack both infected and susceptible colonies (*e.g.* Roy *et al.,* 2001).

During the growing season, additional inoculum may arrive carried by infected immigrants entering the field. Chen and Feng (2004a) reported from China that 0 to 68% of immigrating *S. avenae* are infected by *P. neoaphidis*. These infected immigrants have been proven able to initiate colonies before dying from the fungus and to disseminate the disease in the colony (Chen and Feng, 2004b). In addition, Hatano *et al.* (2012) and Tan *et al.* (2018) showed that *A. pisum* infected with *P. neoaphidis* tend to produce more winged offspring than healthy ones. This behaviour may increase the chance of the progeny to escape the fungus, but it may also increase the dispersion of infected winged individuals.

In our model, fungal inoculum arrives via infected immigrants only. Our argument is that alate *S. avenae* are more susceptible to *P. neoaphidis* than apterous adults (Dromph *et al.,* 2002) and that their capacity for dispersion and colonisation is an important factor for the outbreak of epizootics (White *et al.,* 2000). For simplicity, we assumed that a fixed proportion

of all immigrants was infected (δ ∈ [0; 1]). We chose a wide span for this parameter to represent an expected wide variation in fungus inoculum between sites and years, $\delta = F_{\alpha}(0.1, 0.7)$.

2.7.2 Aphids exposed to *P. neoaphidis*

When aphids become exposed to *P. neoaphidis* in the model, they are taken from the four sub-populations of susceptible aphids $\left(S_j^i\right)$ and transferred to the corresponding four sub-populations of exposed aphids $\left(E_j^i\right)$ (Fig. 1).The exposed sub-populations are kept in 2-D distributed delays to allow two concurrent development processes. In one process, development runs in day-degrees based on T_{min}, T_{max} and T_{opt} defined for the aphid (eq. 2). Indeed, infected aphids grow, age and have the same longevity as susceptible aphids. Hence this development process of exposed aphids is equivalent to that of susceptible aphids.

The other development process of exposed aphids describes the progress of the infection. It runs on a day-degree scale of the fungus with its own T_{min}, T_{max} and T_{opt} (eq. 2). The fungus does not germinate, grow or sporulate below T_{min}= 2 °C and above T_{max} = 30 °C (Nielsen *et al.,* 2001). *Pandora neoaphidis* is a mesophilic species with an optimal temperature (T_{opt}) for growth, lethal time and host mortality between 15 and 25 °C (Barta and Cagan, 2006; Stacey *et al.*, 2003; Nielsen *et al.*, 2001; Morgan *et al.*, 1995; Schmitz *et al.,* 1993). For Entomophthoraceae species in general, T_{opt} depends on the climatic origin of the isolate (*e.g.* Klingen and Nilsen, 2009). Klingen and Nilsen (2009) found that for a Norwegian strain of *Neozygites floridana*, sporulation was higher at 13 and 18 °C compared to 23 °C. To summarise, we set T_{opt} = 18 °C for *P. neoaphidis*.

The time *P. neoaphidis* needs to kill its host is called the lethal time (L_{lethal}, °D). It is highly variable. The median lethal time ranges from 73 to 115 °D (calculated from Saussure *et al.,* 2019; Nielsen *et al.,* 2001; Schmitz *et al.,* 1993). The lethal time differs between *S. avenae* nymphs and adults (Schmitz *et al.,* 1993) but is the same for apterous *vs.* alate morphs of *S. avenae* (Dromph *et al.,* 2002). We chose a range of lethal times to reflect this variability $L_{lethal} = F_{\alpha}(50{,}115)$ °D and applied this across all host life stages and morphs. For those immigrants that arrive already exposed (Fig. 1), we assumed that their exposure was quite recent. Thus, they outlived the whole lethal time on the wheat.

Exposed nymphs may turn into either cadavers or exposed adults. A laboratory experiment on *S. avenae* infected with *P. neoaphidis* showed that exposed nymphs do not reproduce if they reach adulthood (Schmitz *et al.*, 1993). This detail was included in the model, but it is not shown in the model diagram (Fig. 1).

2.7.3 Immunity cost and reproduction capacity of exposed aphids

Exposed *S. avenae* adults can reproduce but most likely at a reduced rate. Thus, infection with *P. neoaphidis* reduces fecundity from 0 to 35% depending on fungal isolate and aphid species (Saussure *et al.,* 2019; Parker *et al.,* 2017; Baverstock *et al.,* 2006). We included this immunity cost ($\nu \in [0;1]$) as a reduction in life time fecundity of exposed compared to susceptible adults. We chose $\nu = F_{\alpha}(0, 0.4)$.

2.7.4 The cadaver unit

When exposed aphids succumb to the infection they turn into cadavers. Cadavers of alate *S. avenae* will produce fewer conidia than those of the apterous morph (Hemmati *et al.,* 2001a). We expect nymphs to produce less conidia than adults due the size difference. Hence, we enumerated the cadaver sub-population in standardised 'cadavers units' counting cadavers as 1 (apterous adults), 0.66 (alate adults) and 0.5 (nymphs). Cadavers are kept in the 1-D distributed delay I_c (Fig. 1).

2.7.5 Non-sporulating and sporulating cadavers

Cadavers are disappearing at a rate that depends on both temperature and moisture. We expressed temperature-dependency on the same day-degree scale as for fungus development in exposed aphids (2.7.2), *i.e.* using eq. 3 with $(T_{min}, T_{opt}, T_{max}) = (2, 18, 30)$ °C. We do not know the longevity of aphid cadavers (L_c). Grasshopper cadavers infected with *Entomophaga grylii* have a median longevity in the field of 2.8 days while 5% last 12.3 days (Sawyer *et al.,* 1997). We chose a longevity of 3-7 days at 18 °C giving $L_c = F_{\alpha}(48, 112)$ °D.

Grasshopper cadavers can go through cycles of dehydration and rehydration according to moisture conditions (Sawyer *et al.,* 1997). We assumed that aphid cadavers also go through such cycles during their lifetime (L_c), producing spores whenever they are hydrated. At 20°C, *S. avenae* cadavers may sporulate for a total period of 2 days (Ardisson *et al.,* 1997) and *Acyrthosiphon pisum* cadavers for 3 days (Bonner *et al.,* 2003). When a cadaver has exhausted its capacity for spore production, it has finished its role, which means that under high moisture conditions it will last shorter than expressed by L_c, which only depends on temperature. We accommodated this effect not by adjusting L_c but by accelerating the development time step ($\Delta\tau$, eq. 3) by a factor (h) under high moisture. We chose $h = F_{\alpha}(1, 3)$.

To trigger sporulation (and germination, see 2.7.6), Entomophthoraceae need a high moisture environment, corresponding to a relative air humidity $H > 80$ % or even $H =$ 100%, depending on the species (see review in Sawyer *et al.,* 1997). The model works with a daily time step but H can vary dramatically during a day, and *P. neoaphidis* needs only 3 h at 18 °C with $H = 95\%$ to sporulate (Ardisson *et al.,* 1997). Therefore, we chose to compare the

daily maximum relative humidity (H_{max}, %) against a threshold value (H^*_{max}, %). For any day with $H_{max} > H^*_{max}$, sporulation was assumed to be ongoing and the acceleration factor h applied on $\Delta\tau$. To reflect uncertainty in the relation between ambient relative humidity and the moisture experienced by the fungus we set $H^*_{max} = F_\alpha(80, 99)$ %.

2.7.6 Virulence and transmission efficiency

The spread of spores from cadavers to susceptible aphids within and between colonies drives the spread of the disease in the host population (Steinkraus, 2006; Sawyer *et al.,* 1994; Steinkraus *et al.*, 1993). Depending on the virulence of the fungus towards its host, spores may have more or less success with infecting a susceptible host. Like Ardisson *et al.* (1997) we describe the whole process of disease transmission by one parameter: the transmission efficiency (ϵ, d^{-1}). Under laboratory conditions with one cadaver per 10 *S. avenae*, they estimated ϵ = 0.0072 h^{-1} = 0.1728 d^{-1}. The aphids used were a mix of life stages kept at a high density (20 per tiller), which would tend to produce alates. The estimate of ϵ can, therefore, be regarded as an average across all stages and morphs.

The simplest model for disease transmission found in the literature is linear. In effect it is a Lotka-Volterra model,

$$\Delta E_i^j = \epsilon I_c S_i^j \Delta t \qquad (5)$$

which computes the density of newly exposed hosts (ΔE_i^j) from the transmission efficiency and the densities of cadavers (I_c) and susceptible aphids (S_i^j) over a time period (Δt) for stage i and morph j (Fig. 1). However, we used the more realistic, classical functional response model of Nicholson and Bailey (1935),

$$\Delta E_i^j = \Delta S_i^j (1 - \exp(-\epsilon I_c \Delta t)) \qquad (6)$$

This model, traditionally used to describe the attack rate of parasitoids, sets a necessary limit to the number of newly infected hosts ($\Delta E_i^j \leq \Delta S_i^j$). Sporulation and spore germination happens only under high moisture conditions. Thus for $H_{max} \leq H^*_{max}$, we set $\epsilon = 0$ d^{-1}, otherwise $\epsilon = F_\alpha(0.05, 0.5)$ d^{-1}. This interval of values includes the estimate of Ardisson *et al.* (1997) and has been widened to account for the many biological processes distilled into just one parameter.

2.8 Sensitivity analysis

The model contains (i) several parameter values based on the literature even if a high variability has been shown in different studies and (ii) arbitrarily chosen parameter values. These values are of different quality and they also differ in how strongly they affect model output. In a sensitivity analysis, the importance of the parameters will be detected. The parameters identified as highly sensitive are the key drivers of the model and effort should be made on the selection of values for these parameters. The less sensitive parameters will almost not affect the model output. These parameters can be fixed within their prior boundaries without affecting the model output, and it is not very important to the output to fix these parameters. We used the Sobol method developed by Saltelli *et al.* (2008). For each parameter studied, two indices were calculated: (1) the importance of the first order effect of the parameter variation on the output variation, and (2) the importance of interactions between variation of the studied parameter and the other parameters. This latter estimation is done indirectly by calculating a total index (first order and interaction effect), and the first order index for each parameter. The difference between the total index and the first order index is the estimation of the interaction importance. Finally, the sum of all first order effects and the sum of all total effects were calculated. The difference between the two sums informs us on the influence of interactions between all the parameters on the output variation.

A total of 12 parameters and the choice of the weather file were implemented in the sensitivity analysis. The parameters studied were linked to the infection process: (1) humidity threshold at which fungal sporulation is triggered, (2) longevity of cadaver, (3) fungus transmission efficiency, (4) proportion of infected aphid immigrants landing daily in our system, (5) fungal lethal time, and (6) immune response cost of the infection. We also ran the sensitivity analysis on two parameters linked to the wheat variety and autumnal development, and on climatic conditions by running the model with different climatic datasets obtained during different years and different locations. Finally, we included the parameter shape k in our analysis. The sensitivity analysis was run on the four model outputs: aphid and fungus populations, the decrease in aphid population due to the fungus and the yield improvement due to the fungus. The range of value studied for each parameter are presented in Table 1 and a total of 15 000 simulations were performed.

Table 1: Variation range of parameter values investigated in the Sobol sensitivity analysis of our tri-trophic model. Name, default value, range of variation, and description of the parameters are listed along with the references justifying our choice.

Name	**Symbol**	**Default value (unit)**	**Range**	**Description**	**References**
fileNumber	-	Rygge 2017	40 scenarii	40 weather files with daily average temperature and maximum relative humidity	Agrometeorology Norway (2019)
CropStart	G_0	20 (GS)	10-30	Wheat GS at the end of winter	Norwegian data
CropHalfway	τ_{50}	750 (GS)	750-850	Wheat GS of maximal growth rate	Norwegian data
SporulationOn	H^*_{max}	95 (%)	80-99	Relative humidity threshold for fungal germination and sporulation	See review in Sawyer *et al.*, 1997
cadaverDuration	L_c	112 (ºD)	32-112	Longevity of aphid cadavers under dry conditions	Sawyer *et al.*, 1997
timeAcceleration	h	2.33	1-3.5	Acceleration of the daily time step of fungal development for sporulating aphid cadavers when H > H^*_{max}	Bonner *et al.*, 2003; Ardisson *et al.*, 1993; Brobyn *et al.*, 1985
propExposedImm	δ	50 (%)	10-70	Ratio of daily immigrants infected with *P. neoaphidis*	Chen and Feng 2004a
immuneCost	ν	30 (%)	0-40	Immune response cost *i.e.* reduction of fecundity in exposed aphids	Saussure *et al.*, 2019; Parker *et al.*, 2017; Baverstock *et al.*, 2006
lethalTimeNymph	L_{lethal}	71 (ºD)	50-115	Lethal time of *P. neoaphidis* infecting nymph *S. avenae*	Schmitz *et al.*, 1993
lethalTimeAdult	L_{lethal}	73 (ºD)	50-115	Lethal time of *P. neoaphidis* infecting adult *S. avenae*	Saussure *et al.*, 2019; Nielsen *et al.*, 2001; Schmitz *et al.*, 1993
Transmission Efficiency	ϵ^*	0.1728 (tiller d^{-1})	0.05-0.5	Transmission efficiency within and between colonies	Ardisson *et al.*, 1997
shapeParameter	k	15	15-30	Shape parameter for biological distributions	Gutierrez *et al.*, 1993; Graf *et al.*, 1990; Carruthers *et al.*, 1986

3 Results

3.1 Simulated population dynamics

The outputs of the model simulation obtained with the default parameter values (Table 1) are shown in Fig. 2. Both aphid and fungus-killed cadaver population dynamics were simulated over the growing season by calculating the density per tiller of each organism every day. Aphid immigration started at the end of May and population built up to reach a peak density of 37 aphids per tiller in the middle of July. Fungus inoculum arrived in the system with the first aphid immigrants. The fungal population started increasing significantly at the beginning of July to reach a peak density of 10 cadavers per tiller at the end of July. Aphid and fungus population in the system crashed down at beginning of August due to wheat ripening. The simulation output aphid-day was calculated as the area under the aphid density curve during the whole season. The output cadaver-day was estimated the same way from the fungus population. This default simulation gave a total aphid-day at 875, while cadaver-day was 101. Finally, aphid damages were estimated every day as a percentage of yield loss depending on aphid density per tiller. The final yield loss at the end of the season was the third output given by the model. The total yield loss at the end of the season for the default simulation was 91% of the expected yield.

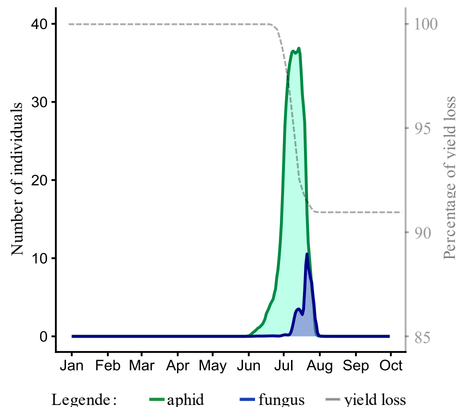

Figure 2: Output of a typical model run. Drak green line: aphid density (m^{-2}). Aphid-days are calculated as the area under the curve (pale green). Dark blue line: cadaver unit density (m^{-2}). Cadaver-days are calculated as the area under the curve (pale blue). Grey dashed line: percentage of yield loss due to aphid damage (Entwistle and Dixon 1987).

3.2 Correlations between trophic levels

The sensitivity analysis was run with 15 000 simulations, each of which used a unique parameter set chosen randomly from the ranges defined in Table 1. Each simulation gave a value for each output: aphid-day, cadaver-day, aphid-day controlled by the fungus ($\Delta A = A_f - A_0$, d m^{-2}, Section 2.4.) and total yield improvement due to the fungus (ΔY_L, % points, Section 2.4.). Correlations between model outputs are shown in Fig. 3. with each point representing one run. Over the 15 000 simulations, cadaver units varied from nearly 0 to 271 (Fig. 3). Aphid-day, which varies between nearly 0 to 1,562, was negatively correlated to cadaver-day (Fig 3A). The decrease in aphid-days due to the fungus called aphid improvement was positively correlated to the number of cadaver-days (Fig. 3B). Aphid-day improvement varied between almost 0 and 1,081. Finally, yield improvement was positively correlated with cadaver-day (Fig. 3C). Yield improvement ranged from 0 to 8.8 %.

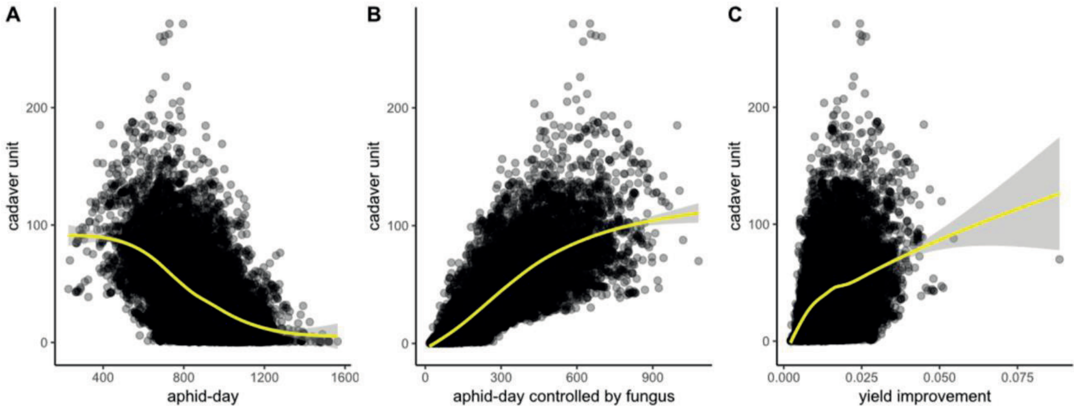

Figure 3: Correlations between model outputs after 15 000 simulations A) between cadaver units and aphid-days, B) between cadaver units and aphid-days improvement due to the fungus, and C) cadaver units and yield improvement. The yellow line is a gam trend line.

3.3 Sensitivity analysis of pest and natural enemy populations

Controlled aphid population

The sensitivity analysis explored the space within the range of parameters expressed in Table 1. The summary statistics of total and first order effects of each parameter were calculated. For each parameter, the total effect on model output variation was calculated as the sum of its first order (or main) effect and its interaction with other parameters. The sum of total effects and first order effects on output variations were both calculated to estimate the importance of interactions between parameters (parameter Sum in figures). Significant parameters are ranked according to their order of importance.

A total of eight parameters significantly influenced the model output aphid-days (Fig. 4). They were in decreasing order of importance: (1) the fungal transmission efficiency (transmissionEfficiency), (2) the weather file (fileNumber), (3) the wheat growth stage at beginning of spring (cropAtStart), (4) the relative humidity threshold (sporulationOn), (5) the cadaver longevity (cadaverDuration), (6) the proportion of infected immigrants (propExposedImmigrants), (7) the acceleration of the cadaver development rate when sporulating (timeAcceleration), and finally (8) the lethal time for nymphs (lethalTimeNymph). All parameters had a significant total and first order effect on controlled aphid-days ($p \leq 0.002$). Few interactions between parameters occurred.

For each highly sensitive parameter, correlations between the model output aphid-day and the parameter value investigated in the sensitivity analysis are shown in Fig. 5. Each point represents the individual model output plotted against the value taken by the studied parameter. A trend line using gam formula and the normal confidence interval is shown. There was a negative correlation between aphid-days and (1) transmissionEfficiency (Fig 5A), cadaverDuration (Fig. 5D) and propExposedImmigrant (Fig. 5E). On the contrary, aphid-days increased with (1) cropAtStart (Fig. 5B), sporulationOn (Fig. 5C), and timeAcceleration (Fig. 5F). The influence of fileNumber is presented with heatmaps in Fig. 12 where, the median aphid-day per weather file (fileNumber) was calculated for each fileNumber. Median aphid-day ranged between 680 and 890 depending on the weather file (Fig. 12A).

Four parameters had no significant influence ($p > 0.05$) on aphid-days: the lethal time for adult aphid (lethalTimeAdult), the decrease in aphid fecundity due to the fungal infection (immuneCost), the inflexion point in the wheat growth speed (cropHalfWay), and the parameter k shaping the biological distributions (shapeParameter).

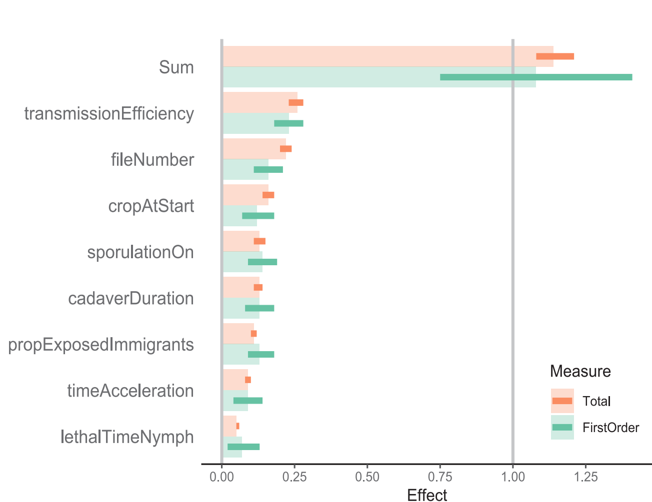

Figure 4: Result from sensitivity analysis showing the most important parameters influencing the model output aphid-days. For each parameter, the total effect was calculated as the sum of its first order effect and its interaction with other parameters. The sum of total effects and first order effects were both calculated to estimate the importance of interactions between parameters on the model output

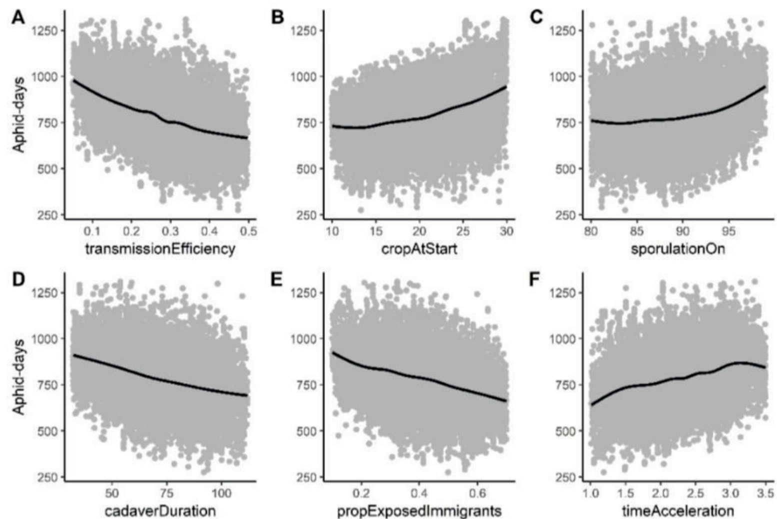

Figure 5: Correlations between the model output aphid-day and A) fungal transmission efficiency, B) wheat growth stage at beginning of spring, C) humidity threshold, D) longevity of fungus-killed cadaver, E) proportion of infected aphid immigrants and F) increase of energy consumption by sporulating cadaver. Each point represents an individual simulation. 15 000 simulations have been run.

Fungus population

Seven parameters significantly influenced the number of cadaver units (Fig. 6). They are in decreasing order: (1) cadaverDuration, (2) timeAcceleration, (3) fileNumber, (4) sporulationOn, (5) transmissionEfficiency, (6) cropAtStart, and (7) lethalTimeNymph. All parameters had a significant total and first order effect ($p < 0.02$) on cadaver units. Interactions occurred between parameter (Sum, Fig. 4) and mostly concerned the first five parameters.

Cadaver units over the whole season increased with (1) cadaverDuration (Fig.7A), (2) transmissionEfficiency (Fig. 7D), and (3) cropAtStart (Fig.7E). However, cadaver units decreased when sporulationOn (Fig. 7C), timeAcceleration (Fig. 7B) and lethalTimeNymph (Fig. 7F) increased. The median cadaver unit per fileNumber ranged from 4.5 to 45 (Fig. 12B).

Five parameters had no significant influence on cadaver units over the season: (1) lethalTimeAdult, (2) propExposedImmigrants, (3) immuneCost, (4) cropHalfWay, and (5) shapeParameter ($p > 0.05$).

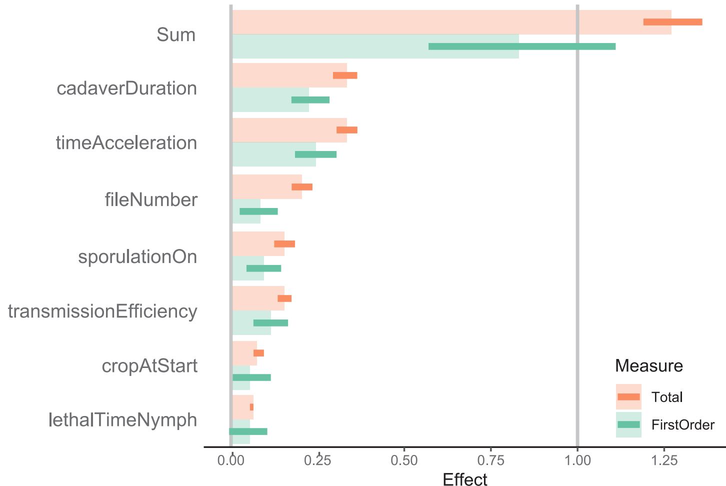

Figure 6: Result from sensitivity analysis showing the most important parameters influencing the model output cadaver-day. For each parameter, the total effect was calculated as the sum of its first order effect and its interaction with other parameters. The sum of total effects and first order effects were both calculated to estimate the importance of interactions between parameters on the model output.

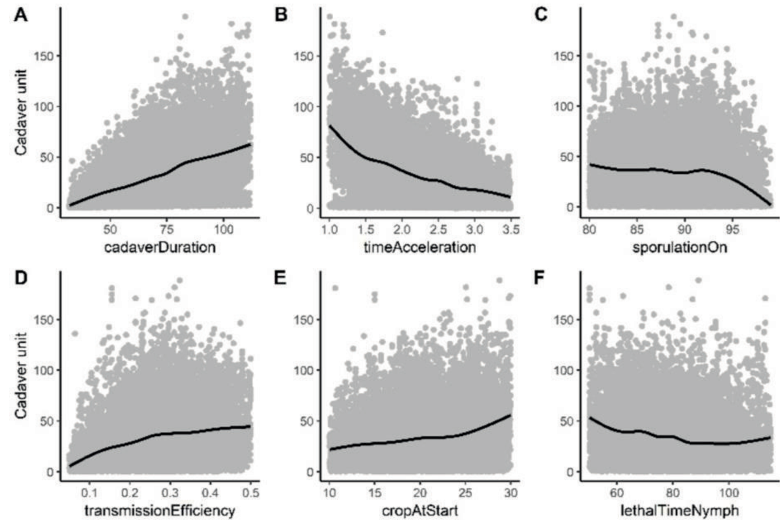

Figure 7: Correlations between the model output cadaver-day and A) cadaver longevity, B) increase of energy consumption by sporulating cadaver, C) humidity threshold, D) fungal transmission efficiency, E) wheat growth stage at beginning of spring and F) lethal time for aphid nymphs. Each point represents an individual simulation. 15 000 simulations have been run.

3.4 Sensitivity analysis and biological control

Decrease of aphid population due to the fungus

For each set of parameters tested, the model was run twice: with and without fungus inoculum. The decrease in aphid-days between the two runs was called aphid-days improvement. Eight parameters influenced aphid-days improvement due to the fungus (Fig. 8). They are in decreasing order of importance: (1) transmissionEfficiency, (2) fileNumber, (3) cadaverDuration, (4) sporulationOn, (5) propExposedImmigrants, (6) timeAcceleration, (7) lethalTimeNymph, and (8) cropAtStart. All parameters had a significant total and first order effect on controlled aphid-days ($p < 0.001$).

The improvement in aphid-days due to the fungus increased with (1) transmissionEfficiency (Fig. 9A), (2) cadaverDuration (Fig. 9C), and (3) propExposedImmigrants (Fig. 9E). However, there was a negative correlation between aphid-day improvement and (1) sporulationOn (Fig. 9B), (2) timeAcceleration (Fig. 9D), and (3) lethalTimeNymph (Fig. 9F). Some interactions occurred between parameters (Sum, Fig. 8) and mostly concerned transmissionEfficiency, fileNumber and sporulationOn. The median aphid-days improvement per fileNumber ranged between 145 and 370 (Fig. 12C).

Four parameters had no significant influence on aphid-days improvement: (1) lethalTimeAdult, (2) immuneCost, (3) cropHalfWay, and (4) shapeParameter ($p > 0.05$).

Damage limitation due to the fungus

Only four parameters significantly influenced the yield improvement due to the fungus: (1) propExposedImmigrants, (2) fileNumber, (3) transmissionEfficiency and (4) sporulationOn (Fig. 10). No interaction occurred between parameters. There was a positive correlation between yield improvement and (1) PropExposedImmigrants (Fig. 11A) and (2) transmissionEfficiency (Fig. 11B). The yield improvement was reduced when sporulationOn increased (Fig. 11C). The median yield improvement per fileNumber ranged from 1.4 to 2.3 % (Fig. 12D).

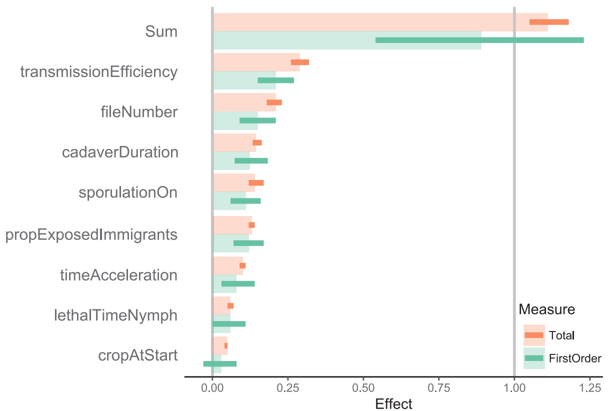

Figure 8: Result from sensitivity analysis showing the most important parameters influencing the model output decrease in aphid-days due to the fungus. For each parameter, the total effect was calculated as the sum of its first order effect and its interaction with other parameters. The sum of total effects and first order effects were both calculated to estimate the importance of interactions between parameters on the model output.

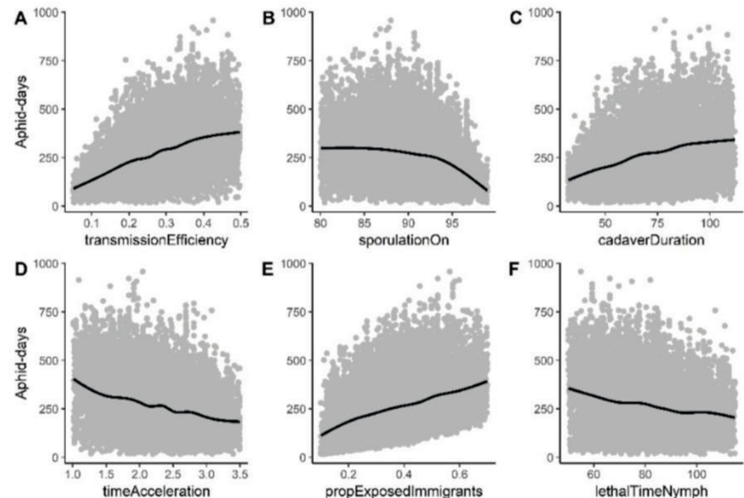

Figure 9: Correlations between the model output aphid-days improvement and A) fungal transmission efficiency, B) humidity threshold, C) cadaver longevity, D) increase of energy consumption by sporulating cadaver, E) proportion of infected aphid immigrants and F) lethal time for aphid nymphs over 15 000 simulations. Each point represents an individual simulation. 15 000 simulations have been run.

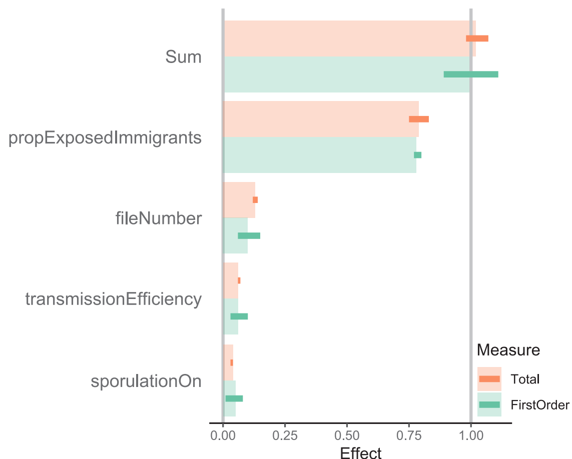

Figure 10: Result from sensitivity analysis showing the most important parameters influencing the model output yield improvement due to the fungus. For each parameter, the total effect was calculated as the sum of its first order effect and its interaction with other parameters. The sum of total effects and first order effects were both calculated to estimate the importance of interactions between parameters on the model output

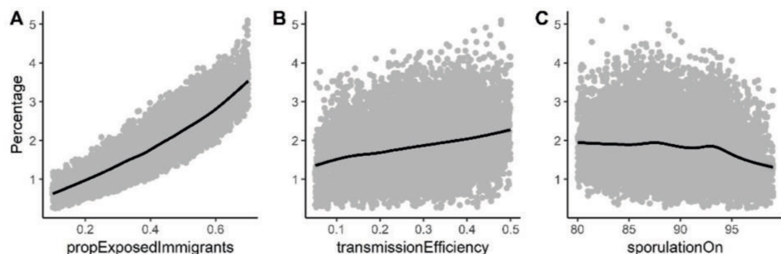

Figure 11: Correlations between the model output yield improvement and A) proportion of infected aphid immigrants, B) fungal transmission efficiency, and C) humidity threshold over 15 000 simulations. Each point represents an individual simulation. 15 000 simulations have been run.

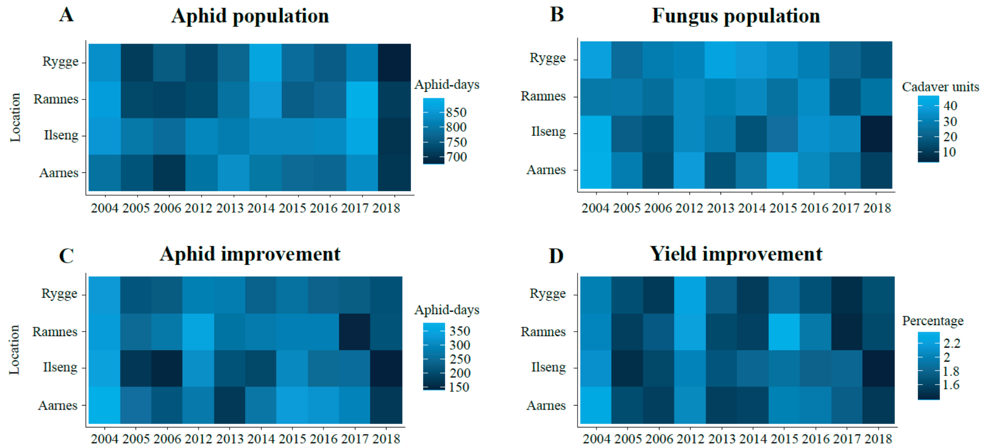

Figure 12: Heatmap of influence of the weather in 4 locations over 10 years on A) aphid population expressed in aphid-days over the growing season, B) fungus population expressed in cadaver units over the growing season and C) the decrease in aphid-days due to the fungus calculates as the difference between aphid-days without and with the fungus.

4 Discussion

In our model, *P. neoaphidis* controlled *S. avenae* by reducing the number of aphid-days and increasing yield improvement over the season. However, the group of most sensitive parameters changed depending on the trophic level studied, aphid or fungus populations; underlining different processes influencing different trophic levels. This was also true for the estimation of biological control calculated as the reduction in aphid population due to the fungus (through aphid-days improvement) or as the reduction of yield loss due to the decrease of the pest (through yield improvement). However, three parameters were always among the most sensitive ones invariantly from the output studied, namely the transmission efficiency (transmissionEfficiency), the weather file (fileNumber) and the humidity threshold that triggers fungal sporulation and germination (sporulationOn). Further, they are the three most important parameters for the aphid population dynamic and the biological control calculated as a decrease in aphid population. These parameters are linked to each other through influence of the environmental humidity on the fungus.

Transmission efficiency is of crucial importance in our model for the three trophic levels (crop, pest, natural enemies). This is in accordance with literature, which has recognised it as a key process in host-pathogen interactions (McCallum *et al.,* 2017; Steinkraus, 2006; McCallum *et al.,* 2001). An active debate exists on how to model the fungal transmission

correctly (e.g. McCallum *et al.,* 2001). Transmission efficiency is a composite parameter that combines different processes such as the probability for a host to get in contact with a pathogen, or the probability of this contact to initiate a disease in the host (McCallum *et al.,* 2017; Reeson *et al.,* 2000). In our model, transmission efficiency is linked to the capacity of a cadaver to sporulate and infect aphids within and between colonies (e.g. Ekesi *et al.,* 2005). This capacity was estimated as constant as suggested by Brown and Hasibuan (1995), even though it might not be the case for all host-pathogen systems (*e.g.* Elder *et al.,* 2008; Reeson *et al.,* 2000). For instance, Thomas et al. (1995) showed that transmission efficiency among grasshopper populations varies through time partially depending on sporulation pattern. When daily maximum relative humidity was below the threshold sporulationOn, transmission efficiency was reduced to zero as humidity influences fungal sporulation, germination, conidia and cadaver longevity (Filotas and Hajek, 2004; Xu and Feng, 2002; Furlong and Pell, 1997; Brown and Hasibuan, 1995; Wilding, 1969). This humidity threshold varies with fungus species and ranges between 80 and 100% among Entomophthoromycotan fungi (see review in Sawyer *et al.,* 1997). We showed that the higher the threshold (parameter sporulationOn), the lower the fungus population and the biological control it confers.

Considering environmental humidity at the leaf boundary layer, where fungi and aphids live, is complex and requires a lot of information and parameters (*e.g.* Fargues *et al.,* 2003). Therefore, many studies investigated the influence of environmental humidity on disease transmission with a rough index such as rainfall (Furlong and Pell 1997; Sawyer *et al.,* 1997), relative humidity (Xu and Feng, 2002; Sawyer *et al.,* 1997; Brown and Hasiun, 1995; Wilding, 1969), leaf wetness (Sawyer *et al.,* 1997; Milner, 1983), free water (Wilding, 1969), or soil moisture content (Furlong and Pell, 1997). In our model we considered the maximum daily relative humidity in weather files (fileNumber), which is a rough and easy estimation of the environmental humidity. However, as discussed in Sawyer *et al.* (1997), all these factors are correlated to the presence of free water in the vicinity of the fungus. Thus, all these factors capture the influence of the humidity on the fungus development. We found that a relative humidity lower than 92% gave better decrease in aphid population and yield improvement. However, because we used a rough estimator of environmental humidity, this threshold might not be the correct at leaf boundary layer.

McCallum *et al.* (2017) rightfully argues for a decomposition of the transmission efficiency parameter to better consider the different steps hidden in this single parameter. This would allow us to integrate various heterogeneity sources such as density-dependence (Dwyer *et al.,* 1997). Since we identified transmission efficiency as among the most important parameters in our model, we encourage the collection of experimental data and further study on how to model it for Entomophthoromycotan infections. Integrating directly influence of

abiotic (*e.g.* humidity) and biotic (*e.g.* fungal virulence or host susceptibility) factors on the fungal transmission efficiency could help us to better understanding this crucial process (McCallum *et al.,* 2017; Steinkraus, 2006).

Five parameters had a variable importance depending on the output studied. Two parameters significantly influenced aphid and fungus populations and the decrease in aphid population due to the fungus. They are the lethal time for nymphs (lethalTimeNymph) and the wheat growth stage at the beginning of spring (CropAtStart). Bonsall (2004) has already demonstrated mathematically the importance of lethal time on disease spreading into host populations. Here we found out that only the lethal time of nymph aphids matters rather than the lethal time of adults. This could be since nymphs are the most abundant aphid stage in the model compared to adults. Consequently, they are weighted more than adults in our model. In laboratory studies lethal time is usually estimated for apterous adults. Schmitz *et al.* (1993) estimated the lethal time for *S. avenae* nymphs infected by *P. neoaphidis*. They found different lethal times depending on nymphal stages. The wheat growth stage in spring was important for three outputs. The higher the crop growth stage at the beginning of spring, the higher the aphid and fungus populations. The faster the wheat growth, the faster flowering occurs, which results in an increase in aphid reproduction (Dean, 1974). This could also influence the fungus population by increasing the susceptible population earlier in season.

The two most important parameters for the fungus population, modelled through cadaver units, are linked to the longevity of the infective units. The most important parameters are the cadaver longevity (CadaverDuration) and the increase of energy consumption due to sporulation which decreases cadaver longevity (timeAcceleration). Fungus-killed cadavers can undergo several cycles of hydration/dehydration as shown in Sawyer *et al.* (1997) with an Entomophthoromycotan fungus infecting grasshoppers. During sporulation, conidiophores are grown, and conidia are actively projected into the environment (Hajek and Meyling, 2018). This requires energy. We modelled cadaver longevity as if sporulation was an acceleration of time or energy consumption though timeAcceleration. Even though, conidia longevity on crop leaves (Brobyn *et al.,* 1985) and *P. neoaphidis* sporulating cadaver longevity have been estimated (Bonner *et al.,* 2003; Ardisson *et al.,* 1997), estimation of cadaver longevity in the field is of prime importance to explain the fungus population dynamics. Conducting experiments similar to those of Sawyer *et al.* (1997) on the species studied here could be useful to estimate longevity of cadavers depending on weather conditions, saprophytic or dislodging of cadavers. Both parameters (cadaverDuration and timeAcceleration) are also important to explain aphid population dynamics and the biological control due to the fungus through the output aphid improvement. Both parameters

influence the transmission efficiency by increase the time of sporulation and the probability for conidia to get into contact with susceptible hosts.

The fungal inoculum expressed as the proportion of infected immigrants landing in the field (propExposedImmigrants) was the most important parameter explaining variability in the yield improvement. The yield improvement did not depend very much on the fungal pathogen dynamic (transmissionEfficiency, sporulationOn), but rather on the fungus controlling the aphid colonisation. Infected aphids landing on a crop can build colonies and transmit the disease in their offspring (Chen and Feng, 2004b). Further, aphid damage is more important around flowering and reducing aphid colonisation may reduce aphid density long enough for the wheat to pass flowering without too much pest pressure. It was also among the important variables for the aphid population and the biological control but not for the fungus population. The fungal inoculum is not crucial for cadaver units to develop compared to the spread of the disease into the host population.

Finally, all model outputs were weakly sensitive to some parameters: the immune response cost (immuneCorst), the lethal time for adult aphid (letahlTimeAdult), the inflexion point in the wheat growth speed (cropHalfWay) and the parameter k shaping biological distributions (shapeParameter). Even though the reproduction of infected aphids has been identified as crucial for aphid population dynamics (*e.g.* Schmitz *et al.,* 1993), in our model immuneCost was not influential for any output. This could be due to the fact that the decrease in fecundity was low (from 10 to 40%) or by the fact that we modelled aphids so their fecundity was higher at the beginning of their adulthood (*e.g.* Dean, 1974). Second, letahlTimeAdult was insignificant. This could be due to the same reason or because there were more nymphs in aphid populations and infected nymphs that become adults cannot reproduce (Schmitz *et al.,* 1993). The inflexion point in wheat growth speed did not affect the trophic levels either. Finally, the shape parameter k is often arbitrarily chosen in modelling (Gutierrez et al., 1993; Graf et al., 1990; Carruthers et al., 1986). However, its influence on population dynamics is usually not tested. Because we find no influence of this parameter on the output, we conclude that modellers could continue the practice of arbitrarily assigning this value.

5 Conclusion

We showed that depending on the trophic level studied (crop, aphid or fungus), different parameters linked to *P. neoaphidis* interactions with its environment were important. Nevertheless, three parameters were among the most important for all trophic levels: transmission efficiency and humidity threshold that triggers fungus sporulation. Further, they were the most important parameters influencing aphid population and its decrease due to the fungus control. The fungus population is mostly influenced by cadaver longevity and how long they can sporulate. Finally, yield improvement due to the biological control of aphids is mostly due to the fungus inoculum as infected immigrants.

6 Acknowledgment

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Errata list

Side	Line	Original text	Corrected text
Introduction p10	24	Consequently, there is a time-delay between the host getting infected and getting infectious (*i.e.* fungal sporulation)	Consequently, there is a time-delay between the host getting infected and dying because of the fungal infection.
Introduction p12	3	It is of dramatic importance to understand their implication on the development of Entomophthoromycotinan infection and spread for biological control purposes.	It is of critical importance to understand their implication on the development of Entomophthoromycotinan infection and spread for biological control purposes.
Introduction p15	9	Further, there is still a vivid debate among disease biologists about how to model disease transmission either as a linear or non-linear function, which probably depends on the system studied (McCallum et al., 2017; McCallum et al., 2001).	Further, there is still a debate among disease biologists about how to model disease transmission either as a linear or non-linear function, which probably depends on the system studied (McCallum et al., 2017; McCallum et al., 2001).
Introduction p16	16-17	This increasing number of players changes the dynamics of the system and adds a dimension to the disease triangle which becomes a disease tetrahedron (Fig. 3B).	This increasing number of players changes the dynamics of the system and the disease triangle becomes a disease square pyramid (Fig.3B).
Paper II p13	2-3	Fungus-killed R. padi cadavers were found close to bud axils, where overwintering R. padi eggs are also usually observed (Fig. 4A, B). When the density of cadavers was high, some were also found on the branch between buds (Fig. 4C).	Fungus-killed R. padi cadavers were found close to bud axils, where overwintering R. padi eggs are also usually observed (Fig 4 A-C). When the density of cadavers was high, some were also found on the branch between buds (Fig. 4D).
Paper II p13	22-23	Ten cadavers were filled with heterogenous hyphal bodies of varying shape and length (Fig. 6C), while one cadaver was filled with homogenous rod shaped hyphal bodies (Fig. 6D).	Ten cadavers were filled with heterogenous hyphal bodies of varying shape and length (Fig. 6D), while one cadaver was filled with homogenous rod shaped hyphal bodies (Fig. 6E).
Paper II p17	Figure 6 Caption	I forgot to label Fig. 6C	Add: “C) smooth and hyaline resting spore without epispore”
Paper IV p5	23	All source code is freely available, together with installation files that will allow anyone to run the model on their own (see File 1).	Deleted (see File 1); the model script has not been included in the thesis.

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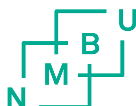

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