Master thesis

Effects of precipitation on fecal-oral transmission of the microsporidium *Nosema lymantriae* in experimental gypsy moth populations

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Abstract

The microsporidium *Nosema lymantriae* is a pathogen of the European gypsy moth, *Lymantria dispar*, a polyphagous insect herbivore that causes defoliation of temperate hardwood forests during mass outbreaks. *N. lymantriae* induces systemic infections during which environmental spores are formed within the host tissues (salivary glands, Malpighian tubules, fat body). Infectious spores are released into the environment from feces and decaying cadavers and provide the source for new infections as they contaminate the host feeding sites on foliage. The present study examined the effect of simulated rain on the transmission of *N. lymantriae* from feces in gypsy moth populations. Experiments were conducted in a two-story cage system under near field conditions. The experimental set-up consisted of (i) a 24-hour contamination phase during which the infected larvae fed foliage in the top cage and rain was simulated twice by spraying water, followed by (ii) a 72-hour transmission phase during which healthy test larvae were left to feed on the same leaves as the infected larvae before. This setup was carried out three times in a row with larvae in early, middle and late stages of disease progression. At an early stage of disease progression, transmission from spore-laden feces was observed only once. Transmission rates increased significantly in the later stages; on average 17% (middle stage) and 7% (late stage) of test larvae acquired infections from feces when dry, but 57 and 68% were infected after the rain events in middle and late stages, respectively. Thus, although fecal pellets from leaf-feeding larvae are firm and non-sticky, they provide an effective source for new infections, especially when raindrops moisten the leaf surface. Rainfall at times when hosts are in late stages of disease may significantly increase the prevalence of infection in the population.

Key words: microsporidia, pathogen, disease transmission, *Lymantria dispar*, precipitation

Zusammenfassung

Das Mikrosporidium *Nosema lymantriae* ist ein Pathogen des Schwammspinners, *Lymantria dispar*, ein polyphages Schadinsekt, das bei Massenbefall temperate Laubwälder kahlfressen kann. *N. lymantriae* verursacht systemische Infektionen, die mit der Bildung von infektiösen Dauersporen in Wirtsgeweben (Spinndrüsen, Malpighische Gefäße, Fettkörper) einhergehen. Diese Sporen werden aus Kot und verwesenden Kadavern freigesetzt und stellen über die Kontamination der Futterpflanze eine Quelle für Neuinfektionen dar. In der vorliegenden Arbeit wurde der Einfluss von simuliertem Regen auf die Übertragung von *N. lymantriae* durch Kot in Schwammspinnerpopulationen untersucht. Die Versuche wurden in einem 2-stöckigen Käfigsystem unter Freilandbedingungen durchgeführt. Der Versuchsablauf umfasste eine 24-stündige Kontaminationsphase mit an Blättern fressenden, infizierten Larven im oberen Käfig mit einer zweimaligen Regengabe, gefolgt von einer 72-stündigen Übertragungsperiode mit gesunden Testraupen auf den Futterpflanzen, auf welchen zuvor die infizierten Raupen gefressen hatten. Diese Prozedur wurde dreimal hintereinander mit Raupen in einem frühen, mittleren und späten Infektionsstadium durchgeführt. In der Frühphase der Infektion kam es in einem einzigen Fall zu einer Infektionsübertragung durch kontaminierten Kot. Die Übertragungsraten stiegen jedoch durch Raupen in späteren Infektionsstadien an. Im Durchschnitt wurden 17 bzw. 7% der gesunden Testraupen durch Kot von Raupen in mittleren bzw. späten Infektionsstadien infiziert; die Übertragungsraten erhöhten sich auf 57 bzw. 68% bei simuliertem Regen. Entsprechend stellen Kotpartikel trotz ihrer festen Konsistenz eine bedeutende Quelle für Neuinfektionen dar, besonders wenn sie durch Regen angefeuchtet sind. Niederschläge zu einem Zeitpunkt, wenn die Krankheit der Raupen bereits stark fortgeschritten ist, können damit die Zahl an Neuinfektionen in einer Population deutlich erhöhen.

Schlagwörter: Mikrosporidien, Pathogen, Krankheitsübertragung, *Lymantria dispar*, Niederschlag

Contents

1. Introduction

Pathogens and parasites act as regulating agents in numerous animal species and are assumed to be the driving force of cyclic population dynamics (Tompkins *et al.*, 2002). This pattern of fluctuation is often described for forest defoliating insects, such as the European gypsy moth, *Lymantria dispar* (Lepidoptera: Erebidae) (Hlásny *et al.*, 2016). Within its area of natural distribution those gradations are usually terminated after one or two years by mostly density-dependent factors (Liebhold *et al.*, 2000; Nierhaus-Wunderwald & Wermelinger, 2001). As an invasive pest species in North America, however, *L. dispar* has been causing significant damage due to favourable environmental conditions and a lack of natural enemies in this region (McManus & Csóka, 2007). Amongst other predators, parasitoids and pathogens, several species of Microsporidia are known to act as natural enemies of the gypsy moth in Europe (McManus & Solter, 2003). While often not instantly lethal, microsporidiosis can still negatively affect infected individuals and is thought to have a significant impact on population cycles (Maddox *et al.*, 1998). Furthermore, the adverse effect on insect fitness can have implications for other mortality factors (Solter & Becnel, 2003; van Frankenhuyzen *et al*., 2007).

The aim of present study is to gain insight in the transmission of the microsporidium *Nosema lymantriae* within its host *L. dispar* and how it is affected by the environmental factor rain. Hoch & Goertz (2009) state that feces of infected larvae contain high numbers of environmental spores and play an important role in disease transmission. Steyer (2010) already showed that transmission of this microsporidian species via feces is significantly increased by simulated rain.

In the present work, these findings are tested under simulation of light rain. Furthermore, the impact of disease progression on transmission success is considered.

1.1 *Lymantria dispar*

1.1.1 Morphology and biology

Lymantria dispar, commonly referred to as the (European) gypsy moth, belongs to the subfamily Lymantriinae and was assigned to the lepidopteran family of Erebidae in 2011 (Zahiri *et al.*, 2012).

Adult moths are characterized by their typical tectiform way of folding their wings when resting and a strong sexual dimorphism. Females are white-brownish with black antennae, tarsae and tibiae. Their wings bear four brown stripes and have a span of

50-80 mm. Typically, their cloddy abdomen is covered with thick, light brown hair. Males are of darker colours and considerably smaller with a wing span of 35-50 mm (Wellenstein & Schwenke, 1978). The antennae of males are plumose and play a key role in female pheromone perception. Female gypsy moths of the European strain are flightless. To attract males, that are excellent flyers, they release a blend of sexual pheromones from a small gland at the tip of their abdomen (Leonard, 1981). *L. dispar* fly at midday in July and August. Soon after mating, females lay approximately 500 eggs in a single mass on tree stems and cover them with abdominal hair (Wellenstein & Schwenke, 1978). After a week the adult moths die (Leonard, 1981).

Within the eggs, larvae finish embryonic development within three to four weeks (Wellenstein & Schwenke, 1978). Fully developed larvae enter diapause and overwinter inside the egg. The period between oviposition and hatching of larvae lasts about nine months. *L. dispar* is obligatorily univoltine (Leonard, 1981).

In Central Europe larvae hatch in April or May. They are greyish with dark dots and feature six rows of hairy warts. All larval stages are able to produce silk (Wellenstein & Schwenke, 1978).

First instar larvae are important for dispersal, especially when population densities are high (Mason & McManus, 1981). They produce long silk strands upon which they slide down to hang in mid-air. The larvae are tiny, light and covered by long hair that enables them to be carried by wind over long distances (Wellenstein & Schwenke, 1978). This behaviour is called ‘ballooning‘ (Mason & McManus, 1981).

First to third instars, usually feed gregariously during the day. Later instars feed at night and usually rest in the day time hidden in bark crevices on the stem or in the leaf litter (Wellenstein & Schwenke, 1978; Hajek *et al.*, 2003). Starting with the fourth instar, larvae exhibit a very wasteful fashion of feeding. In the course of larval development, each gypsy moth larva consumes approximately 0.1 m² of foliage (Andrae, 2013). Adult gypsy moths do not feed at all (Leonard, 1981).

Male larvae molt four or five times, females typically five or six times before they pupate. The pupal stage lasts 10-23 days. To compensate for the longer larval development of females, the pupal stage of males lasts a few days longer than that of females (Wellenstein & Schwenke, 1978).

1.1.2 Distribution

L. dispar is native to Europe, Asia and North Africa. Its range of natural distribution stretches like a band from Great Britain to Japan (Wellenstein & Schwenke, 1978). While the southern border is formed by the Mediterranean Islands, Tunisia, Algeria and northern Morocco (McManus & Csóka, 2007), the distribution area reaches up to Mid-Sweden and Moscow in the north (Wellenstein & Schwenke, 1978).

L. dispar prefers light and warm forest stands. Within Europe, especially the Balkans provides optimal conditions for the gypsy moth. Outside of those xeric regions in southern and south-eastern Europe, *L. dispar* is, therefore, restricted to warm and dry regions and gradations are often observed after exceptionally warm years (Wellenstein & Schwenke, 1978).

In the late 1860s, *L. dispar* was introduced to North America (Hajek *et al.*, 2003) where it has been continuously expanding its area of distribution (McManus & McIntyre, 1981). *L. dispar* is still considered to be one of the most important pest insects in deciduous forests in the eastern United States (McManus & Csóka, 2007).

1.1.3 Host plants

While *L. dispar* larvae are generally considered to be polyphagous, they show host plant preferences during phases of low population densities. In wide areas of Europe and North America, *Quercus* spp., *Carpinus betulus*, *Salix* spp. and *Populus* spp. are the most important host plants (Wellenstein & Schwenke, 1978).

Mass outbreaks and defoliation of trees due to the excessive larval feeding can occur on all potential host plants. Therefore, also forests or areas that differ in tree species composition from the preferred hosts can be economically affected as well. In Lithuania, for example, *L. dispar* is reported to occur on birch (*Betula* sp.) and alder (*Alnus* sp.) (McManus & Csóka, 2007). In Hungary *L. dispar* is causing problems in poplar (*Populus* sp.) stands (Andrae, 2013), in Switzerland, amongst others, it has been described on chestnut (Nierhaus-Wunderwald & Wermelinger, 2001) and in eastern Austria *L. dispar* outbreaks occur on *Quercus petraea* and *Quercus cerris* (Schopf & Hoch, 1997). In some exceptional cases, *L. dispar* populations have been found to successfully establish on rather unusual host plants: in Croatia, for example, larvae feed – amongst others – on the evergreen *Quercus ilex* (Andrae, 2013).

During mass outbreaks, intraspecific competition and shortage of food forces foraging larvae to start feeding on non-host plants (Leonard, 1981). For the development of

larvae this alternative diet, however, is not beneficial. Feeding on non-host plants can lead to prolonged development, reduced fecundity or preliminary death (Wellenstein & Schwenke, 1978; Lobinger & Skatulla, 2001).

The considerably wide range of potential host plants, the ability to adapt to pure stands on a regional level and the fact that wide areas in Europe and North America are covered by preferred host tree species are some explanations for the successful distribution and range expansion of *L. dispar* (McManus & Csóka, 2007). Furthermore, elevated atmospheric CO_2 levels change the host plant quality, which might influence host plant preferences of *L. dispar* (Hättenschwiler & Schafellner, 2004).

1.1.4 Gradations

L. dispar shows population dynamics of the eruptive type. Especially in North America, outbreaks occur irregularly and are hard to predict (Liebhold *et al.*, 2000).

Warm and dry weather conditions (Wellenstein & Schwenke, 1978), especially in the spring (April- June) (Hoch *et al.*, 2006; Kalbacher, 2008), are favourable for gypsy moth larval development and survival and, therefore, promote gradations. Wellenstein & Schwenke (1978) declare two factors responsible. Firstly, larval development is accelerated by elevated temperatures. A shorter larval period means a shorter exposure to potential mortality factors (e.g. predation) (Leonard, 1981). Secondly, the food available is of better quality (Wellenstein & Schwenke, 1978). The latter statement refers to the plant stress hypothesis, which suggests that plants respond to stress – e.g. caused by water deficiency – by an increase in soluble nitrogen and free amino acids in tissues. This could, indeed, be an advantage for herbivore development as nitrogen is often the limiting factor for insect growth (Mattson, 1980).

In some areas, such as Croatia and Bulgaria, mass outbreaks of *L. dispar* occur periodically (Pernek *et al.*, 2008; Hrašovec *et al.*, 2013; Zúbrik *et al.*, 2016). Cyclic population dynamics with gradations occurring every 8-13 years have also been described for the Carpathian Mountains in central and south-eastern Europe (Hlásny *et al.*, 2016).

In Europe, mass outbreaks are usually terminated after one or two years (Nierhaus-Wunderwald & Wermelinger, 2001). Gradations are limited by density-dependent mortality factors (Liebhold *et al.*, 2000), such as shortage of food or increased susceptibility to diseases, especially viruses (Wellenstein & Schwenke, 1978). Also adverse abiotic conditions, like rain or unfavourable temperatures, can terminate mass

outbreaks (Wellenstein & Schwenke, 1978; Pernek *et al.*, 2008). Generally, gradations occur at elevations below 1,000 m above sea level (Nierhaus-Wunderwald & Wermelinger, 2001).

Several gypsy moth gradations have already been documented for oak stands in eastern Austria. The most recent ones occurred during the years 1992-1994 and 2003-2005 (Hoch *et al.*, 2001; 2006).

1.1.5 Damage

The previously described characteristics – a polyphagous lifestyle, a wasteful manner of feeding and occurrence of mass outbreaks – make the gypsy moth a dreaded pest of deciduous forests. Regularly occurring defoliation causes severe damage, economically and, in areas where it is invasive, ecologically (McManus & Csóka, 2007).

Repeated defoliation during mass outbreaks impairs tree vigour and results in a reduction of wood increment or even tree mortality (Muzika & Liebhold, 2001; McManus & Csóka, 2007). Secondary pest agents such as *Armillaria mellea* (Agaricales: Physalacriaceae) or *Agrilus bigutattus* (Coleoptera: Buprestidae) might be promoted by *L. dispar* calamities. Furthermore, endemic herbivorous species are sometimes outcompeted for food resources or sun exposure due to defoliation that leads to unfavourable changes of the microhabitat (Hajek *et al.*, 2003). Also nutrient cycling in soils is affected by changes in microbial activity due to a rapid input of larval feces (Speight *et al.*, 2008).

Moreover, non-host plants can be negatively affected by the gypsy moth. Even though larvae might not be able to complete their life cycle on an unsuitable host, defoliation is still possible and might be harmful for the plant (Muzika & Liebhold, 2001). The actual consequence of defoliation and the degree of damage, however, depends on the tree-species-specific sensibility (Muzika & Liebhold, 2001; McManus & Csóka, 2007).

Comparative studies suggest that damage is more severe in North America than in Europe because tree mortality after repeated defoliation generally seems to be higher, regardless of species-specific susceptibility (McManus & Csóka, 2007) and due to the lower abundance of natural enemies (Alalouni *et al.*, 2013).

1.1.6 Gypsy moth and climatic conditions/weather

Within the European distribution area of *L. dispar*, two climatic zones can be discriminated. On the one hand, there are the temperate hardwood forests in the west,

mid and north of the continent. On the other hand, there are the Mediterranean forests in the south (Wellenstein & Schwenke, 1978; McManus & Csóka, 2007). As a consequence of divergent temperature and weather conditions regional differences in the dynamics of gypsy moth populations can be observed (e.g. in Hoch *et al.*, 2001). Even though populations are frequently denser in regions along the Adriatic Sea, outbreaks do not necessarily occur (Weiser, 1998). Therefore, the relevance of *L. dispar* as a forest pest is unequally distributed throughout Europe and some countries regularly see the need to undertake control measures (e.g. Croatia), (Hrašovec *et al.*, 2013) while others do not (e.g. Austria) (Hoch *et al.*, 2001).

The effects of climate change on *L. dispar* populations are uncertain. Klapwijk *et al.* (2013) evaluated data on defoliation of Hungarian forests of the years 1961-2009 and did not find cyclic mass outbreaks being affected by the continuous increase in mean monthly temperature. Even though insect development is accelerated by higher temperatures, supraoptimal temperature conditions led to slower development and might arrest the dispersal of *L. dispar* into warmer regions in North America (Tobin *et al.,* 2014).

1.1.7 Parasitoids, Predators

Within the area of natural distribution, there are several natural enemies known for each life stage of *L. dispar*. Through their simultaneous and/or successive interference with gypsy moth populations, they provide effective control. Their individual impact on *L. dispar* populations, however, is variable with regard to the geographic location, various phases of population dynamics (Reardon, 1981) and interaction among each other (e.g. vertebrate predators suppress predation by invertebrates (in Gschwantner *et al.*, 2002)).

Several species have been described as important parasitoids of *L. dispar* larvae. Relevant parasitoid wasp species in Austrian gypsy moth populations are (among others) the braconid species *Glyptapanteles liparidis, Glyptapanteles porthetriae* and *Cotesia melanoscela* in early larval instars and the ichneumonid *Phobocampe* spp. in early to mid instars, especially in retrogradation years (Hoch *et al.*, 2001). During culmination, however, mortality caused by parasitoids seems to be rather low compared to that caused by other factors (Kalbacher, 2008).

Due to its ability to deposit eggs on all (but the first) larval instars, the tachinid species *Parasitigena silvestris* is a rather successful and dominant parasite at elevated host densities (Hoch *et al.*, 2001), and during the early post-culmination phase (Hoch *et al.*,

2006). *Blepharipa pratensis*, another tachinid species, is frequently found in *L. dispar* pupae in Austria and Slovakia, especially in the first year after culmination (Hoch *et al.*, 2001). The parasite fly, *Compsilura concinnata* (Diptera: Tachinidae) is an important parasitoid fly species of pupae in North American gypsy moth populations (Hajek & Tobin, 2011).

The mite *Allothrombium wolffi* (Prostigmata: Trombidiidae) is a predator of gypsy moth eggs in Austria (Wellenstein & Schwenke, 1978). Birds, like pheasant and cuckoos, prey on eggs and larvae (Wellenstein & Schwenke, 1978; Smith & Lautenschläger, 1981) but not on pupae (Gschwantner *et al.,* 2002). Another predator of larvae is the carrion beetle *Xylodrepa quadripunctata* (Coleoptera: Silphidae) (Nierhaus-Wunderwald & Wermelinger, 2001). Small mammals are considered the most important predators of pupae (Gschwantner *et al.,* 2002; Liebhold *et al.,* 2005) also during times of low gypsy moth population densities (Liebhold *et al.*, 2000).

The carabid beetles *Calosoma sycophanta* and *Calosoma inquisitor* effectively prey on *L. dispar* larvae and pupae (Gschwantner *et al.,* 2002). In North America generalist predators and parasitoids seem to play a bigger role as natural enemies of *L. dispar* due to the lack of specialized species (Alalouni *et al.,* 2013).

1.1.8 Pathogens

Pathogens consistently play a major role in the collapse of gypsy moth gradations (Nierhaus-Wunderwald & Wermelinger, 2001). Especially the gypsy moth nucleopolyhedrosis virus (LdNPV) is an important factor for terminating mass outbreaks in both, Europe and North America (Weiser, 1998; Hoch *et al.*, 2001; Hajek *et al.*, 2003). Infections occur either after ingestion of virus particles or through transmission by ovipositing parasitoids. The virus is dispersed by young infected larvae but also by predators and parasitoids of the gypsy moth (Hajek & Tobin, 2011).

Since 1989, the entomopathogenic fungus *E. maimaiga* has gradually been taking over the role of the dominant pathogen of *L. dispar* in North America (Hajek *et al.*, 2003). In 1999, *E. maimaiga* was deliberately introduced in Bulgaria, and, for the first time, successfully established in an European gypsy moth population. After this and several other releases of the pathogen, *E. maimaiga* spread into many countries of Europe, mostly in the southeast (Serbia, Hungary etc.). There, like in North America, the fungus seems to become the most important natural enemy of *L. dispar* (Zúbrik *et al.*, 2016).

E. maimaiga is specific to Lepidoptera, within which the tussock moths show the highest degree of susceptibility (Hajek *et al.*, 2003). Different from the LdNPV, *E. maimaiga* does not have to be ingested by the larvae. If spores of the fungus land on and successfully attach to the surface of a host insect, they can actively penetrate the cuticle through a combination of enzymatic digestion and pressure (Hajek, 2004). *E. maimaiga* can also reach a high level of prevalence in low density populations of the gypsy moth, thus, prevent mass outbreaks (Zúbrik *et al.*, 2016). Rainfall and temperature strongly influence the fungus (Hajek & Tobin, 2011). While moisture has a positive influence on *E. maimaiga*, temperatures above 30 °C have a negative effect, which might be of importance considering the further spread of this pathogen (Reilly *et al.*, 2014). In North America, *E. maimaiga* seems to gradually replace other natural enemies of the gypsy moth (Hajek *et al.*, 2015). In Europe, its impact on other pathogens and predators of *L. dispar* is still uncertain (Zúbrik *et al.*, 2016).

1.2 Microsporidia

Microsporidia are a group of ubiquitous unicellular, spore-forming parasites found in vertebrates and invertebrates (Vávra & Lukeš, 2013). Out of 1,200 known species (Vossbrinck & Debrunner-Vossbrinck, 2005), approximately 700 are described as pathogens of insects. Some are important natural enemies of particular insect species (Solter & Becnel, 2003).

There are a few exceptional cases of epidemically occurring microsporidia like *Nosema bombycis* in the silk worm, *Bombyx mori* (Lepidoptera: Bombycidae), or *Nosema apis* in the honey bee, *Apis mellifera* (Hymenoptera: Apidae). Other than that, microsporidia are often characterized by low virulence, thus, causing rather chronic than acute diseases in their hosts (Vávra & Lukeš, 2013).

Nevertheless, microsporidiosis can have adverse effects on insect fitness and, therefore, amplify the effect of other mortality factors (Solter & Becnel, 2003; van Frankenhuyzen *et al*., 2007). Although they seldom cause epizootics, they are thoroughly researched and thought to exert a significant impact on population cycles as natural enemies of forest defoliating lepidopteran species (Maddox *et al.,* 1998).

1.2.1 Taxonomy

The taxonomic status of Microsporidia is still unresolved. Due to their extreme lifestyle they exhibit a highly reduced genome and lack cell organelles such as mitochondria.

Due to the reduction of both, physiology and genome, they are not easy to place within a group. Regardless of morphological dissimilarity, phylogenetic studies point to a relatedness to Fungi (Hibbett *et al.*, 2007; Solter & Becnel, 2007). Specifically they are hypothesized to belong on the earliest diverging branch of the fungal phylogenetic tree (James *et al.*, 2006).

Traditionally, classification of microsporidia is based on morphological traits detected by light- and electron-microscopy (Vávra & Lukeš, 2013). Over the past decades, however, a large database of ssrDNA (small subunit ribosomal DNA) sequences was generated, that contains numerous taxa and facilitates identification of microsporidian species. On grounds of these data, Vossbrinck & Debrunner-Vossbrinck (2005) suggest a classification reflecting host and habitats of microsporidian species (3 groups: Aquasporidia, Marinosporidia, Terresporidia).

Microsporidia possess numerous ribosomes that facilitate rapid proteosynthesis. Microsporidia have mitosomes instead of mitochondria and lack several other organelles usually found in eukaryotic cells. Their reduced appearance might result from their life style: They are truly intracytoplasmatic parasites that complete their life cycle entirely within the host cell. They depend on the host cell's energy resources and metabolism (Vávra & Lukeš, 2013).

1.2.2 Life cycle

Microsporidia disseminate as infective environmental spores, the only ontogenetic stage that can persist outside the host organism (Undeen & Vávra, 1997; Vávra & Lukeš, 2013). Environmental spores comprise of a complex, two-layered cell wall that encloses the sporoplast (Vávra & Lukeš, 2013). The sporoplast is basically a highly reduced eukaryotic cell that contains, aside from numerous ribosomes, only a few organelles and species-specifically one or two nuclei (Vávra & Lukeš, 2013). The one feature that all microsporidian spores have in common is their unique germination apparatus (Weiser, 1961). It consists, essentially, of a membrane storage organelle, the polar plast, an injection tube and a posterior vacuole. Upon germination, the pressure built up in the vacuole triggers the ejection of the polar filament, through which the microsporidian sporoplast gets injected into the cytoplasm of a host cell (Vávra & Lukeš, 2013). While the microsporidian life cycle is generally rather complicated, some species form one and others several different spore types throughout their individual development (Undeen & Vávra, 1997). Exemplarily, the life cycle of *N. lymantriae* will be discussed in further detail below (see 1.2.6). Basically, once the parasite reaches the host cytoplasm, it quickly starts proteosynthesis, proliferation – the so called merogony – and intrahost dissemination (Undeen & Vávra, 1997; Vávra & Lukeš, 2013). While in principle any kind of tissue can be infected (Undeen & Vávra, 1997), microsporidia usually have a species-specific preference for certain hosts and within those for certain tissues (Solter & Becnel, 2003). At the beginning of their intracytoplasmatic development, microsporidia do not cause visible harm to the host cell (Vávra & Lukeš, 2013). Upon spore formation, however, infected cells start to disintegrate and appear hypertrophied (Undeen & Vávra, 1997). Also, the host itself does not necessarily exhibit obvious signs of infection. Often, infected hosts are observed to feed and behave like uninfected ones but die during molt or pupation as a result of the exhaustion of their energy reserves by the parasite (Vávra & Lukeš, 2013). Those chronic forms of disease progression may lead to death within days or

even weeks after infection (Solter & Becnel, 2003) but are important due to reduction of both fecundity and longevity of the host (Undeen & Vávra, 1997).

1.2.3 Transmission pathways

Interhost dissemination always happens through environmental spores (Vávra & Lukeš, 2013). Horizontal transmission occurs within one generation of hosts. Usually, spores have to be ingested by the susceptible host with food. Within the digestive system a combination of stimuli triggers the germination of the spores (Weiser, 1961; Vávra & Lukeš, 2013). Further, parasitoids can act as vectors of microsporidian infections when they mechanically injure the host during oviposition (Solter & Becnel, 2003).

The second route is vertical transmission where infected adult hosts pass on microsporidiosis to their progeny. Some microsporidian species can be transmitted transovarially. In this case, the eggs and resulting embryos of infected adults already bear the infection. Also contaminated egg surface of infected adults can lead to ingestion of infective spores by the young instars (Goertz & Hoch, 2008b).

1.2.4 Host specificity

The tight relationship between microsporidia and their specific hosts is the result of co-evolution and co-speciation of the organisms (Vávra & Lukeš, 2013). Undeen & Vávra (1997) even state that hosts are a suitable aid for identification of microsporidia.

For better estimation of non-target effects of microsporidia used in bio control, several studies on host specificity of lepidopteran microsporidia have been undertaken. Numerous species were inoculated with spores to observe pathogen development within non-hosts. The inoculations led to roughly three different reactions in non-host Lepidoptera: host-like infections, atypical infections and no infections (Solter *et al.*, 1997; Solter *et al.*, 2000; McManus & Solter, 2003). Sometimes spores even pass the digestive system of non-host organisms completely undamaged (Weiser, 1961). Although in a few cases reinfection with spores formed in non-hosts during experimental infections might be possible, the ecological host-range of microsporidian species seems rather narrow (Solter & Becnel, 2003).

A narrow host range is a desired trait for microbial pesticides. Nevertheless, microsporidia have been found to be mostly unsuitable for two reasons (Solter & Becnel, 2003). Firstly, many microsporidia have a complicated life cycle that prevents efficient mass production of spores. Secondly, the disease appears slowly, due to the long latent period of the pathogen (Solter & Becnel, 2003; 2007). Still their role as

naturally occurring enemies of certain pest species and their effect on population dynamics should not be overlooked (Maddox *et al.,* 1998).

1.2.5 Microsporidia in *Lymantria dispar*

Several microsporidian species are thought to have a natural regulating effect on gypsy moth populations (Solter & Becnel, 2007). The first report of microsporidia isolated from *L. dispar* dates back to the description of *Plistophora* (=*Endoreticulatus*) *schubergi* by Zwölfer (1927). Since then, numerous samplings have been undertaken and several species and various biotypes have been gathered over the years (Weiser, 1998). McManus and Solter (2003) list five microsporidian species of *L. dispar* as properly described in literature. In 1993, a year with rather low population prevalence in European gypsy moth populations, they collected several biotypes of various microsporidian species. As all of these isolates belonged to one of the known three genera – *Vairimorpha, Nosema, Endoreticulatus* – they concluded that they were the relevant ones in Europe. Furthermore, they state that no microsporidia had been detected in North American populations of the gypsy moth.

Phylogenetic studies unveiled that microsporidia isolated from *L. dispar* are often closely related, regardless of the assigned genus. According to McManus & Solter (2003), *Nosema* and *Vairimorpha* isolates from gypsy moths are closer related to each other than to species of the same genus found in other insect species (examples: *Nosema bombycis* and *Vairimorpha necatrix*).

Concerning transmission pathways and virulence, however, huge differences have been described even within the same genus of microsporidia isolated from *L. dispar* (Goertz & Hoch, 2008a; 2008b).

1.2.6 *Nosema lymantriae*

N. lymantriae was first described by Weiser (1957) who isolated it from gypsy moth larvae collected during a gradation in Slovakia in 1956.

Spores in the *Nosema*-group are described as oval, in the case of *N. lymantriae* even spindle-shaped. Their cell walls appear smooth and shiny with a rather uniform and refractive surface (Weiser, 1961; 1998). Spores of *N. lymantriae* germinate inside the host's gut lumen within several hours after ingestion. As they enter the gut cells, merogony begins (Weiser, 1961). After experimental inoculation with 1,000 spores, Goertz and Hoch (2008a) first detected meronts in the midgut cells two days post inoculation (dpi). *N. lymantriae* is characterized by a systemic infection where several

organs are infected, more or less, subsequently (Goertz & Hoch, 2008a). When sporogony starts, primary spores are formed in the affected organs. They are easily recognized by the visible vacuole at one end of the spore (Weiser, 1998).

After approximately two weeks, silk glands are entirely filled with spores. Environmental spores can also be found in the fat body and the Malpighian tubules. Only a few days later, these organs are filled with spores, too (Goertz & Hoch, 2008a). Infected larvae usually die after three to five weeks (Weiser, 1961; 1998; Pollan *et al.,* 2009).

Experimental inoculations showed no significant impact of the number of spores ingested on the time period after which the infected larvae die (Goertz & Hoch, 2008a). The day until death is, however, significantly altered by ambient temperature. Larvae reared at 18 °C die 13-18 days later than those kept at 24 °C (Pollan, 2009). Furthermore, Pollan *et al.* (2009) showed a significant acceleration of both, larval and pathogen development, at elevated temperatures. Not only death occurs earlier, also severe infection of organs commences sooner.

N. lymantriae efficiently uses the resources provided by the host organs, producing an average amount of 5×10^9 spores per cadaver (Hoch & Goertz, 2009), regardless of the inoculation dose (Goertz & Hoch, 2008a). It is unclear what triggers microsporidian sporogony and what makes the pathogen recognize the host‘s parasite-carrying capacity (Vávra & Lukeš, 2013). Interestingly, *Vairimorpha* sp. achieved a significantly higher spore yield in gypsy moth larvae parasitized by the parasitic wasp *Glyptapanteles liparidis* than in unparasitized control larvae (Hoch & Schopf, 2001). Polydnavirus and venom injected into larvae during parasitisation lead to an increase of glycogen in host insect tissues which might support parasite development (Hoch *et al.*, 2009) Therefore, *N. lymantriae* might also benefit from wasp parasitisation. Cadavers of infected larvae, however, serve as a storage for spores and thus as an inoculum for new infections. Approximately 50% of susceptible test larvae acquired infection after one or two hours of exposure to infected cadavers (Goertz & Hoch, 2008a). Under favourable conditions environmental spores of *N. lymantriae* contained in cadavers can even stay viable until the proximate year facilitating persistence in the environment to the next host generation (Goertz & Hoch, 2008b).

Another important factor in pathogen persistence between generations is vertical transmission. When larvae get infected with microsporidiosis during late instars, they usually complete development to the adult stage. Infected females transmit *N. lymantriae* transovarially to over 70% of their progeny. Males do not transmit infection.

Mating of two infected individuals does not produce any offspring (Goertz & Hoch, 2008b).

Also, living larvae can pass on infection to their conspecifics. A newly acquired infection is followed by several days of latency during which larvae are not infective. Hoch *et al.* (2008) conducted experiments with inoculated larvae and healthy test larvae on caged oak trees. Choosing population densities that are typical during gradations, they did not observe transmission earlier than 20 days after inoculation, i.e. a 16-days-exposure period. Furthermore, horizontal transmission of *N. lymantriae* in gypsy moth populations is strongly influenced by the density of infected hosts (Hoch *et al.*, 2008; Steyer, 2010).

Further studies revealed that horizontal transmission from living larvae is mainly caused by two factors: spores released with feces and direct contact between infected and susceptible larvae (Goertz & Hoch, 2008a; Pollan *et al.,* 2009; Steyer, 2010). More than 40% of healthy test larvae had acquired microsporidiosis after being exposed to infected individuals for two hours (Goertz & Hoch, 2008a). At approximately the same time environmental spores first appear in the Malpighian tubules, larvae begin to release infective spores with feces. Gypsy moth larvae inoculated as third instars, release approximately 2.7×10^7 spores with feces throughout their lifetime (Goertz & Hoch, 2008a). The onset of spore release with feces depends significantly on the temperature during larval development. Pollan *et al.* (2009) showed that spores first occurred in feces 13 days after inoculation, when the rearing temperature is 24 °C, while latency was extended by 7 days at 18 °C. Model calculations designed for better estimation of pathogen-host dynamics also suggest that feces are an important factor in disease transmission for *N. lymantriae* in gypsy moth populations (Goertz & Hoch, 2001).

In the course of disease progression, silk glands soon get entirely filled with spores, which makes them lose their function. Infected larvae cease silk production (Weiser, 1961; Goertz & Hoch, 2008a). Hence, silk does not play a significant role in transmission of *N. lymantriae* (Goertz & Hoch, 2008a) as opposed to the closely related *Nosema portugal* (Jeffords *et al.*, 1987).

When third instars of *L. dispar* are inoculated with 1,000 spores, mortality is observed in more than 90% with only very few to no individuals reaching the adult stage (Goertz & Hoch, 2008a; Pollan *et al.*, 2009). Despite this high degree of virulence, *N. lymantriae* is not used as biopesticide in a conventional way since latency is too long.

Nonetheless, the pathogen can have an impact on host population dynamics as a naturally occurring enemy (Hoch & Goertz, 2009).

1.2.7 Environmental factors affecting microsporidian transmission

The only ontogenetic stage of microsporidia that is found outside the host is the environmental spore. It enables microsporidian dissemination and is therefore the agent of disease transmission (Undeen & Vávra, 1997; Vávra & Lukeš, 2013). Environmental spores are rather sensitive to abiotic factors, like UV-radiation, heat or low temperatures. Further, they lose their viability when exposed to dehydration (Weiser, 1961; Solter & Becnel, 2003). Due to their sensitivity to external factors and their need to enter the host gut before germination, a large number of spores is required to secure pathogen survival (Weiser, 1961; Vávra & Lukeš, 2013).

Feces of larvae infected with *N. lymantriae* contain large numbers of environmental spores and contribute to horizontal transmission (Goertz & Hoch, 2008a; Steyer, 2010). Feces of larvae feeding foliage are hard, dry and unlikely to stick to leaf surfaces. Presumably, the presence of moisture, for example after precipitation, can lead to improved adherence of fecal particles and thus to stronger contamination of foliage. This could ultimately lead to higher prevalence of the pathogen in gypsy moth populations.

1.3 Aims and rationale

Simulation of rain increases the transmission of *N. lymantriae* from feces with hard and non-sticky texture (Steyer, 2010). This study aims to further elucidate the impact of simulated rain on disease transmission in gypsy moth larvae.

A two-story cage system was constructed to provide near-natural distribution of spore-laden feces on foliage. Furthermore, it ensured that leaves contained in the bottom cage were only contaminated by fecal particles and not through direct contact with infected larvae. The water amount used for the simulation of rain was reduced to amounts that allowed studying the effects of light rain on transmission success. Furthermore, three stages of disease progression in experimentally inoculated larvae were chosen to estimate the effects of disease progression on transmission. To determine the potential transmission from contaminated foliage, populations of healthy larvae were tested.

The setup described above aimed to test the following hypotheses:

1. Transmission of the microsporidium *N. lymantriae* through spores from infectious feces is positively affected by the simulation of light rain.

2. Host larvae in later stages of disease progression cause a higher number of new infections in healthy larvae than infected individuals in an early stage of disease progression.

2. Material and Methods

2.1 Insects

L. dispar larvae (New Jersey Standard Strain) used in this study were provided in form of egg masses by the USDA-APHIS Otis Method Development Centre, Cape Cod, Massachusetts, USA.

Egg masses and larvae were incubated under controlled conditions in a climate chamber at 21 ± 1 °C with a photoperiod of 16 hours light and 8 hours dark (16L:8D). Groups of newly hatched larvae were transferred to 250 ml plastic cups and fed wheat germ diet (Bell *et al.,* 1981) until premolt to third instar.

On the first day of the third instar, larvae were inoculated with microsporidian spores and incubated for 12, 16 and 20 days (first trial) and 12, 15 and 18 days (second and third trial), respectively. The climate chamber was set to 21 °C and a photoperiod of 16L:8D. As indicated by a thermometer placed inside the climate chamber, however, the temperature was not constant during the entire incubation period but deviated from the desired 21 °C to up to 24 °C.

Test larvae were transferred to the semi-field-condition cage system at the first day of the third instar.

2.2 Pathogen

The microsporidium *Nosema lymantriae* „Levishte“ (Isolate No. 1996-A) was used in the experiments. This isolate is stored in the microsporidia germ-plasma collection of the Illinois Natural History Survey (INHS), Urbana-Champaign, Illinois, USA. Spore samples of *N. lymantriae* are kept in liquid nitrogen and propagated for experimental use in the laboratory of the Institute of Forest Entomology, Forest Pathology and Forest Protection, BOKU Vienna.

To ensure optimal infectivity of the microsporidium used in the experiment, fresh spores were produced by inoculating *L. dispar* larvae on the first day of the third instar with a dose of 1,000 spores each. Inoculated larvae were then incubated for 18 days. Spores were harvested from silk glands and fat body. Infected organs were separated, homogenized in tap water and filtered through cellulose tissue (Bauer *et al.,* 1998). The suspension was centrifuged at 3500 rpm for 15 minutes, the supernatant was discarded. The resulting pellet was resuspended in water, mixed with glycerol at a ratio

of 50:50 and divided into five 1 ml vials for storage in liquid nitrogen. The longest period of storage of spores used for the experiment was six weeks.

2.3 Experimental inoculation

Larvae that are referred to as inoculated larvae were treated as follows. *L. dispar* larvae were selected in premolt to third instar (indicated by a slippage of the head capsule to the front), grouped in cups and kept in the climate chamber (21 °C, 16L:8D) over night. Next day, freshly molted larvae were used for inoculation.

Prepared spores were thawed and suspended in water. To produce a suspension containing 1,000 spores per microlitre, the concentration of the mixture was determined by spore count in a Neubauer haemocytometer followed by dilution with water as appropriate.

Following the protocols of Goertz *et al.* (2007) and Pollan *et al.* (2009), wheat germ diet was cut into cubes of approximately 2 mm^3. The cubes were placed singly in the wells of 24-well microtiter plates and inoculated with 1 µl of above described spore suspension containing 1,000 spores.

Larvae were placed separately in the wells and kept in the climate chamber over night. For subsequent experiments only larvae that had consumed the entire diet cube until next morning were used. The ingested number of 1,000 spores was supposed to cause infections in 100% of the inoculated larvae and lead to death after 27-34 days (Goertz & Hoch, 2008a; Pollan, 2009; Pollan *et al*., 2009).

Successfully inoculated larvae were reared on wheat germ diet in groups of 9-11 individuals per cup in a climate chamber (21 °C, 16L:8D). Group size was reduced to 5 as larvae reached larger stages. Wheat germ diet was renewed when necessary.

2.4 Incubation periods and stages of disease progression

During the first trial, the dates for the experimental set-up were 12, 16 and 20 days post inoculation (dpi) considering the development of *N. lymantriae* in *L. dispar* larvae inoculated with 1,000 spores at the beginning of third instar and subsequent incubation at 21 °C (Pollan *et al.*, 2009). Pollan *et al.* (2009) found that the presence of spores in the Malpighian tubules approximately coincided with the beginning of spore release via feces. Moreover, spore release was only observed after larvae had molted to fifth instar and thus spores should be easily detected in the Malpighian tubules 12 dpi. For present experiments, larvae that had reached the fifth instar on day 12 after inoculation

were chosen for the early setup variant. 16 dpi was chosen following the protocol of Steyer (2010), representing the middle stage of infection. 20 dpi was chosen as the late-stage variant representing a terminal stage of infection before death of the larvae. At the end of each setup, all inoculated larvae were dissected to confirm success of infection.

After completion of the first trial, it became clear that most larvae had reached a very advanced stage of disease by day 20. Many inoculated larvae had died before the experimental setup on day 20; those surviving until the day of setup died during experiments. Time intervals between dates were, therefore, reduced from four to three days, resulting in the pattern 12, 15 and 18 dpi.

2.5 Dietary adjustment: change from wheat germ diet to foliage

An important factor in these experiments was the texture of feces of inoculated larvae. Wheat germ diet results in rather soft and wet excrement whereas foliage leads to firm and dry fecal pellets. To achieve close to natural conditions, Steyer (2010) put the larvae through a change of diet. Seven days prior to experimental setup, larvae were taken off wheat germ diet and left to feed on oak foliage. For the present test series, this approach was adopted with adjustments. Instead of oak, hornbeam (*Carpinus betulus*) leaves were used as the near-natural food source. Furthermore, the time period of dietary adjustment was reduced to three days.

The adjustment was conducted as follows. Three days before experimental setup, groups of inoculated larvae were transferred to hornbeam foliage. Therefore, twigs were cut off a hornbeam tree in the garden of the institute. To remove potentially present arthropods, leaves were rinsed with water and shaken vigorously. Twigs were bundled in a vase with tap water to keep them fresh. The vases were sealed with parafilm and placed in plastic boxes. 15-17 larvae were put into each box and incubated in a climate chamber under previously described conditions. Additional fresh hornbeam twigs were provided when necessary.

2.6 Cage system

To simulate two layers within a tree canopy a cage system was constructed. On the top branch, infected larvae fed leaves, defecated and released spores. The lower branch was not colonized by the larvae, but was exposed to contamination by feces falling down. Infected and uninfected larvae entered the cage system subsequently for

defined time periods (Fig. 1). The design chosen for this purpose was adopted from the experiments conducted by Steyer (2010).

Cylindrical Plexiglas cages (28 cm high with a diameter of 19.4 cm) were equipped with twigs of hornbeam that represented the corresponding layers of branches. The hornbeam twigs were taken from a tree in the garden of the institute at the day of experimental setup. Branches were rinsed with water and vigorously shaken to remove unwanted occupants. Small bouquets were assembled to create an approximate leaf surface area of 15-20 fully grown hornbeam leaves for each cage. To keep the leaves fresh throughout the experimental phase, the twigs were arranged in vases filled up with water. The vases were sealed with parafilm to prevent larvae from falling into the water.

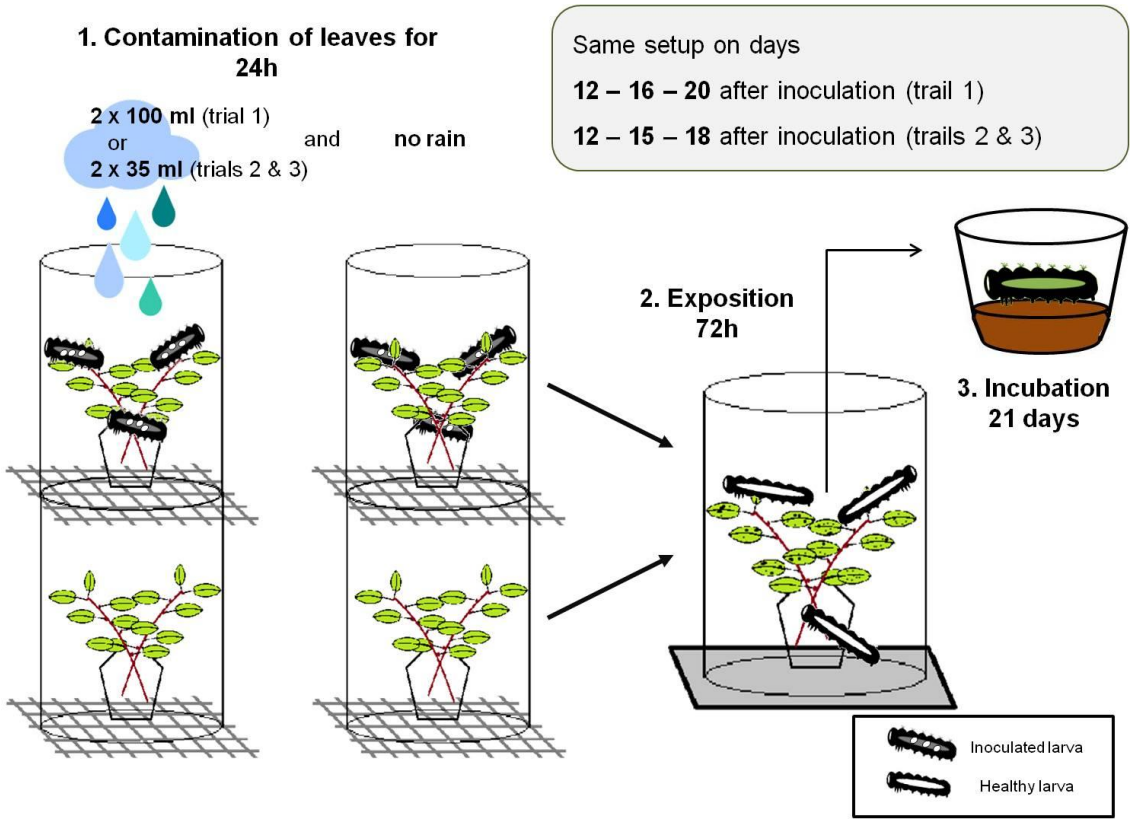

Fig. 1 Scheme of the experimental procedure. Five inoculated larvae were placed on hornbeam twigs for 24 hours. Afterwards, 10 healthy test larvae were placed in top and bottom cages and left to feed for 72 hours, followed by an individual incubation phase on wheat germ diet. Time interval of setup dates was four days in the first and three days in the second and third trial.

2.6.1 Contamination phase

The experiment was set up in the garden of the institute on days 12, 16 and 20 post inoculation (in the first trial) and days 12, 15 and 18 post inoculation (in trials two and three), respectively. To shield the cages from wind and rain, they were placed under a roof.

Two Plexiglas cages were stacked, separated by a steel grid. The grid kept inoculated larvae from entering the bottom cage while, at the same time, facilitating the passage of fecal pellets into the bottom cage.

This two-story cage system was placed on grids on bowls filled with water. This construction on one hand allowed water from rain-simulation to run off and on the other hand kept ants from entering the cages during the contamination phase. For additional stability, the two-story constructs were placed in larger, wooden cages to prevent them from toppling over (Fig. 2).

Five inoculated larvae were placed onto the leaves within each of the top cages. To prevent them from escaping, the top cages were covered with gauze and fixed with wire rings.

Inoculated larvae entered the cage system in the late afternoon. After 24 hours the larvae were removed and placed in the freezer for preservation until examination.

Fig. 2 Cage system during contamination phase.

2.6.2 Simulation of rain

During the contamination phase, rain was simulated twice in half of the cage systems. Tap water was sprayed with a spray bottle from above into the top cages, once at 6 p.m. and once at 8 a.m. the following day. During the first trial, 100 ml of water were used per cage for each rain event (approximately 6.8 mm precipitation within 24 hours). This large amount was reduced to 35 ml for the second and third trials, representing a precipitation of 2.4 mm within 24 hours.

2.6.3 Transmission phase

At the end of the contamination phase, inoculated larvae were removed and the two-story cage-system was deconstructed. Each Plexiglas cage, along with the containing vase, was transferred onto a Plexiglas bottom plate and attached to it with duct tape on the outside. The bouquets of hornbeam-twigs were handled carefully to keep them in their original state of contamination. The cages were placed on a wood palette under a roof at outdoor conditions (Fig. 3).

Ten healthy test larvae on their first day of third instar were placed into each of the resulting cages, regardless of former position (top or bottom). Each cage was covered with a piece of gauze. To keep ants from climbing up and entering the cages, a stripe of petroleum jelly was spread all around each Plexiglas cylinder.

The freshly molted test larvae were left to feed on the contaminated leaves for three days. Then they were individually put into small cups and reared on wheat germ diet for at least 21 days at room temperature to allow possible microsporidian infection to establish and to facilitate microscopic diagnosis. Fresh diet was provided as required during incubation.

Fig. 3 Cage system during transmission phase. Inoculated larvae were replaced by 10 healthy test larvae.

2.6.4 Control group

Alongside each repetition, a group of ten healthy third instar larvae was fed with untreated foliage in order to rule out the possibility of microsporidian contamination of hornbeam leaves. This control group was also kept in a Plexiglas cage under outdoor conditions for three days. Like test larvae, they were incubated on wheat germ diet for 21 days and dissected for diagnosis.

2.7 Temperature measurement

During the periods of the experiments in the institute garden, air temperature was recorded with a Hobo Pendant data logger. During the contamination phase, the data logger was put inside the wooden cage together with the Plexiglas cylinders. For the transmission phase, the data logger was transferred to a small paperboard case (protection from direct sunlight) and placed between the cages.

2.8 Dissection for diagnosis

L. dispar larvae were dissected longitudinally on the ventral side, fresh smears were prepared from silk glands and the fat body. Diagnosis of infection of *L. dispar* pupal stage during incubation was carried out as follows. A cross section of the middle thoracal ring was conducted. From each pupa three tissue samples were taken, smeared onto a glass slide with a drop of water and observed with phase contrast microscopy at 400-fold magnification.

2.9 Analysis of fecal particles

Fecal particles were collected during the contamination phase with gauze tissue attached underneath the lower steel grid. At deconstruction of the two-story cages, they were collected in plastic zip bags and stored in a freezer until examination.

Frozen excrements were transferred to 2 ml (bigger samples: 10 ml) tubes. Open tubes were placed in a drying cabinet for 5 days at 40 °C together with a bowl of silica gel. Subsequently, the tubes were closed and stored in an exsiccator (also equipped with silica gel) until the day of spore extraction.

Dried samples were taken out of the exsiccator and weighed on a microbalance (Mettler Toledo MT5), particles were counted. The size (length, width) of approximately 10 particles per sample was determined using millimetre paper. Then deionised water was added to the tubes and feces were left to soak for approximately two hours. To extract microsporidian spores, feces were homogenized and filtered through two layers of cellulose. The resulting extract was centrifuged at 3,500 rpm for 15 minutes. The supernatant was discarded, the pellet was resuspended in 0.75 ml of deionised water and layered on 0.75 ml of 60% sucrose solution. This was followed by another centrifugation step, this time at 1,650 rpm for 15 minutes. Again supernatant was discarded and the pellet was mixed with 1 ml of deionised water (Goertz & Hoch,

2008a). The resulting suspension was counted using a Neubauer haemocytometer; 5 large squares were counted in 6-fold repetition. Results were averaged.

2.10 Data analysis

Data analysis was performed with IBM SPSS Statistics Version 21.0 (Copyright: IBM Corporation 2012), arithmetic means and standard deviations were calculated with Excel (Microsoft Office Professional Plus 2010). All experiments were repeated three times. Results from trial 2 were excluded from the statistical analysis as the outcome of the experiments was biased by extremely high ambient temperatures and strong insulation in June 2015. Further details on this issue will be discussed below (see 4.3.2). For analysis, each cage represented a replicate. Therefore, n = 6 for each variant with one exception: n = 4 in the late stage variant without rain. This is due to the fact that many of the inoculated larvae died before they had reached the intended ‘late stage of infection‘. Transmission was calculated as percentage of infected individuals within the test larvae population in one cage. The two-story cage system resulted in two different types of contamination on foliage. Leaves in top cages were contaminated by the presence of inoculated larvae and their feces while the bottom cages were solely contaminated by infectious feces. The resulting percent infection were paired samples, therefore comparison of contamination type was conducted via Wilcoxon Matched-Pairs Test. Paired comparison of 15 and 16 dpi as well as 18 and 20 dpi via the Mann-Whitney *U* Test did not show any significant difference between the groups. Therefore 15 and 16 dpi data were combined as ‘middle stage of infection‘ and 18 and 20 dpi data form the dataset ‘late stage of infection‘. All inoculated larvae in the group ‘early stage of infection‘ entered the cage system 12 dpi.

Kolmogorov-Smirnov Test indicated no normal distribution for most of the obtained data. Therefore, non-parametric tests were chosen for data analysis. To investigate whether transmission rate in dry cage systems differed from that in irrigated ones, pairwise Mann-Whitney *U* Tests were performed for all three infection-stages separately. To examine effects of disease progression on transmission rates Kruskal-Wallis *H* Test was conducted. When significant differences were indicated, a pairwise comparison with Mann-Whitney *U* was performed. To control for type I errors, the level of significance was adjusted with the Bonferroni-correction. Furthermore, Pearson Correlation Coefficients and corresponding significance levels for the change of transmission in the course of disease progression were calculated in Excel. Therefore, percent infection was arcus-sinus-transformed and assigned to the corresponding dpi (not grouped as early, middle, late).

If a data set showed a high degree of correlation, also regression was analyzed and linear and logarithmic curves were calculated.

Mean dry weights of fecal particles were pooled to variants ‘early‘, ‘middle‘ and ‘late‘. Only data of the first and third trial were used for statistical analysis. Differences between stages were calculated using the Kruskal Wallis *H*-test followed by the Mann-Whitney *U*-test with Bonferroni-corrected α. To determine significant differences between variants ‘rain‘ and ‘no rain‘, the Mann-Whitney *U*-test was applied.

3. Results

3.1 Disease progression in inoculated larvae

Dissection of inoculated larvae showed that in some cases only four out of five larvae had actually been infected. Furthermore, for the middle- and late-stage setup, several larvae died in the cage system during the contamination phase. Table 2 gives detailed information on the number and status of inoculated larvae and the resulting percent infection in corresponding test larvae populations.

Despite given circumstances, all replicates of trials 1 and 3 were included in statistical analysis to maintain a proper sample size.

3.1.1 Early stage of disease progression: 12 days post inoculation

Silk glands of infected larvae were hypertrophied and swollen areas were filled with environmental spores (Fig. 4). Life stages of *N. lymantriae* were also detectable in the fat body. Here, primary spores prevailed but environmental spores were also easily detected (Fig. 5). In the Malpighian tubules only a few spores were present (Fig. 6).

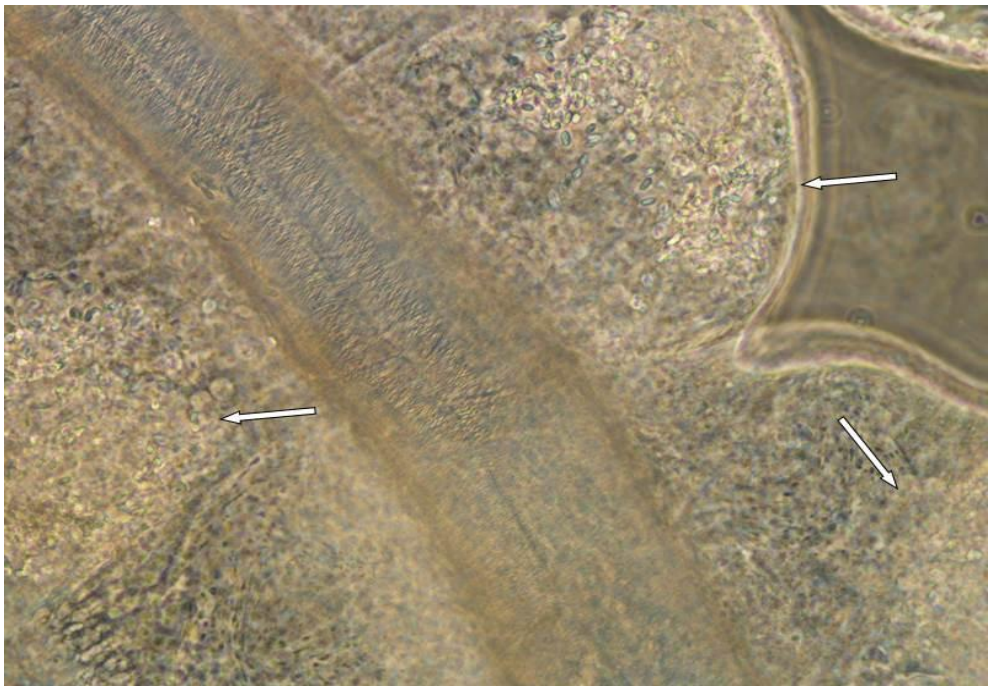

Fig. 4 12 dpi: Silk glands of *L. dispar* larva infected with *N. lymantriae*. Arrows indicate hypertrophied areas filled with spores.

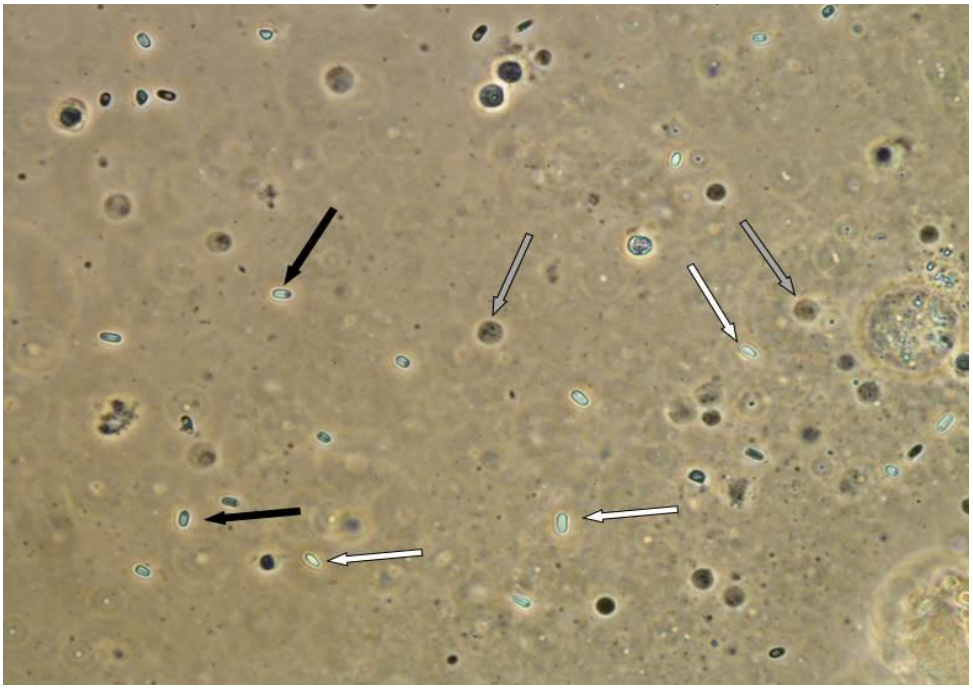

Fig. 5 12 dpi. All stages of *N. lymantriae* could be found in the fat body: grey arrows: meronts; black arrows: primary spores; white arrows: environmental spores.

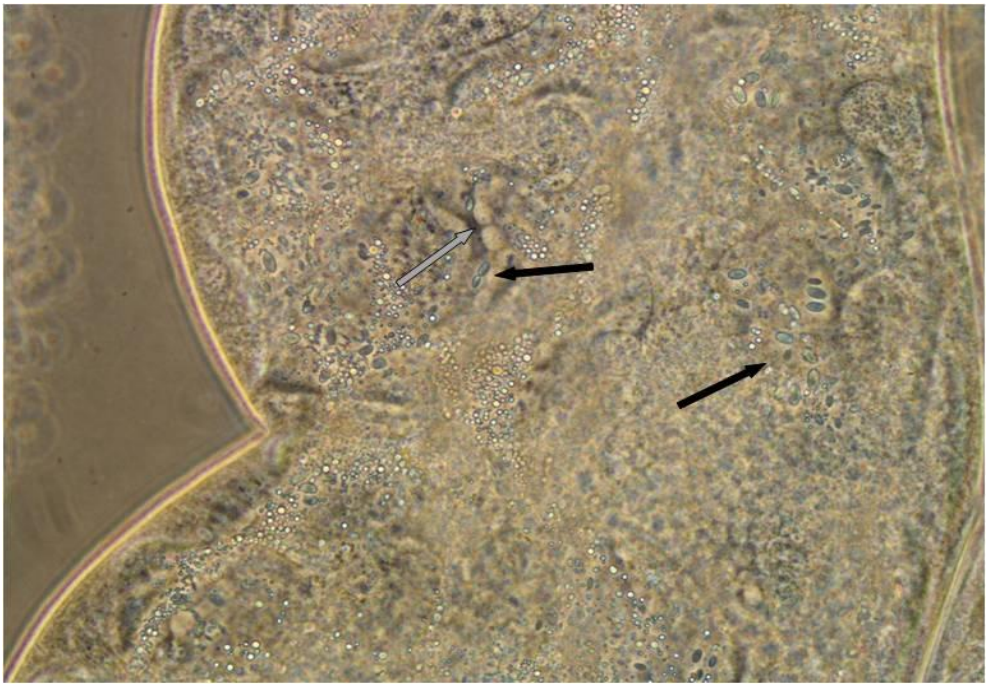

Fig. 6 12 dpi. Malpighian tubules of larvae were filled with uric acid crystals. Grey arrow: group of meronts; black arrows: primary spores.

3.1.2 Middle stage of disease progression: 15 and 16 days post inoculation

At 15 and 16 days after inoculation, silk glands were completely filled with environmental spores (Fig. 7). Also in the fat body the number of environmental spores had increased, still numerous primary spores could be found (Fig. 8). Although the content of the Malpighian tubules had increased in density, spores were more easily distinguishable than before (Fig. 9).

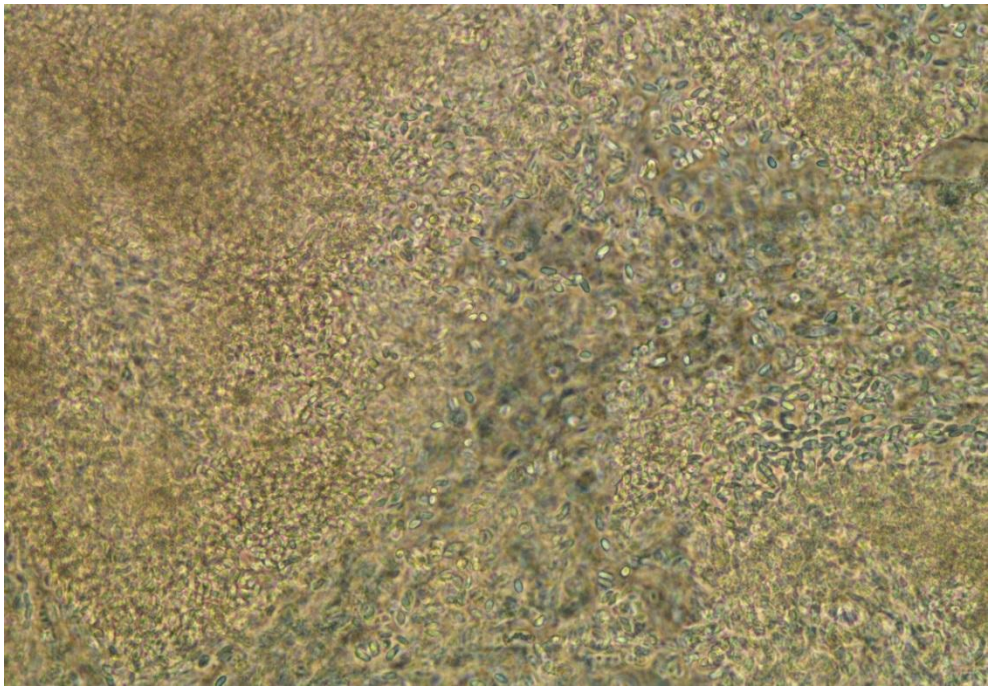

Fig. 7 16 dpi. Silk glands are completely filled with spores.

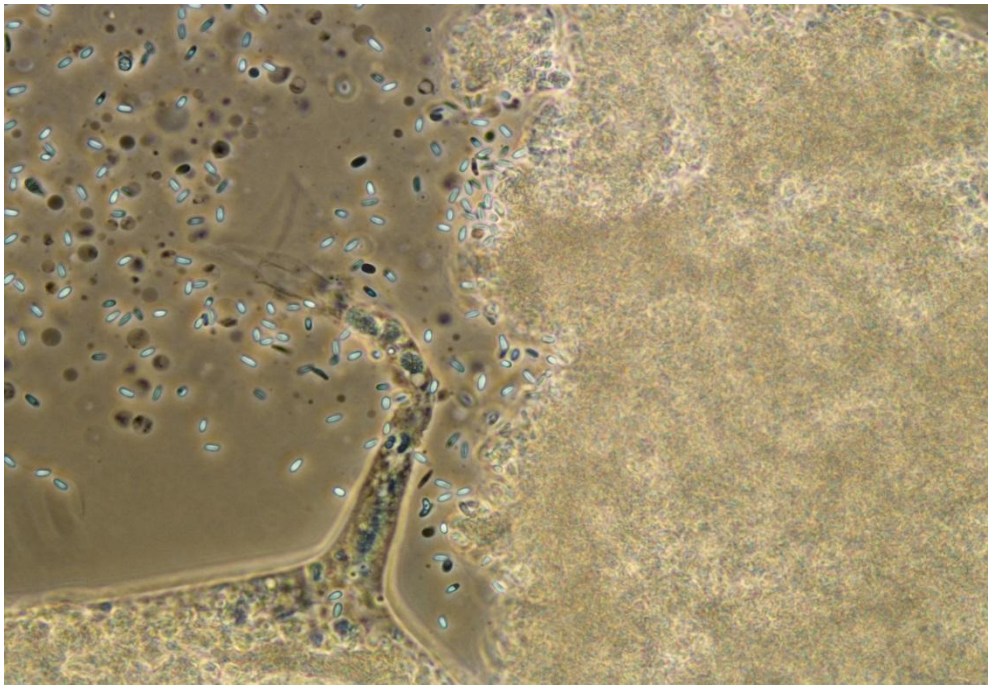

Fig. 8 16 dpi. Large numbers of environmental spores had developed in the fat body.

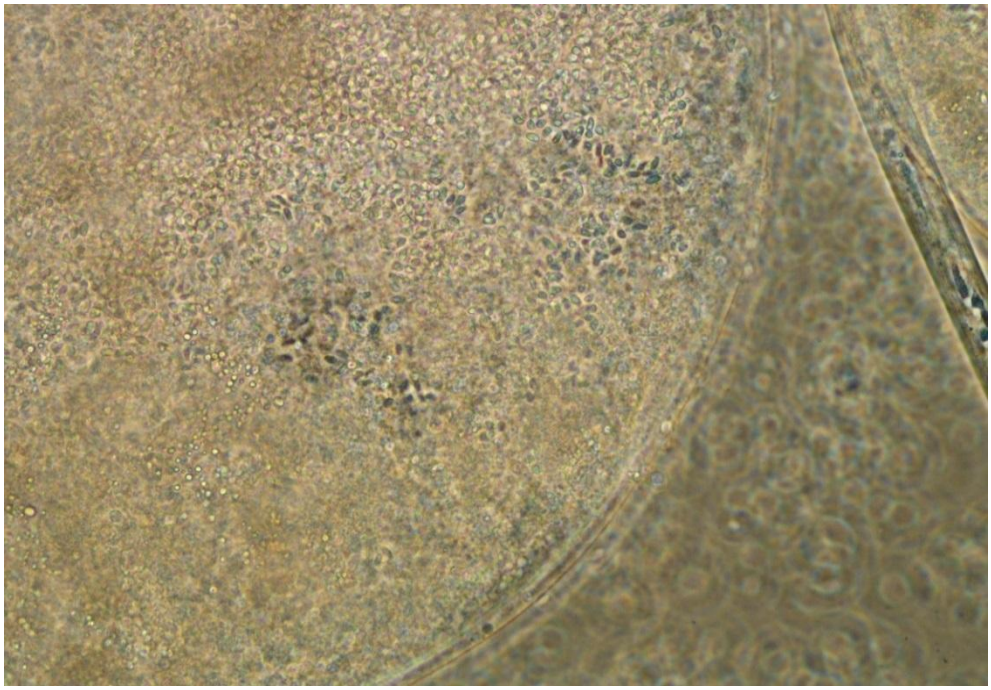

Fig. 9 16 dpi. Malpighian tubules were densely filled, microsporidian spores were easily distinguishable.

3.1.3 Late stage of disease progression: 18 and 20 days post inoculation

At the late stage of disease progression all organs were densely filled with environmental spores (Fig. 10-12). Heavy infection affected larval movement and behaviour visibly.

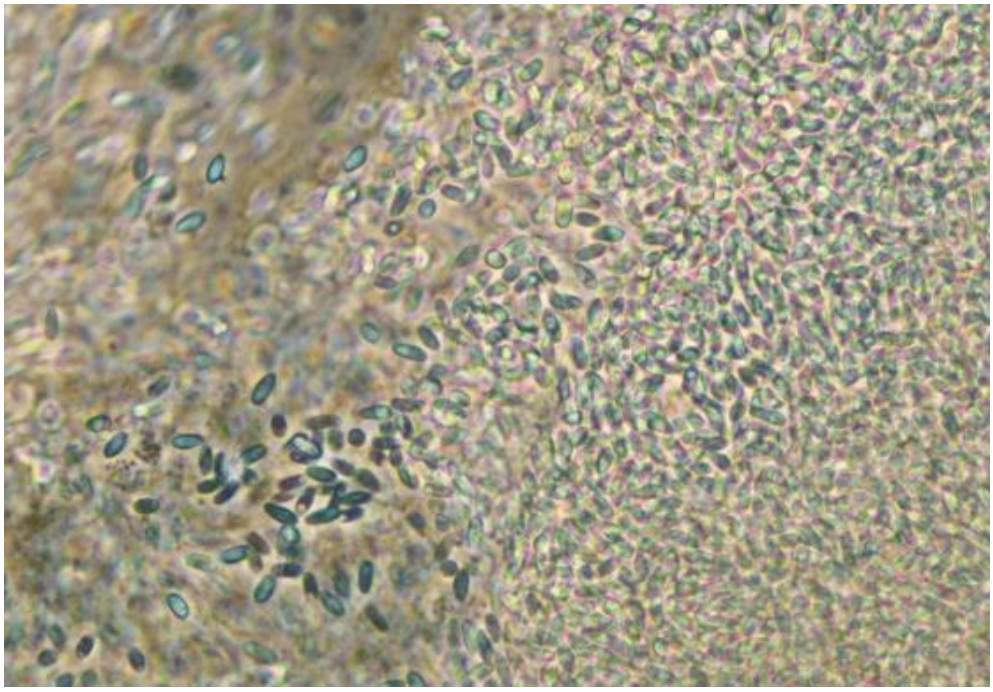

Fig. 10 20 dpi: Tissue of silk glands was densely filled with spores.

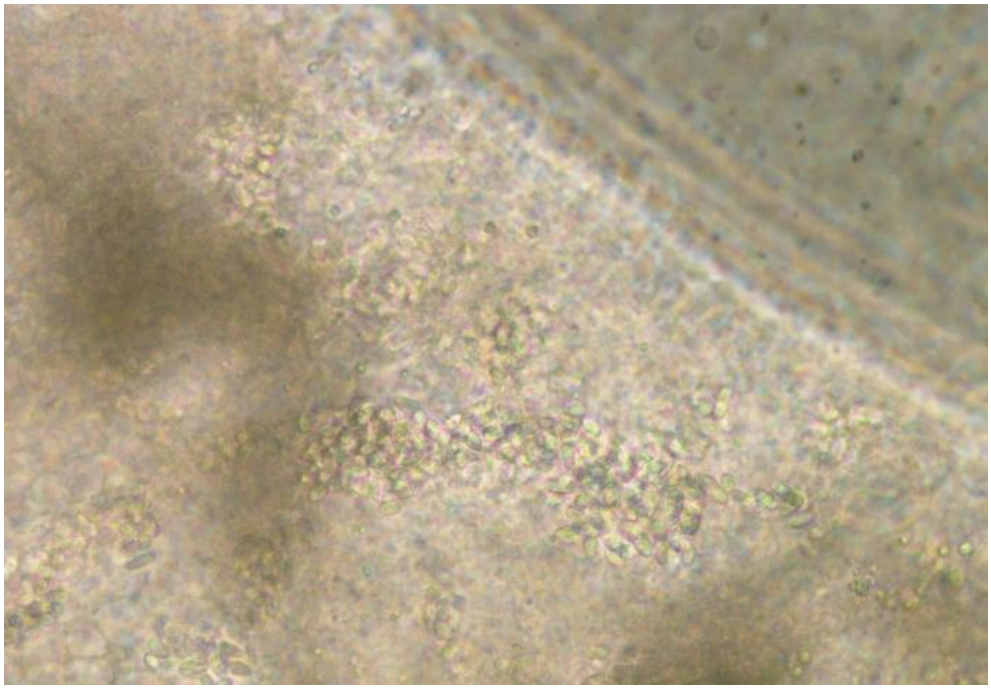

Fig. 11 20 dpi: Malpighian tubules were full of environmental spores.

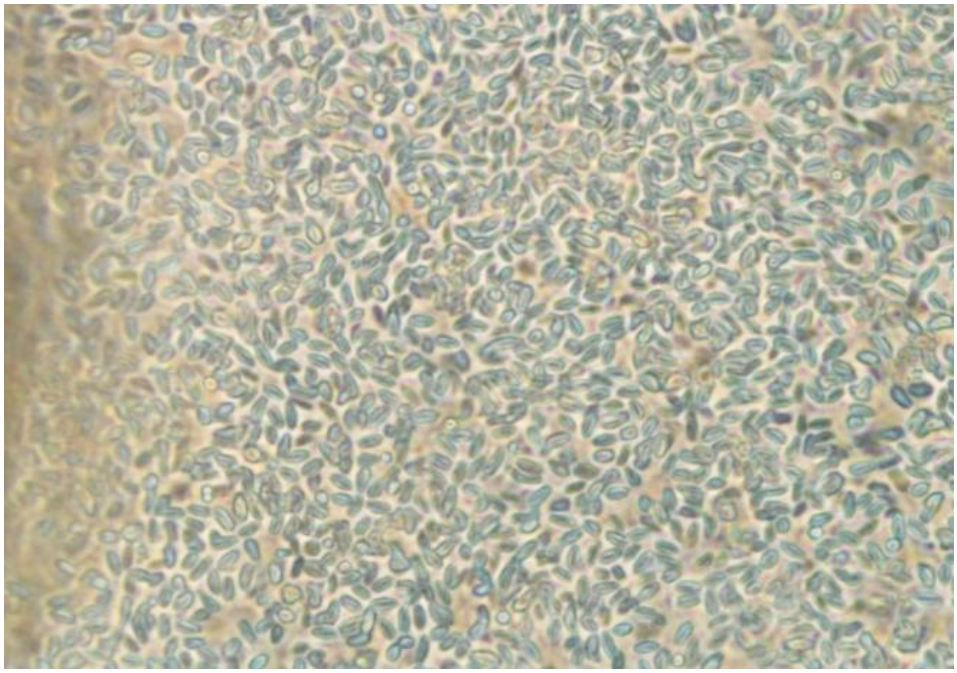

Fig. 12 20 dpi: Fat body tissue was filled with spores entirely.

3.2 Disease transmission in experimental populations of healthy test larvae

Out of 88 recovered and dissected control larvae none were found to be infected by microsporidia. Thus, leaves used in this experimental series were not contaminated with environmental spores.

3.2.1 Contamination in top and bottom cages

Percent infection was always higher in the top cages than in the bottom cages. The difference was statistically significant with two exceptions, which are probably due to the high variability of results and low sample size. Details on the observed percent infection are listed in tables 1 and 2.

Table 1 Percent infection (mean value ± standard deviation). P-value calculated for the pairwise comparison of top and bottom cages by Wilcoxon matched-pairs test. Sample size (n) indicates the number of cages; α = 0.05. Significant differences are indicated in bold letters.

		Mean percent infection		p	n
		Top Cage	Bottom Cage		
Early	**Rain**	41.7 ± 36.0	3.3 ± 8.2	**0.042**	6
	No rain	30.0 ± 39.0	0	0.063	6
Middle	**Rain**	93.3 ± 8.2	57.4 ± 31.0	**0.046**	6
	No rain	73.3 ± 25.0	16.7 ± 36.2	**0.046**	6
Late	**Rain**	88.3 ± 20.4	68.3 ± 34.9	**0.020**	6
	No rain	87.5 ± 9.6	7.5 ± 9.6	0.065	4

3.2.2 Leaf contamination in top cages

3.2.2.1 Effect of simulated rain on transmission

When leaves were contaminated by inoculated larvae in an early stage of disease progression (12 dpi), 30.0 ± 39.0% of test larvae became infected under dry conditions and 41.7 ± 36.0% when rain was simulated (Mann-Whitney *U* Test: p= 0.4).

Contamination of leaves during the middle phase of disease progression (15 and 16 dpi) led to 73.3 ± 25.0% infection when dry and 93.3 ± 8.2% infection when sprayed with water (Mann-Whitney *U* Test: p= 0.1).

Presence of larvae and feces of the last infection stage (18 and 20 dpi) resulted in 87.5 ± 9.6% infection on dry and 88.3 ± 20.4% infection on wet leaves (Mann-Whitney *U* Test: p=0.6).

As indicated by the p-values pairwise comparisons of obtained data via Mann-Whitney *U* Test did not show any significant differences between dry and irrigated groups (Fig. 13). Percent infection, however, was always higher in rain treatments.

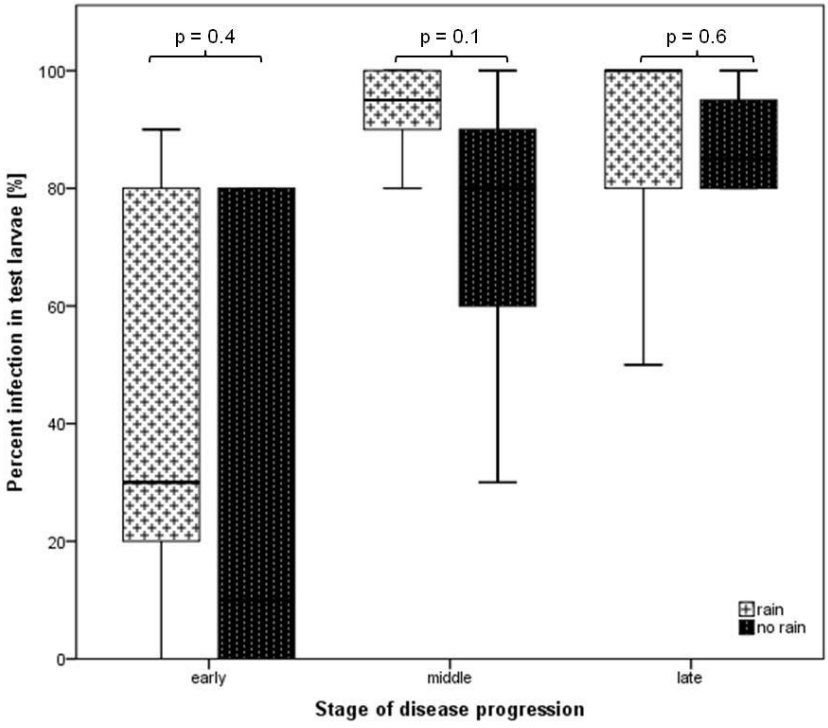

Fig. 13: Percent infection in test larvae with leaves contaminated by inoculated larvae and their feces. Pairwise Mann-Whitney *U* Tests showed no significant differences between variants with and without rain. α = 0.05, n = 6 (except for 'late, no rain', n = 4)

3.2.2.2 Effects of disease progression on transmission

Rain did not affect transmission success significantly in cages with contaminated larvae and their feces. Therefore, data of the variants with and without rain were pooled for investigation of the effects of disease progression.

With 83.3 ± 20.6% infection in the middle stage and 88 ± 16.16% infection in the late stage, the variants caused a higher percent infection than the early stage (35.8 ±

36.3%). Because Kruskal-Wallis *H* Test indicated significant differences between groups (*H* Test: $\chi^2 = 14.26$, df = 2, p = 0.001) pairwise Mann-Whitney *U* Tests were carried out. While there was no difference between the percent infection of middle and late stages, both were significantly higher than that of the early stage (*U* Test: early-middle: p = 0.001; early-late p = 0.001, Bonferroni corrected $\alpha = 0.0167$).

Pooled data of variants with and without rain showed a correlation of r = 0.58 (p < 0.000). When dividing data into two sets – 'rain' and 'no rain' – correlation was even stronger in the variant without rain (Pearson Correlation: 'no rain': r = 0.64, p = 0.008; 'rain': r = 0.53, p = 0.02).

Regression analysis of pooled data did not yield a high coefficient of determination for the applied regression models. Neither the linear ($R^2 = 0.335$; F (1, 32) = 16.146; p < 0.000) nor the logarithmic equation ($R^2 = 0.336$; F (1,32) = 18.44; p < 0.000), seemed to sufficiently describe the observed change in transmission (Fig. 14).

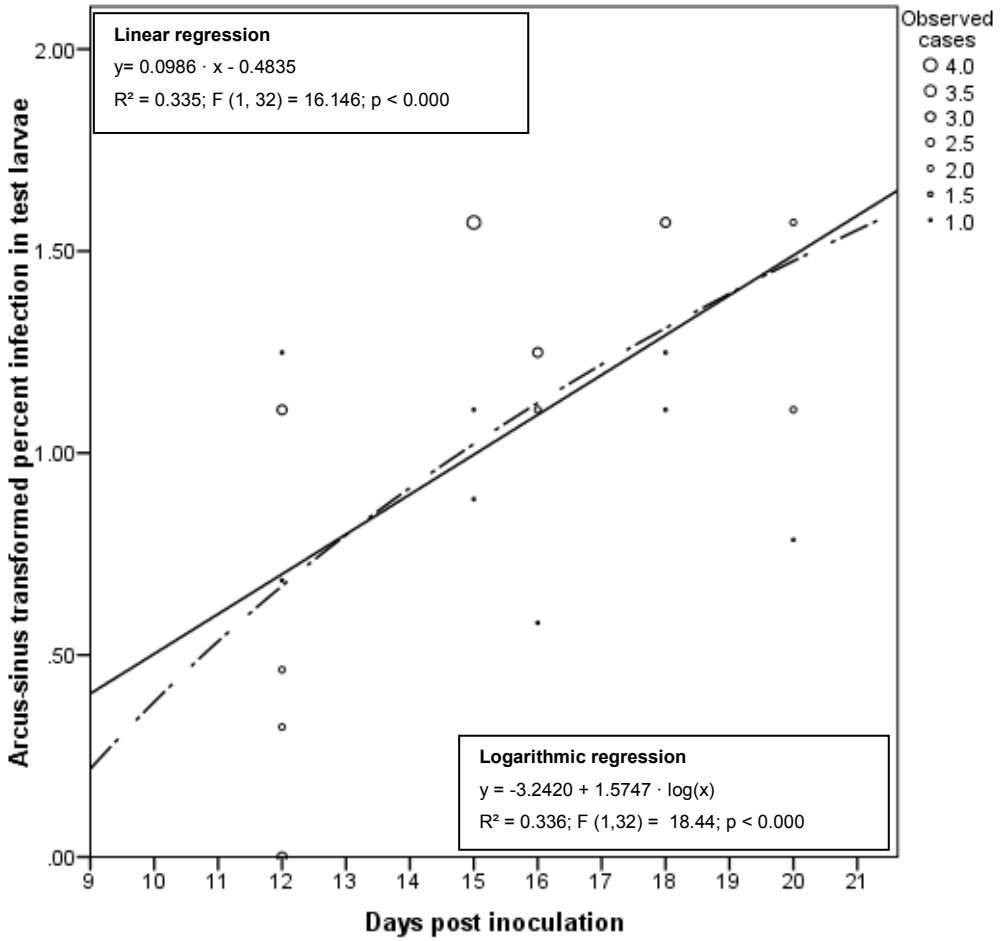

Fig. 14 Linear and logarithmic regression of percent infection over time in top cages. Pooled data of trials 1 & 3 and both variants, with and without rain.

3.2.3 Leaf contamination in bottom cage

3.2.3.1 Effect of simulated rain on transmission

When leaves were contaminated with feces only, no transmission to test larvae was observed during the early stage of infection (all dry cages and five out of six cages sprayed with water). One of the cages with simulated rain, however, resulted in 20% infection (Mann-Whitney *U*: p = 0.7).

In the middle and late stage of disease progression, transmission of microsporidia from feces of larvae was significantly affected by simulated rain (Fig. 15). In the middle stage, feces led to 16.7 ± 36.2% infection in the dry and 57.4 ± 31% in the irrigated variant, in the late stage mean values of 7.5 ± 9.6% and 68.3 ± 34.9% were observed (*U* Test: for middle stage: p = 0.04; for late stage: p = 0.02).

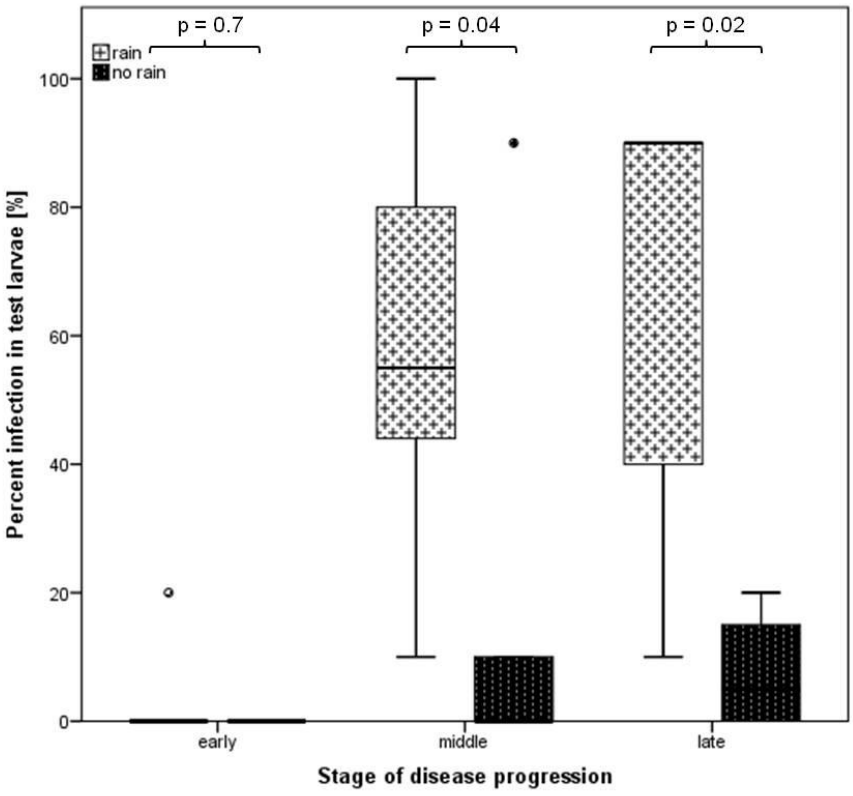

Fig. 15 Percent infection in test larvae with leaves contaminated only with feces of inoculated larvae. Pairwise Mann-Whitney *U* Tests showed no significant difference for the early infection stages but significant differences for the middle and the late infection stage. α = 0.05, n = 6 (except for 'late, no rain', n = 4). Circles indicate outliers.

3.2.3.2 Effects of disease progression on transmission

Data of variants ‘with rain‘ and ‘no rain‘ were analyzed separately.

When no rain was simulated, percent infection in test larvae population did not change significantly in the course of disease progression (Kruskal-Wallis *H* Test: $\chi^2 = 3.163$, df = 2, p = 0.2). Almost no transmission occurred in the early stage variants, 16.6 ± 36.2% infection was caused by middle stage and 7.5 ± 9.6% infection by the late stage, respectively.

Transmission success increased with disease progression in treatments with simulated rain (Kruskal-Wallis *H* Test: $\chi^2 = 10.623$, df = 2, p = 0.005). Both, pairwise comparison of early (3.3 ± 8.2%) and late stage of infection (68.3 ± 34.9%), as well as early and middle stage (57.4 ± 31%) revealed a significant difference (Mann-Whitney *U* Test: early-middle: p=0.004; early-late p = 0.004, Bonferroni corrected $\alpha = 0.0167$).

Arcus-sinus transformed percent infection correlated significantly with disease progression in the variant ‘rain‘ (Pearson: r = 0.65, p = 0.003), but not in that without rain (Pearson: r = 0.2, p = 0.46).

Regression analysis was only performed for the variant with simulated rain. Both models, linear ($R^2 = 0.424$; F (1, 16) = 11.762; p = 0.003) and logarithmic ($R^2 = 0.470$; F(1, 16) = 14.208; p = 0.002), showed a low coefficient of determination (Fig. 16).

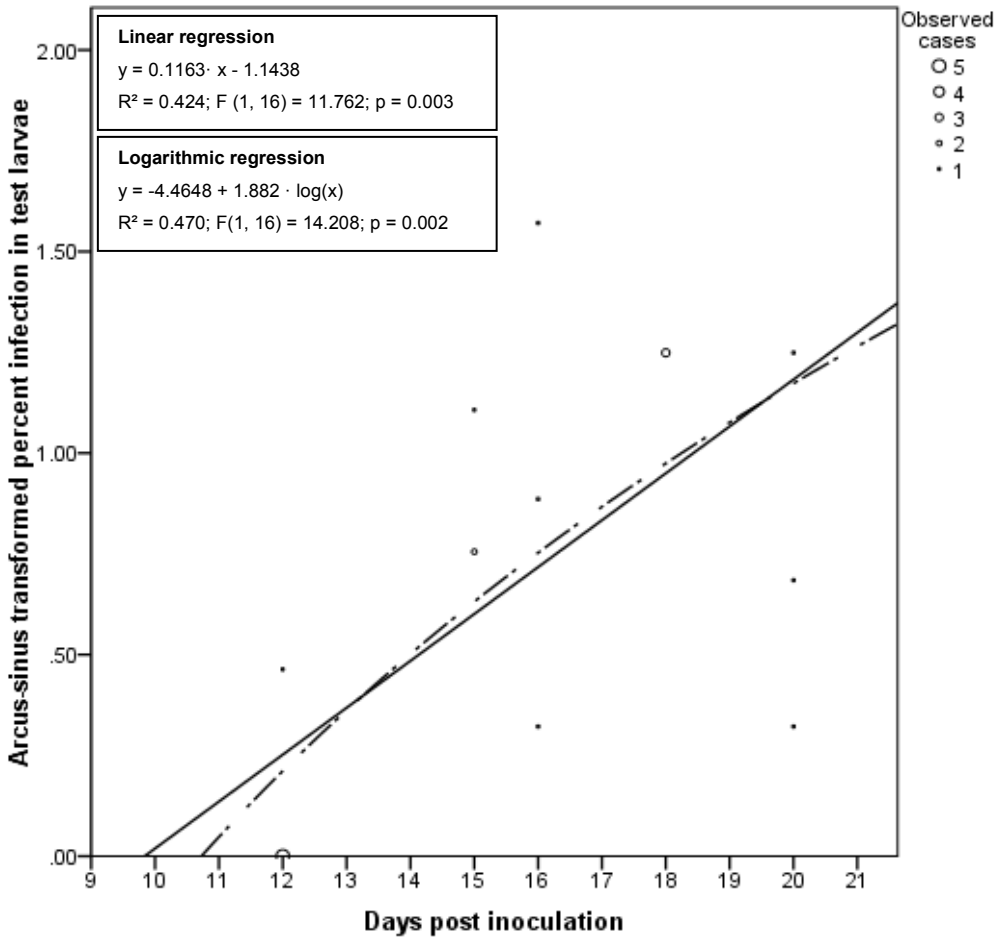

Fig. 16 Linear and logarithmic regression of change in transmission over time in cages with simulated rain and feces contamination.

Table 2 Infection in test larvae (%) when leaves were contaminated with inoculated larvae and their feces or by feces alone. Data from all 3 trials are presented, only trials 1 and 3 were used for statistical analysis and the data presented in this chapter (3.2).

		Inoculated larvae and feces		Feces	
	Trial	Rain	No rain	Rain	No rain
Early stage of infection (12 dpi)	**1**	20	10	0	0
		0	0	0	0
		40	0	0	0
	2	0	0	0	0
		0	0	0	0
		0	0	0	0
	3	90	10	20	0
		20	80	0	0
		80	80	0	0
Middle stage of infection (15 & 16 dpi)	**1**	90	90 [2]	60	0 [2]
		80 [2]	30	10 [2]	0
		90 [2]	80 [1]	100 [2]	0 [1]
	2	0	10	0	0
		10	0	0	0
		0	20	0	0
	3	100	100	44	0
		100 [1]	80 [1]	80 [1]	90 [1]
		100	60	50	10
Late stage of infection (18 & 20 dpi)	**1**	100 [3]	80 [2]	90 [3]	0 [2]
		50 [1]	100 [1]	40 [1]	10 [1]
		80		10 [3]	
	2	100	100	0	0
		100	60	0	0
		10	100	25	0
	3	100 [2]	80	90 [2]	0
		100 [1]	90 [1]	90 [1]	20 [1]
		100 [1]		90 [1]	

Status of inoculated larvae	
Symbol	**Meaning**
shaded	4 out of 5 inoculated larvae were infected
[1]	1 inoculated larva died during the contamination phase
[2]	2 inoculated larvae died during the contamination phase
[3]	3 inoculated larvae died during the contamination phase

3.3 Feces

Results of analysis below contain pooled data of trials 1 and 3. ‘Early‘ refers to 12 dpi, ‘middle‘ sums up 15 and 16 dpi and ‘late‘ contains data of 18 and 20 dpi.

The weight of fecal particles increased steadily: early stages produced the lightest pellets and late stages the heaviest. A difference between stages was indicated for the dry weight of fecal pellets in cages without simulated rain (Kruskal Wallis *H*-test: χ^2= 13.25, p = 0.001). Mann Whitney *U* showed significant differences between all pairs (early-late: p = 0.010; early-middle: p = 0.002; middle-late: p = 0.010; Bonferroni corrected α = 0.0167).

For the variant with rain, dry weights were also significantly different between stages, (*H*-test: χ^2= 6.382, p = 0.041), but only the pairwise comparisons of early and middle stage of infection were significantly different (*U*-test: p = 0.009)

Pellets from dry cages were rather firm and compact which was in contrast to the friable texture of pellets exposed to rain. Pairwise comparison of fecal dry weights of variants with and without rain was conducted using the Mann-Whitney *U*-test. Mean dry weights are listed in table 3. In the early stage, fecal particles collected in cages with simulated rain were significantly lighter than pellets from the dry variant (*U*-test p = 0.009). In the middle stage, no significant difference was observed (p = 0.537). In the late stage, the decrease was again significant (*U*-test p = 0.016) (Fig. 17).

Table 3 Dry weight per fecal pellet. Different lowercase letters indicate significant differences between the variants ‘rain‘ and ‘no rain‘. Different uppercase letters indicate significant differences between the early, middle and late stage of disease progression.

	Rain	**No rain**
Early	0.66 ± 0.11 (n = 6) *A a*	0.88 ± 0.08 (n = 6) *A b*
Middle	1.23 ± 0.56 (n = 5) *B a*	1.15 ± 0.05 (n = 6) *B a*
Late	1.10 ± 0.44 (n = 5) *B a*	2.21 ± 0.44 (n = 4) *C b*

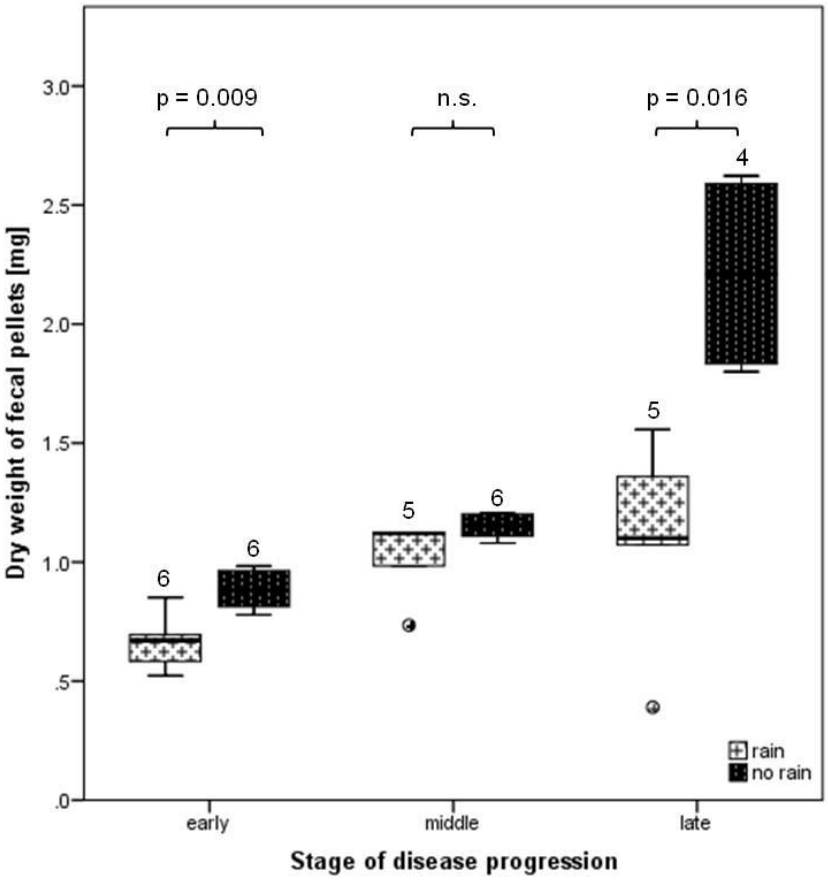

Fig. 17 Dry weight of fecal particles [mg] in the course of disease progression. Significance of difference was determined using the Mann-Whitney *U*-test, sample size (n) is indicated by the number above each box, α=0.05. Circles indicate outliers.

The decrease of dry weight caused by simulated rain was variable. Fig. 18 shows the mean dry weights of fecal pellets for all trials and stages. Fecal particles of the larvae used for the middle setup of the second trial were found to be heavier than those collected during the other two trials.

It was not possible to reliably quantify spore content of the fecal pellets. The method applied for spore separation was not suitable for samples that previously had been frozen and then heat dried. Details can be found in the discussion section.

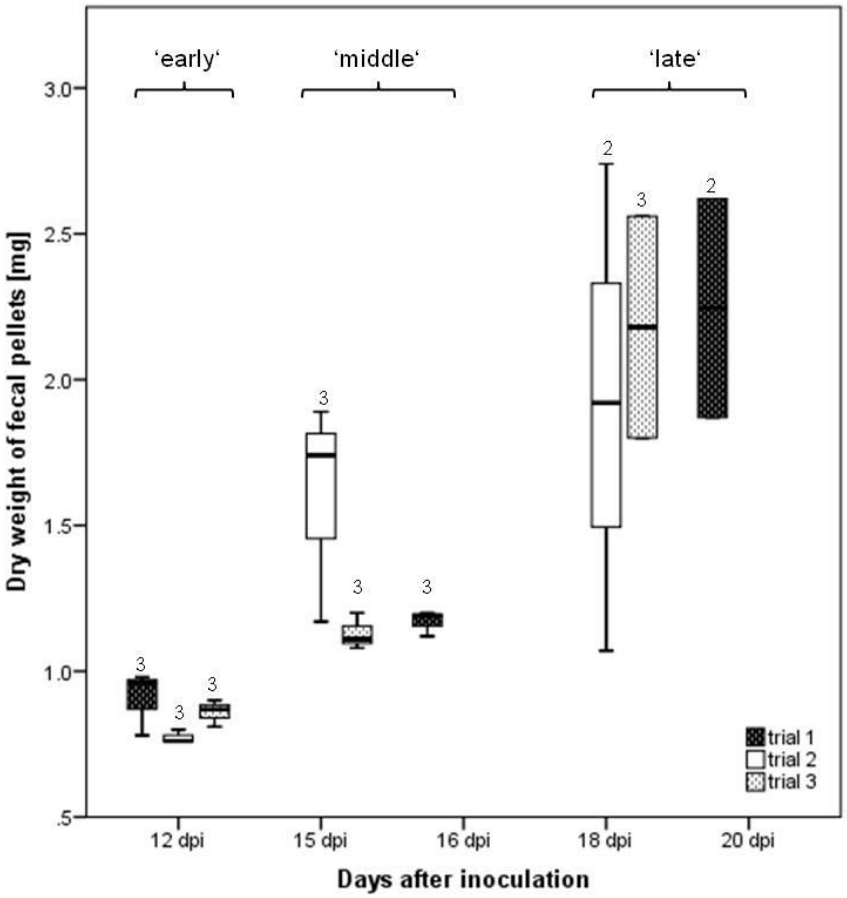

Fig. 18 Mean dry weight [mg] per fecal particle of the variant without rain for all trials and corresponding larval age (measured in days post inoculation). Early, middle and late indicate the corresponding disease progression stage. Sample size (n) is indicated by the number above each box.

3.4 Temperature profiles

3.4.1 First trial

During the first trial (13-25 May 2015), ambient temperatures were rather moderate. The daily mean temperatures (measured once per hour) lay between 11.4 and 21.4 °C. A peak temperature of 31.3 °C was recorded on 19 May at midday. The lowest value measured during this trial was 9.9 °C.

3.4.2 Second trial

The second trial was setup on 28 May and completed on 7 June 2015. The temperatures increased continuously and peaked at 43.6 °C on 3 June 2015. The lowest temperature during this time was 8.5 °C on 29 May. Average daily temperatures ranged from 13.0-25.7 °C.

3.4.3 Third trial

During the third trial from 18 June to 28 June, average daily temperatures ranged between 14.4-22.8 °C. The highest temperature in the experimental area was 30.2 °C, the lowest 10.6 °C.

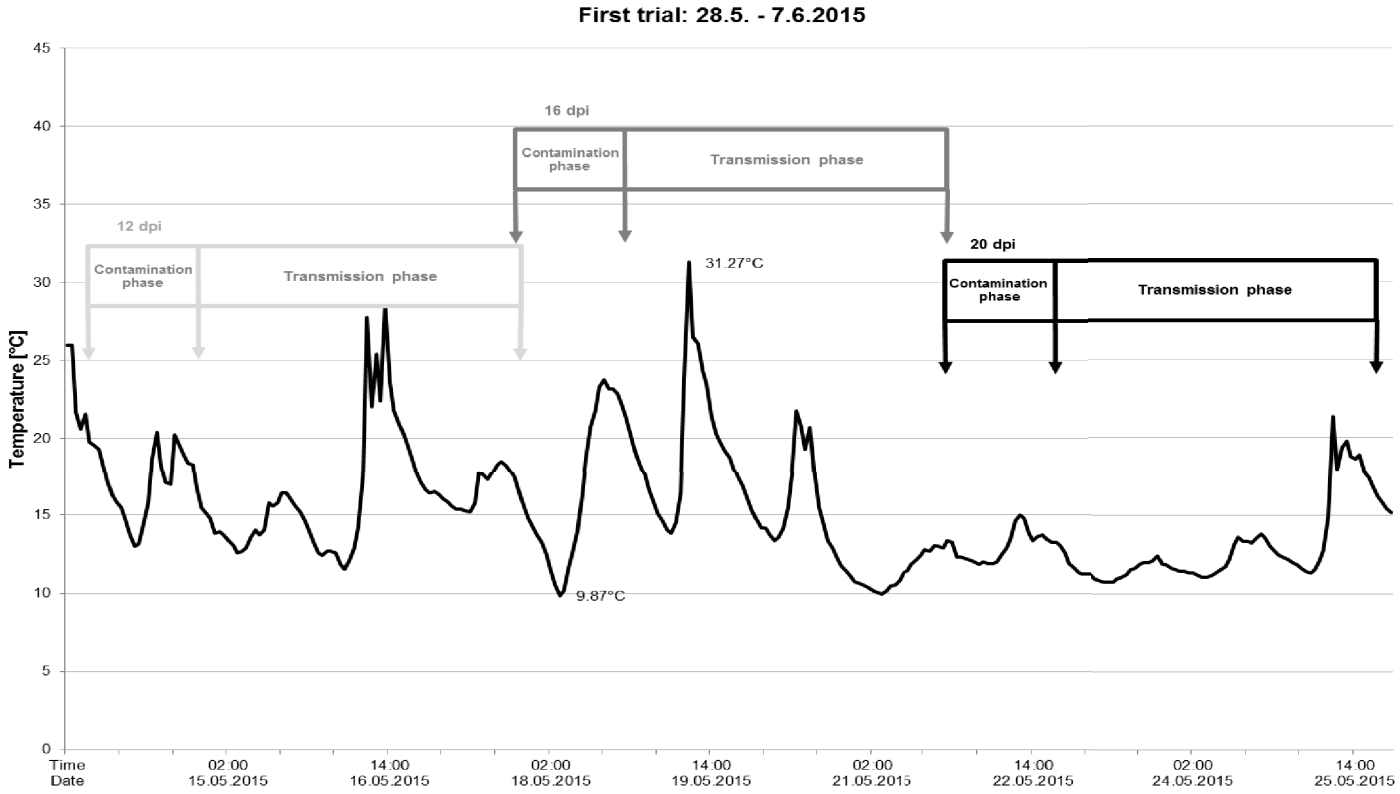

Fig. 19 Temperature profile during the first trial of the outdoor experiment. 12 dpi: contamination phase (cp): 13.-14.5., transmission phase (tp): 14.-17.5., 16 dpI: cp: 17.-18.5., tp: 18.-21.5.; 20 dpi: cp: 21.-22.5., tp: 22.-25.5.2015.

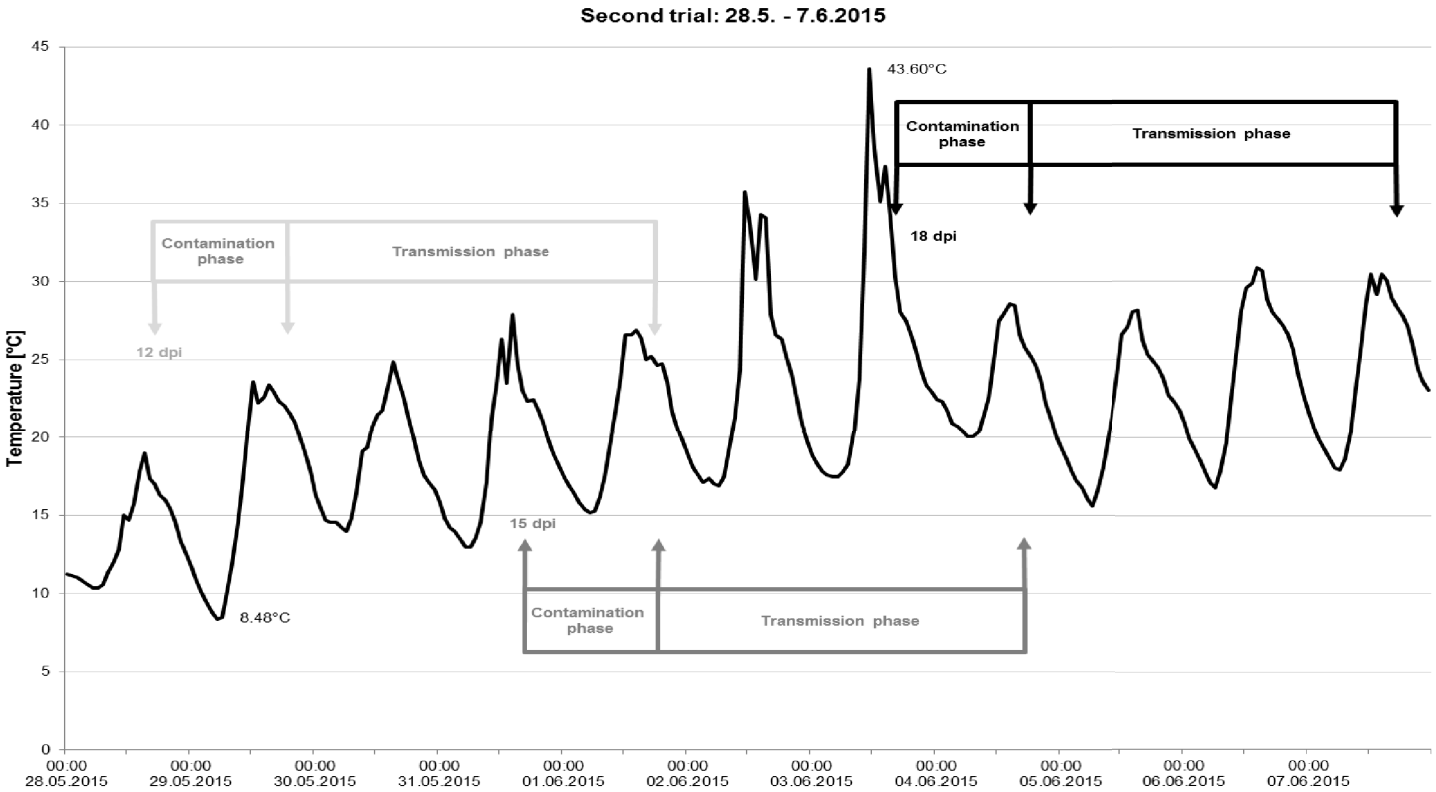

Fig. 20 Temperature profile during the second trial of the outdoor experiment; 12 dpi: contamination phase (cp): 28.-29.5., transmission phase (tp): 28.5.-1.6.; 15 dpI: cp: 31.5.-1.6., tp: 1.6.-4.6.; 18 dpi: cp: 3.6.-4.6., tp: 4.6.-7.6.2015.

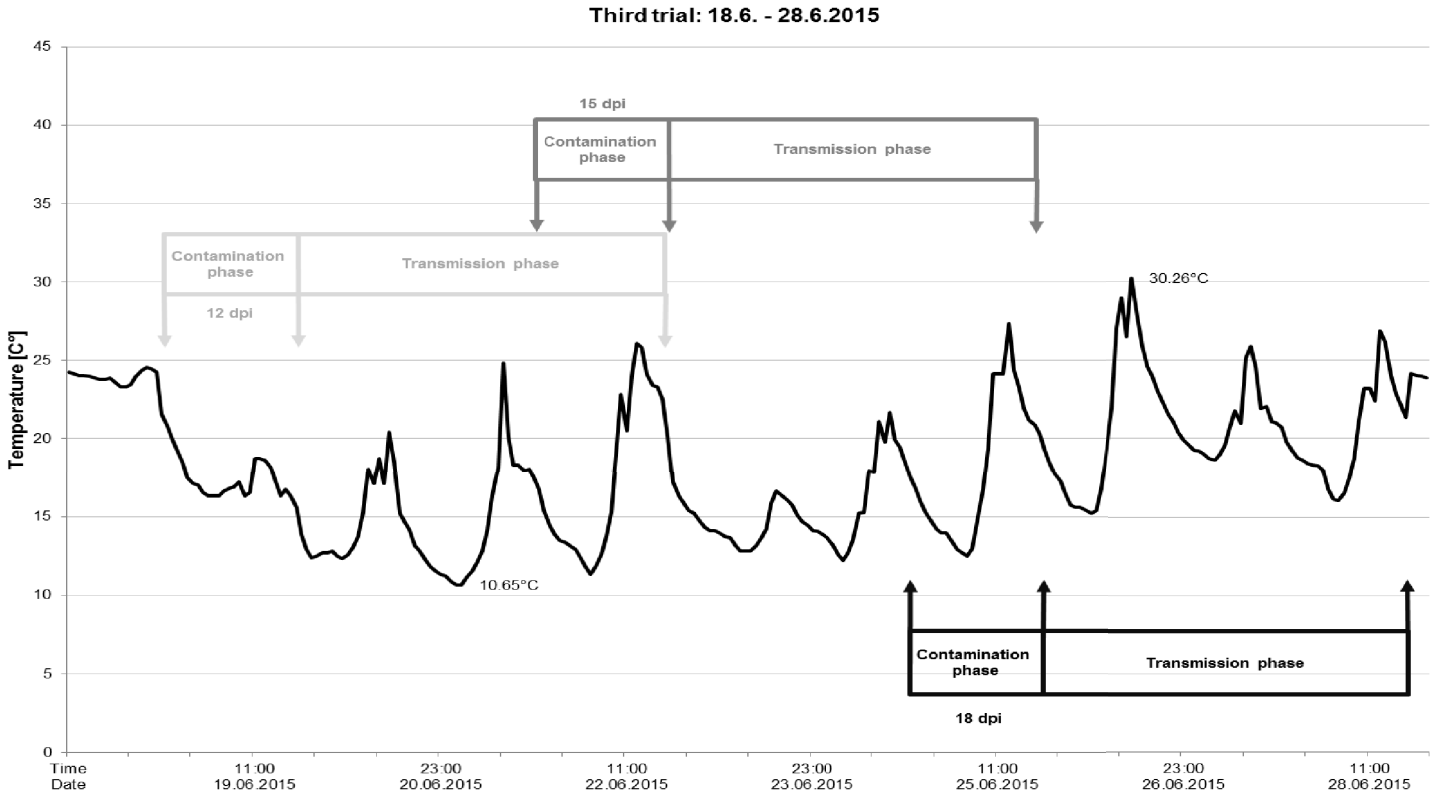

Fig. 21 Temperature profile during the first trial of the outdoor experiment; 12 dpi: contamination phase (cp): 18.6.-19.6., transmission phase (tp): 19.6.-22.6.; 15 dpI: cp: 21.6.-22.6., tp: 19.6.-22.6.; 18 dpi: cp: 24.6.-25.6., tp: 25.6.-28.6.2015.

4. Discussion

4.1 Disease progression in inoculated larvae

The first organs of inoculated larvae to be heavily infected are the silk glands. Twelve days after inoculation, environmental spores had already formed in large numbers. Hypertrophied areas of the tissue appeared white and could even be recognized by the naked eye. At this stage, also the fat body contained numerous primary spores. In the Malpighian tubules meronts could be found. 15 and 16 days post inoculation disease had markedly progressed. All organs already contained a much higher amount of environmental spores and the increase of spore numbers continued. All investigated organs were filled with spores at the late stage of infection. During the first trial 20 days post inoculation was chosen as the setup date of the ‘late stage‘ variant. In this trial larvae were impaired in their movement. When being placed on the hornbeam twigs or sprayed with water they were too weak to hold on tight and kept falling off. Also, high mortality dramatically reduced the number of inoculated larvae available for the late experimental setup. Several inoculated larvae even died during the contamination period in the cage.

Development of microsporidia in the host corresponded with the disease progression described by Pollan (2009). Observed spore distribution in the host organs is rather similar at the two warmer temperature regimes studied by Pollan (2009), 21 °C and 24 °C. The difference is more pronounced in the cumulative mortality rates than in the spore content of affected organs. 20 days after inoculation, Pollan (2009) observed a mortality rate of more than 50% when larvae were reared at 24 °C. On average it took 20.7-23.5 days until death at 24 °C. At the same time, the 21 °C group had suffered a cumulative mortality of less than 25%. Although data for cumulative mortality was not collected in present study, death dates of larvae and temperatures recorded in the climate chamber used in the inoculation phase indicated that the actual rearing temperature was higher than the intended 21 °C.

Accordingly, the experimental setup was slightly modified after the first trial. Time periods between setup variants were shortened from four to three days to avoid premature mortality of inoculated larvae. Furthermore, the amount of simulated rain was reduced because the already weakened larvae suffered when being sprayed with water for too long.

4.2 Disease transmission

4.2.1 Transmission via contaminated larvae and feces

A high percentage of test larvae became infected when leaves were contaminated by both the presence of inoculated larvae and their feces. Simulated rain did not cause a significant change in the transmission rate of microsporidia – it was equally high in the dry and the wet cages. This result confirms the findings of Steyer (2010).

Through a different setup in her experimental series, Steyer (2010) showed that even in the presence of infected larvae spore-laden feces were a major source for new infections. Shaking fecal pellets off contaminated leaves previous to introduction of healthy test larvae reduced transmission. Nevertheless, some other yet unknown transmission pathways seem to be important especially during the early infectious stages. Fecal pellets of young instars are significantly lighter than those of older larvae and contain a smaller amount of spores in comparison to later stages (Goertz & Hoch, 2008a). In the present experiment transmission took place in top cages of the early setup (12 dpi) but virtually not in bottom cages. Microsporidian infection can also be acquired when susceptible larvae are exposed to direct contact with infectious conspecifics (Goertz & Hoch, 2008a). It is, however, unclear what exactly the source of spores is in this case. Silk does not play a role in disease transmission for *N. lymantriae* because infection impairs the glands and no silk is produced (Goertz & Hoch, 2008a). Probably, spores ooze from the mouth of feeding infected larvae, thus, contaminating the leaf surface. Furthermore, both healthy and infected larvae used in this study have been observed to regurgitate as a reaction to irritation. This behaviour could serve as an inoculum for new microsporidian infections, too.

Transmission of *N. lymantriae* in *L. dispar* populations is strongly density-dependent (Hoch *et al.*, 2008). Larvae in the middle and late stage of infection caused 100% transmission to test larvae. Eventually a lower number of inoculated larvae would have shown a clearer distinction between the middle and late stages of infection.

A correlation between disease progression of inoculated larvae and percent infection in test larvae was indicated. Subsequent regression analysis yielded a rather low coefficient of determination for the models tested. This is most probably due to the fact that the data were obtained from two subsequent trials conducted under only slightly altered conditions. Still a clear trend was visible pointing to an increase of transmission with disease progression, which has also been reported for *N. lymantriae* under field conditions (Hoch *et al.*, 2008).

4.2.2 Transmission via contaminated feces

Larvae in this experimental setup contaminated foliage in the bottom cages with feces but direct contact with test larvae was prevented. This design created a near-natural distribution of fecal pellets on the leaves. As mentioned earlier, practically no transmission was observed at twelve days post inoculation – regardless of rain simulation.

Transmission via feces was affected by both disease progression of infectious larvae and by rain. Although the difference between the middle and the late stages was not statistically significant, a slight increase of transmission could still be observed. In the variant with simulated rain also a correlation was indicated between percent infection and disease progression. In this case, the coefficient of determination was high indicating an increase in transmission with disease progression. Furthermore, dry weight of fecal pellets increased significantly between stages, increasing the amount of inoculum. These findings are in accordance with previous reports on the importance of feces as a source of new infections with *N. lymantriae* (Goertz & Hoch, 2008a; Hoch & Goertz, 2009; Pollan *et al.,* 2009; Steyer, 2010). According to Goertz & Hoch (2008a), the increase of spore content of feces follows a logarithmic trend in the course of disease progression.

When studying the transmission of the microsporidium *Nosema pyrausta* in populations of the European corn borer *Ostrinia nubilalis* (Lepidoptera: Crambidae), Andreadis (1987) also observed an increase of transmission with disease progression. The bivoltin corn borer formed two generations of larvae during named study, one in early July and one in late August. Healthy larvae became infected in up to 80% of the cases when feeding on corn stalks contaminated by the feces of late-stage infected conspecifics. In both generations earlier samplings showed lower transmission rates. Accordingly, increased disease transmission in later stages of disease progression occurs also under field conditions.

Feces are a potential source of spores for the microsporidium *Thelohania solenopsae*, an important pathogen of the Red Imported Fire Ants *Solenopsis invicta* (Hymenoptera: Formicidae) (Chen *et al.*, 2004). Infected workers play a major role in the transmission of microsporidiosis to queens. The influence of spores in their fecal droppings on actual transmission success is still unclear.

Although it might not have an impact on the transmission effect, it is still interesting that environmental spores in the feces of infected individuals seem to have species

specifically different origin within the host body. In the case of *N. lymantriae* and another *Nosema* sp. pathogenic to *L. dispar* the spores found in feces probably originated from infected Malpighian tubules (Goertz *et al.*, 2007; Goertz & Hoch, 2008a). *Endoreticulatus schubergi*, another pathogen to the gypsy moth, seems to produce environmental spores within the gut (Zwölfer, 1927; Goertz & Hoch, 2008a).

Microsporidian spores often pass the digestive system of non-hosts undamaged (Weiser, 1961). Hence, contamination of foliage with feces of host predators (like predacious bugs and beetles) can mediate the transmission of microsporidian infections (Kaya, 1979; Down *et al.*, 2004).

4.2.3 Effects of rain on disease transmission

Simulation of rain strongly affected disease transmission when feces were the sole contaminant on foliage. In middle and late stages of disease progression, percent infection in test larvae increased significantly. Steyer (2010), who worked with a similar experimental setup but used a larger amount of water found similar outcomes. The water quantity used in her study was equivalent to 13.6 mm precipitation per day, applied in two administrations which must be considered as heavy rainfall (UWZ, 2016). Furthermore, the average rain quantity per day, for eastern Austria in May and June, is usually below the calculated values (Meteo-Boku, 2016; ZAMG, 2016). This in mind, a reduction of water amount seemed reasonable. At first, half the quantity was applied by spraying into the cages from above. As it turned out larvae in the late stage of infection were already very weak and kept falling off the wet leaves. Therefore, the amount of rain was further reduced to an equivalent of about 2.4 mm per day. As shown by the results, transmission success was not different than before and still agreed with Steyer (2010). Thus, rain favours transmission success even when precipitation is low. Further experiments with larger or even smaller quantities of water could reveal the limits of this promoting effect.

Previous studies on transmission pathways were conducted with larvae on meridic diet (e.g. Goertz & Hoch, 2008a). Feces produced after consumption of wheat germ diet are soft and rather sticky. Larvae naturally feed on foliage therefore their fecal particles are hard, dry and unlikely to stick to leaf surfaces. Thus, humidity is an important factor when considering possible contamination from feces in the field. Wet surfaces lead to increased adherence of feces, resulting in the promoting effect on transmission. The dietary adjustment conducted before the experimental setup ensured this near-natural texture of the frass pellets (Steyer, 2010).

During phases of defoliation, the composition of dissolved material in rainwater that reaches the forest soil is altered. Precipitation leaches nutrients from damaged leaves and herbivore frass (Speight *et al.,* 2008). Therefore, it can be assumed that not only nutrients but also spores get eluted and transported during rainfall. Simulated rain in the present experiments even caused a significant reduction of the frass pellet dry weights.

The effects of rain on both disease transmission and spore dispersion have been studied in other host-pathogen systems. Inyang *et al.* (2000) observed a reduction of host infections of over 50% when simulating rain in an oilseed rape field treated with spores of the entomopathogenic fungus *Metarhizium anisopliae*. Similar results were yielded when the persistence of *Beauveria bassiana* spores on alfalfa and wheat crops were studied (Inglis *et al.,* 1995). Simulation of rain reduced the spore concentration on leaves by up to 60%. A major difference to the present experiments is the duration of rain simulation. In the studies mentioned above the rain events lasted half an hour or even an hour, considerably longer than in the present experiments, were spraying lasted only a few minutes. Furthermore, spore application in both systems was different: the fungal spores were dispersed in liquid solutions and sprayed on the plants in a one-time application. In the present test series, living hosts continuously excreted infectious spores – partly embedded in fecal pellets – even after the simulation of rain. Thus, the system using infected and living organisms has the advantage of repeated spore release and dispersal by the movement of infected individuals. Therefore, it is likely that also longer durations of rainfall would not necessarily reduce transmission through a wash-off effect, as long as infectious larvae are still alive.

The advantage of a living and moving source of spores is also obvious when considering the experiments conducted by D'Amico & Elkinton (1995) on the effects of simulated rain on the transmission of LdNPV from infected *L. dispar* cadavers. Similar to the present experiments, they investigated the effects of rain on the transmission rate of the pathogen in two layers of the tree canopy. When they fed contaminated leaves from both layers to healthy larvae it became clear that rain had increased transmission in the lower branches and reduced it in upper branches. The virus inclusion bodies were obviously washed from the upper layer of foliage onto the leaves beneath. By contrast, no negative effects of simulated light rain on transmission were observed in the top cages in present study. Still it is likely that increased precipitation – both in amount and duration – would wash the spores off, too.

4.3 Problems encountered during the experiment

4.3.1 Spore extraction from feces

Spores from fecal pellets were extracted following the protocol by Goertz & Hoch (2008a). A major difference between their work and the present study was that feces here were not fresh but stored in the freezer and heat-dried prior to extraction. Microsporidian species differ in their sensitivity to environmental conditions. According to Weiser (1961) moisture is the limiting factor for the viability of spores. Dehydration occurs via freezing and drying. Both procedures were here applied subsequently. Since no data is available concerning the heat-stability of *N. lymantriae*, it cannot be ruled out that spores in feces were altered in both appearance and properties which might explain why visual recognition of the spores was so difficult in the extracts. Furthermore, Undeen & Solter (1996) showed that dead and viable spores of the same microsporidian species move to different bands in a Ludox density gradient. In the present study dead spores might have been lost before counting as they might have been discarded together with supernatant after centrifugation. For future experiments, the use of a different protocol for spore extraction is strongly recommended, including different centrifugation times and probably additional detection methods for environmental spores, e.g. staining. Spore extractions should be carried out with fresh feces and any kind of storage or heat-treatment should be avoided.

To estimate the potential inoculum for the present experimental setup, theoretical spore release from feces was calculated with the regression equation given by Goertz & Hoch (2008a). They counted spores extracted from fresh feces collected over several days. For the conditions in present study they predict that the total spore release of 5 inoculated larvae between 12 and 13 dpi would be 6.5×10^4 spores, between 15 and 16 dpi 4.4×10^5 spores and 5.1×10^6 spores between 18 and 19 dpi.

4.3.2 Ambient temperature during trial 2

In the second trial, almost no transmission of microsporidia occurred during the early- and the middle-stage setups. Temperatures during the transmission phase (15 dpi) reached 43 °C. When test larvae were transferred to wheat germ diet for incubation, altogether 55 out of 124 (including control larvae) had entered premolt to the fourth instar and therefore did not feed during most of the transmission period and thus were unlikely to ingest spores. During none of the other experimental setups or trials larvae had reached premolt, not even at the end of the transmission phase. Presumably, development was accelerated by the high temperatures. Depending on the instar, an

increase in temperature by 6 °C accelerates the time between two ecdyses by three to eight days (Pollan, 2009).

The physiological alteration of the larvae during the second trial is also manifested in the dry weights of the fecal pellets. Fecal particles during the middle-stage setup were different from the other two trials and suggest that the inoculated larvae must have been under altered conditions previous to the contamination phase. Although the climate chamber used for rearing larvae after inoculation was set at 21 °C, the thermometer put inside showed that the temperature exceeded this value regularly. The fast disease progression of inoculated larvae agrees with the observed disease development at 24 °C by Pollan *et al.* (2009). Furthermore, inoculated larvae died earlier than expected, which also indicates elevated temperatures. Inoculation dose, however, does not seem to affect the dates of death in inoculated larvae (Goertz & Hoch, 2008a).

Not only were the larvae affected by the high temperatures. The hornbeam leaves within the cages wilked until end of the transmission phase. It is quite reasonable to assume that microsporidian spores were negatively affected as well. As mentioned earlier, temperatures above 40 °C and dry conditions pose a threat to spore survival (Solter & Becnel, 2003).

These deviations from the experimental conditions likely explain the low transmission success in trial 2 which was excluded from the analysis.

4.4 Conclusion

Feces are assumed to be one of the main transmission pathways for the microsporidium *N. lymantriae* in populations of its host *L. dispar* (Goertz & Hoch, 2008a). Horizontal transmission takes place when infected individuals excrete spores and healthy conspecifics take up the spores orally (Weiser, 1961; Vávra & Lukeš, 2013). Hence, radius of disease transmission could possibly increase when spore-laden feces fall onto leaves below feeding sites of infected larvae. Under natural conditions, however, feces of larvae feeding foliage are hard dry pellets that do not stick to leaf surfaces. Chances for contamination are assumed to increase on wet foliage.

This study proves that spore-containing feces with a dry and non-sticky texture can act as an inoculum for new *N. lymantriae* infections. The strongest transmission effect was observed when contamination was caused by larvae in a late stage of disease

progression. Furthermore, simulation of light rain led to a significant increase of disease transmission because of improved adherence of fecal pellets to leaf surfaces. These results suggest that light rainfall may increase disease prevalence in gypsy moth populations considerably, especially when infected larvae are already in an advanced state of microsporidiosis.

5. References

Alalouni, U., Schädler, M., Brandl, R., 2013. Natural enemies and environmental factors affecting the population dynamics of the gypsy moth. Journal of Applied Entomology 137, 721-738.

Andrae, A., 2013. Development *of Lymantria dispar* larvae from different populations depending on their host plants. Master thesis, BOKU University of Natural Resources and Applied Life Sciences, Vienna, 85 pp.

Andreadis, T.G., 1987. Horizontal transmission of *Nosema pyrausta* (Microsporida: Nosematidae) in the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae). Environmental Entomology 16, 1124-1129.

Bauer, L.S., Miller, D.L., Maddox, J.V., McManus, M.L., 1998. Interactions between a *Nosema* sp. (Microsporidia: Nosematidae) and nuclear polyhedrosis virus infecting the gypsy moth, *Lymantria dispar* (Lepidoptera: Lymantriidae). Journal of Invertebrate Pathology 74, 147-153.

Bell, R.A., Owens, C.D., Shapiro, M., Tardi, V.J.R., 1981. Mass rearing and virus production. In: Doane, C.C., McManus, M.L., (Eds.), The gypsy moth: Research toward integrated pest management. Technical Bulletin 1584. USDA, Washington, D.C., 599-633.

Chen, J.S.C., Snowden, K., Forrest, M., Sokolova, J., Fuxa, J., Vinson, S.B., 2004. Sources of spores for the possible horizontal transmission of *Thelohania solenopsae* (Microspora: Thelohaniidae) in the red imported fire ants, *Solenopsis invicta*. Journal of Invertebrate Pathology 85, 139-145.

D`Amico, V., Elkinton, J. S., 1995. Rainfall effects on transmission of gypsy moth (Lepidoptera: Lymantriidae) nuclear polyhedrosis virus. Environmental Entomology 24, 1144-1149.

Down, R.E., Bell, H.A., Matthews, H.J., Kirkbride-Smith, A.E., Edwards, J.P., 2004. Dissemination of the biocontrol agent *Vairimorpha necatrix* by the spined soldier bug, *Podisus maculiventris*. Entomologia Experimentalis et Applicata 110, 103-114.

Gschwantner, T., Hoch, G., Schopf, A., 2002. Impact of predators on artificially augmented populations of *Lymantria dispar* L. pupae (Lep., Lymantriidae). Journal of Applied Entomology 126, 66-73.

Goertz, D., Hoch, G., 2008a. Horizontal transmission pathways of terrestrial microsporidia: A quantitative comparison of three pathogens infecting different organs of *Lymantria dispar* L. (Lep.: Lymantriidae) larvae. Biological Control 44, 196-206.

Goertz, D., Hoch, G., 2008b. Vertical transmission and overwintering of microsporidia in the gypsy moth, *Lymantria dispar*. Journal of Invertebrate Pathology 99, 43-48.

Goertz, D., Hoch, G., 2011. Modelling horizontal transmission of microsporidia infecting gypsy moth, *Lymantria dispar* (L.), larvae. Biological control 56, 263-270.

Goertz, D., Solter, L.F., Linde, A., 2007: Horizontal and vertical transmission of a *Nosema* sp. (Microsporidia) from *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae). Journal of Invertebrate Pathology 95, 9-16.

Hajek, A.E., 2004. Natural enemies: An introduction to biological control. Cambridge University Press, Cambridge, 378 pp.

Hajek, A.E., Delalibera, I., Butler, L., 2003. Entomopathogenic fungi as classical biological control agents. In: Hokkanen, H.M.T., Hajek, A.E., (Eds.), Environmental Impacts of microbial insecticides. Kluwer Academic Publishers, Dordrecht, 15-34.

Hajek, A.E., Tobin, P.C. 2011. Introduced pathogens follow the invasion front of a speeding alien host. Journal of Animal Ecology 80, 1217-1226.

Hajek, A.E., Tobin, P.C., Haynes, K.J., 2015. Replacement of a dominant viral pathogen by a fungal pathogen does not alter the collapse of a regional forest insect outbreak. Oecologia 177, 785–797.

Hättenschwiler, S., Schafellner C., 2004. Gypsy moth feeding in the canopy of a CO_2-enriched mature forest. Global Change Biology 10, 1899-1908.

Hibbett, D.S., Binder, M., Bischoff, J.F., Blackwell, M., Cannon, P.F., Eriksson, O.E., Huhndorf, S., James, T., Kirk, P.M., Lücking, R., Thorsten Lumbsch, H., Lutzoni, F., Matheny, P.B., McLaughlin, D.J., Powell, M.J., Redhead, S., Schoch, C.L., Spatafora, J.W., Stalpers, J.A., Vilgalys, R., Aime, M.C., Aptroot, A., Bauer, R., Begerow, D., Benny, G.L., Castlebury, L.A., Crous, P.W., Dai, Y.C., Gams, W., Geiser, D.M., Griffith, G.W., Gueidan, C., Hawksworth, D.L., Hestmark, G., Hosaka, K., Humber, R.A., Hyde, K.D., Ironside, J.E., Kõljalg, U., Kurtzman, C.P., Larsson, K.H., Lichtwardt, R., Longcore, J., Miadlikowska, J., Miller, A., Moncalvo, J.M., Mozley-Standridge, S., Oberwinkler, F., Parmasto, E., Reeb, V., Rogers, J.D., Roux, C., Ryvarden, L., Sampaio, J.P., Schüssler, A., Sugiyama, J., Thorn, R.G., Tibell, L., Untereiner, W.A.,

Walker, C., Wang, Z., Weir, A., Weiss, M., White, M.M., Winka, K., Yao, Y.J., Zhang, N., 2007. A higher-level phylogenetic classification of the Fungi. Mycological research 111, 509-547.

Hlásny, T., Trombik, J., Holuša, J., Lukášová, K., Grendár, M., Turčáni, M., Zúbrik, M., Tabaković-Tošić, M., Hirka, A., Buksha, I., Modlinger, R., Kacprzyk, M., Csóka, G., 2016. Multi-decade patterns of gypsy moth fluctuations in the Carpathian Mountains and options for outbreak forecasting. Journal of Pest Science 89, 413-425.

Hoch, G., D'Amico, V., Solter, L.F., Zúbrik, M., McManus, M.L., 2008. Quantifying horizontal transmission of *Nosema lymantriae*, a microsporidian pathogen of the gypsy moth, *Lymantria dispar* (Lep., Lymantriidae) in field cage studies. Journal of Invertebrate Pathology 99, 146-150.

Hoch, G., Goertz, D., 2009. Understanding transmission of microsporidia: A prerequisite for their use in inoculative biological control of forest insects. Mitteilungen der Deutschen Gesellschaft für allgemeine und angewandte Entomologie 17, 89-93.

Hoch, G., Kalbacher, G., Schopf A., 2006. Gypsy moth revisited: Studies on the natural enemy complex of *Lymantria dispar* L. (Lep., Lymantriidae) during an outbreak in a well known gypsy moth area. Mitteilungen der Deutschen Gesellschaft für allgemeine und angewandte Entomologie 15, 201-204.

Hoch, G., Marktl, R.C., Schopf, A., 2009. Gamma radiation-induced pseudoparasitization as a tool to study interactions between host insects and parasitoids in the system *Lymantria dispar* (Lep., Lymantriidae)-*Glyptapanteles liparidis* (Hym., Braconidae). Biocontrol Science and Technology 19, 23-34.

Hoch, G., Schopf, A., 2001. Effects of *Glyptapanteles liparidis* (Hym.: Braconidae) parasitism, polydnavirus, and venom on development of microsporidia-infected and uninfected *Lymantria dispar* (Lep.: Lymantriidae) larvae. Journal of Invertebrate Pathology 77, 37-43.

Hoch, G., Zúbrik, M., Novotny, J., Schopf, A., 2001. The natural enemy complex of the gypsy moth, *Lymantria dispar* (Lep., Lymantriidae) in different phases of its population dynamics in eastern Austria and Slovakia – a comparative study. Journal of Applied Entomology 125, 217-227.

Hrašovec, B., Pernek, M., Lukic, I., Milotic, M., Diminic, D., Franjevic, M., Hajek, A., Linde, A., Pilarska, D., 2013. First record of the pathogenic fungi *Entomophaga*

maimaiga Humber, Shimazu, and Soper (Entomophthorales: Entomophthoraceae) within an outbreak populations of *Lymantria dispar* (Lepidoptera: Erebidae) in Croatia. Periodicum Biologorum 115(3), 379-384.

Inglis, G.D., Johnson, D.L., Goettel, M.S., 1995. Effects of simulated rain on the persistence of *Beauveria bassiana* conidia on leaves of alfalfa and wheat. Biocontrol Science and Technology, 5, 365-370.

Inyang, E.N., McCartney, H.A., Oyejola, B., Ibrahim, L., Pye, B.J., Archer, S.A., Butt, T.M., 2000. Effects of formulation, application and rain on the persistence of the entomogenous fungus *Metarhizium anisopliae* on oilseed rape. Mycological Research 104, 653-661.

James, T.Y., Kauff, F., Schoch, C.L., Hofstetter, V., Cox, C.J., Celio, G., Gueidan, C., Fraker, E., Miadlikowska, J., Lumbsch, H.T., Rauhut, A., Reeb, V., Arnold, A.E., Amtoft, A., Stajich, J.E., Hosaka, K., Sung, G.H., Johnson, D., O'Rourke, B., Crockett, M., Binder, M., Curtis, J.M., Slot, J.C., Wang, Z., Wilson, A.W., Schüssler, A., Longcore, J.E., O'Donnell, K., Mozley-Standridge, S., Porter, D., Letcher, P.M., Powell, M.J., Taylor, J.W., White, M.M., Griffith, G.W., Davies, D.R., Humber, R.A., Morton, J.B., Sugiyama, J., Rossman, A.Y., Rogers, J.D., Pfister, D.H., Hewitt, D., Hansen, K., Hambleton, S., Shoemaker, R.A., Kohlmeyer, J., Volkmann-Kohlmeyer, B., Spotts, R.A., Serdani, M., Crous, P.W., Hughes, K.W., Matsuura, K., Langer, E., Langer, G., Untereiner, W.A., Lücking, R., Büdel, B., Geiser, D.M., Aptroot, A., Diederich, P., Schmitt, I., Schultz, M., Yahr, R., Hibbett, D.S., Lutzoni, F., McLaughlin, D.J., Spatafora, J.W., Vilgalys, R., 2006. Reconstructing the early evolution of fungi using a six-gene phylogeny. Nature 443, 818-822.

Jeffords, M.R., Maddox, J.V., O'Hayer, K.W., 1987. Microsporidian spores in gypsy moth silk: A possible route of horizontal transmission. Journal of Invertebrate Pathology 49, 332-333.

Kalbacher, G., 2008. Untersuchungen zum Parasitoidenkomplex des Schwammspinners, *Lymantria dispar* (Lep., Lymantriidae), in seiner Progradations- und Kulminationsphase. Diploma thesis, BOKU University of Natural Resources and Applied Life Sciences, Vienna, 41 pp.

Kaya, H.K., 1979. Microsporidian spores: Retention of infectivity after passage through the gut of the assassin bug, *Zelus exsanguis* (Stål). Proceedings, Hawaiian Entomological Society 13, 91-94.

Klapwijk, M.J., Csóka, G., Hirka, A., Björkman, C., 2013. Forest insects and climate change: long-term trends in herbivore damage. Ecology and Evolution 3 (12), 4183-4196.

Leonard, D.E., 1981. Bioecology of the Gypsy Moth. Doane, C.C., McManus, M.L., (Eds.), The gypsy moth: Research toward integrated pest management. Technical Bulletin 1584. USDA, Washington, D.C., 9-29.

Liebhold, A., Elkinton J., Williams, D., Muzika, R.-M., 2000. What causes outbreaks of the gypsy moth in North America? Population Ecology 42, 257-266.

Liebhold, A.M., Raffa, K.F., Diss, A.L., 2005. Forest types affect predation on gypsy moth pupae. Agricultural and Forest Entomology 7, 179-185.

Lobinger, G., Skatulla, U., 2001. Untersuchungen zur Überlebensfähigkeit und Entwicklung der Larven zweier Herkünfte des Schwammspinners *Lymantria dispar* L. (Lep.: Lymantriidae) in Abhängigkeit von der Fraßpflanze. Journal of Pest Science 74, 89-93.

Maddox, J.V., McManus, M.L., Solter, L.F., 1998. Microsporidia Affecting Forest Lepidoptera. In: McManus, M.L., Liebhold, A.M., (Eds.), Proceedings: Populations dynamics, impacts, and integrated management of forest defoliating insects. USDA Forest Service General Technical Report NE-247, 187-197.

Mason, C.J., McManus, M.L., 1981. Larval dispersal of the gypsy moth. In: Doane, C.C., McManus, M.L., (Eds.), The gypsy moth: Research toward integrated pest management. Technical Bulletin 1584. USDA, Washington, D.C., 161-214.

Mattson, W.J. Jr., 1980. Herbivory in relation to plant nitrogen content. Annual Reviews of Ecology and Systematics 11, 119-161.

McManus, M., Csóka, G., 2007. History and impact of gypsy moth in North America and comparison to recent outbreaks in Europe. Acta Silvatica Lignaria Hungarica 3, 47-64.

McManus, M.L., McIntyre, T., 1981. Introduction. In: Doane, C.C., McManus, M.L., (Eds.), The gypsy moth: Research toward integrated pest management. Technical Bulletin 1584. USDA, Washington, D.C., 1-7.

McManus, M.L., Solter, L.F., 2003. Microsporidian pathogens in European gypsy moth populations. In: McManus, M.L., Liebhold, A.M., (Eds.), Proceedings: Ecology, Survey

and Management of Forest Insects. Sept. 1-5, 2002. Krakow, Poland. Gen.Tech.Rep.NE-311. USDA Forest Service, Newton Square, Pennsylvania, 44-51.

Meteo-Boku, 2016. Wetterstation BOKU-Met: Monatsübersichten ab 03/2005. Institut für Meteorologie, BOKU University of Natural Resources and Applied Life Sciences, Vienna, https://meteo.boku.ac.at/wetter/mon-archiv/2016/201610/201610.html, accessed: 01.10.2016

Muzika, R.M., Liebhold, A.M., 2001. Effects of gypsy moth defoliation in oak-pine forests in the northeastern United States. In: Liebhold, A.M., McManus, M.L., Otvos, I.S., Fosbroke, S.L.C. (Eds.), 2001. Proceedings: integrated management and dynamics of forest defoliating insects. 1999 August 15-19, Victoria, BC. Gen. Tech. Rep. NE-277. Newton Square, PA, U.S. Department of Agriculture, Forest Service, Northeastern Research Station, 117-123.

Nierhaus-Wunderwald, D., Wermelinger, B., 2001. Der Schwammspinner (*Lymantria dispar*). Merkblatt für die Praxis 34, 8 pp.

Pernek, M., Pilas, I., Vrbek, B., Benko, M., Hrašovec, B., Milkovic, J., 2008. Forecasting the impact of the gypsy moth on lowland hardwood forests by analyzing the cyclical pattern of population and climate data series. Forest Ecology and Management 225, 1740-1748.

Pollan, S., 2009: Effect of temperature on development of the microsporidium *Nosema lymantriae* and disease progress in the host *Lymantria dispar*. Master thesis, BOKU University of Natural Resources and Applied Life Sciences, Vienna, 54 pp.

Pollan, S., Goertz, D., Hoch, G., 2009. Effect of temperature on development of the microsporidium *Nosema lymantriae* and disease progression in the host *Lymantria dispar* (L. 1758). Mitteilungen der Deutschen Gesellschaft für allgemeine und angewandte Entomologie 17, 173-178.

Reardon, R.C., 1981. Summary. In: Doane, C.C., McManus, M.L., (Eds.), The gypsy moth: Research toward integrated pest management. Technical Bulletin 1584. USDA, Washington, D.C., 412-413.

Reilly, J.R., Hajek, A.E., Liebhold A.M., Plymale, R., 2014. Impact of *Entomophaga maimaiga* (Entomophtorales: Entomophtoraceae) on outbreak gypsy moth populations (Lepidoptera: Erebidae): The role of weather. Environmental Entomology 43, 632-641.

Schopf, A., Hoch, G., 1997. Zur Bionomie und Bedeutung von *Glyptapanteles liparidis* (Hym., Braconidae) als Regulator von *Lymantria dispar* (Lep., Lymantriidae) in Gebieten mit unterschiedlichen Populationsdichten. Journal of Applied Entomology 121, 195-203.

Smith, H.R., Lautenschlager, R.A., 1981. Gypsy moth predators. In: Doane, C.C., McManus, M.L., (Eds.), The gypsy moth: Research toward integrated pest management. Technical Bulletin 1584. USDA, Washington, D.C., 96-125.

Solter, L.F., Becnel, J.J., 2003. Environmental safety of microsporidia. In: Hokkanen, H.M.T., Hajek, A.E., (Eds.), Environmental impacts of microbial insecticides. Kluwer Academic Publishers, Dordrecht, 93-118.

Solter, L.F., Becnel, J.J., 2007. Entomopathogenic microsporidia. In: Lacey, L.A., Kaya, H.K., (Eds.), Field manual of techniques in invertebrate pathology, Springer, Dordrecht, 199-221.

Solter, L.F., Maddox, J.V., McManus, M.L., 1997. Host specificity of microsporidia (Protista: Microspora) from European populations of *Lymantria dispar* (Lepidoptera: Lymantriidae) to indigenous North American Lepidoptera. Journal of Invertebrate Pathology 69, 135-150.

Solter, L.F., Pilarska, D.K., Vossbrinck, C.F., 2000. Host specificity of microsporidia pathogenic to forest Lepidoptera. Biological Control 19, 48-56.

Solter, L.F., Siegel, J.P., Pilarska, D.K., Higgs, M.C., 2002. The impact of mixed infections of three species of microsporidia isolated from the gypsy moth, *Lymantria dispar* L. (Lepidoptera: Lymantriidae). Journal of Invertebrate Pathology 81, 103-113.

Speight, M.R., Hunter, M.D., Watt, A.D., 2008. Ecology of insects: Concepts and applications. Whiley-Blackwell, West Sussex, 628 pp.

Steyer, C., 2010. Die Rolle von Faeces und simuliertem Regen bei der Übertragung der Mikrosporidien *Nosema lymantriae* und *Endoreticulatus schubergi* bei *Lymantria dispar*. Master thesis, BOKU University of Natural Resources and Applied Life Sciences, Vienna, 55 pp.

Tobin, P.C., Gray, D.R., Liebhold, A.M., 2014. Supraoptimal temperatures influence the range dynamics of a non-native insect. Diversity and Distributions 20, 813-823.

Tompkins, D.M., Dobson, A.P., Arneberg, P., Begon, M.E., Cattadori, I.M., Greenman, J.V., Heesterbeek, J.A.P., Hudson, P.J., Newborn, D., Pugliese, A., Rizzoli, A.P., Rosá, R., Rosso, F., Wilson, K., 2002. Parasites and host population dynamics. In: Hudson, P.J., Rizzoli, A., Grenfell, B.T., Heesterneek, H., Dobson A.P., (Eds.), The ecology of wildlife disease. Oxford University Press, Oxford, 45-62.

Undeen, A.H., Solter, L.F., 1996. The sugar content and density of living and dead microsporidian (Protozoa: Microspora) spores. Journal of Invertebrate Pathology 67, 80-91.

Undeen, A.H., Vávra, J., 1997. Research methods for entomopathogenic protozoa. In: Lacey, L.A., (Ed.), Manual of techniques in insect pathology. 1997, Academic Press, San Diego, 117-151.

UWZ, 2016: Unwetterarten: Was versteht man unter Starkregen? Österreichische Unwetterzentrale, Vienna, http://www.uwz.at/at/de/unwetterarten/starkregen, accessed: 30.09.2016.

van Frankenhuyzen, K., Nystom, C., Liu, Y., 2007. Vertical transmission of *Nosema fumiveranae* (Microsporidia: Nosematidae) and consequences for distribution, post-diapause emergence and dispersal of second-instar larvae of the spruce budworm, *Choristoneura fumiferana* (Clem.) (Lepidoptera: Tortricidae). Journal of Invertebrate Pathology 96, 173-182.

Vávra, J., Lukeš, J, 2013. Microsporidia and ‚the art of living together'. In: Rollison, D., (Ed.), Advances in Parasitology 82, Academic Press, 253-320.

Vossbrinck, C.R., Debrunner-Vossbrinck, B.A., 2005. Molecular phylogeny of the Microsporidia: ecological, ultrastructural and taxonomic considerations. Folia Parasitologica 52, 131-142.

Weiser, J., 1957. Mikrosporidien des Schwammspinners und Goldafters. Zeitschrift für Angewandte Entomologie 40, 509-527.

Weiser, J., 1961. Die Mikrosporidien als Parasiten der Insekten. In: Zwölfer, W., (Ed.) 1961, Monographien zur angewandten Entomologie, 17. Parey, Berlin & Hamburg, 149 pp.

Weiser, J., 1998. Pathogens of the gypsy moth in Central Europe: Host range and interactions. In: McManus, M.L. and Liebhold, A.M., (Eds.), Proceedings: Population

dynamics, impacts, and integrated management of forest defoliating insects. USDA Forest Service General Technical Report NE-247, 322-333.

Wellenstein, G., Schwenke, W., 1978. *Lymantria dispar* L., Schwammspinner. In: Schwenke, W. (Ed.), Die Forstschädlinge Europas Bd. III, Parey, Hamburg, Berlin, 335-349.

Zahiri, R., Holloway, J.D., Kitching, I.J., Lafontaine, J.D., Mutanen, M., Wahlberg, N., 2012. Molecular phylogenetics of Erebidae (Lepidoptera, Noctuoidae). Systematic Entomology 37, 102-124.

ZAMG, 2016: Klimaübersicht. Zentralanstalt für Meteorologie und Geodynamik, Vienna, http://www.zamg.ac.at/cms/de/klima/klimauebersichten, accessed: 01.10.2016.

ZAMG, 2015: Rückblick Mai: Einer der trübsten der letzten 25 Jahre. Zentralanstalt für Meteorologie und Geodynamik, Vienna, https://www.zamg.ac.at/cms/de/klima/news/mai-2015-einer-der-truebsten-der-letzten-25-jahre, accessed: 06.06.2016.

ZAMG, 2015: Juni 2015: überdurchschnittlich warm, sonnig und trocken. Zentralanstalt für Meteorologie und Geodynamik, Vienna. https://www.zamg.ac.at/cms/de/klima/news/juni-2015-ueberdurchschnittlich-warm-sonnig-und-trocken, accessed: 06.06.2016.

Zúbrik, M., Hajek, A., Pilarska, D., Špilda, I., Georgiev, G., Hrašovec, B., Hirka, A., Goertz, D., Hoch, G., Barta, M., Sangia, M., Kunca, A., Nikolov, C., Vakula, J., Galko, J., Pilarski, P., Csóka, G., 2016. The potential of *Entomophaga maimaiga* to regulate gypsy moth *Lymantria dispar* (L.) (Lepidoptera: Erebidae) in Europe. Journal of Applied Entomology, 140, 565-579.

Zwölfer, W., 1927. Die Pebrine des Schwammspinners (*Porthetria dispar* L.) und Goldafters (*Nygmia phaeorrhea* Don. = *Euproctis chrysprrhea* L.), eine neue wirtschaftlich bedeutungsvolle Infektionskrankheit. Verhandlungen der Deutschen Gesellschaft für angewandte Entomologie 6, 98-109.

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