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Effect of temperature on development of the microsporidium *Nosema lymantriae* and disease progress in the host *Lymantria dispar*

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Master thesis

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Kurzfassung

Wirkung der Temperatur auf die Entwicklung der Mikrosporidie *Nosema lymantriae* und den Krankheitsverlauf im Wirtsinsekt *Lymantria dispar*

In der vorliegenden Studie untersuchte ich den Einfluss der Temperatur auf die Entwicklung der entomopathogenen Mikrosporidie *Nosema lymantriae* und den Krankheitsverlauf im Schwammspinner, *Lymantria dispar* (Lep., Lymantriidae). Larven wurden mit einer Dosis von 1x10³ Sporen infiziert und bei 18 ± 1°C, 21 ± 1°C bzw. 24 ± 1°C und 16 h L/8 h D gezüchtet. Die Temperatur wirkte sich signifikant auf den Infektionsverlauf aus. Bei höheren Temperaturen waren die verschiedenen Entwicklungsstadien der Mikrosporidie früher in Spinndrüsen, Fettkörper und Malpighischen Gefäßen vorhanden. Die Ausscheidung von Sporen durch den Kot erkrankter Larven erfolgte bei 24°C sieben Tage eher als bei 18°C sowie zwei Tage früher als bei 21°C. Ebenso war die Menge freigesetzter Sporen signifikant von der Temperatur beeinflusst. Sie betrug in den ersten sechs Tagen des fünften Stadiums $2.7\times10^7 \pm 7.0\times10^6$ bei 24°C, $8.9\times10^6 \pm 2.1\times10^6$ bei 21°C und $3.1\times10^6 \pm 1.5\times10^6$ bei 18°C. Während nicht infizierte Larven mit steigenden Temperaturen mehr konsumierten und schneller wuchsen, war dieser Effekt bei infizierten Larven oft nicht signifikant. Darüber hinaus konsumierten infizierte Larven weniger und wuchsen langsamer als ihre uninfizierten Kontrollen. Infizierte Larven starben bei 24°C etwa zwei Wochen früher als bei 21° und 18°C. Der schnellere Krankheitsfortschritt hat Bedeutung für beide wichtigen Wege der horizontalen Übertragung von *N. lymantriae*, die einerseits mittels sporenhaltigem Kot infektiöser Larven, sowie durch Sporen aus Kadavern verstorbener Wirtstiere geschieht. Der Übertragungserfolg wird unter anderem vom Zeitpunkt der ersten Sporenfreisetzung beeinflusst.

Schlagwörter: Entwicklung, *Lymantria dispar*, Microsporidia, *Nosema lymantriae*, Temperatur, Wachstum

Abstract

In the present study, I investigated the influence of temperature on the development of the entomopathogenic microsporidium *Nosema lymantriae* and the disease progress in the gypsy moth, *Lymantria dispar* (Lep., Lymantriidae). Larvae were infected with a dose of $1x10^3$ spores and reared at 18 ± 1°C, 21 ± 1°C and 24 ± 1°C, respectively, and 16 h L/8 h D. Temperature had a significant effect on the course of infection. At higher temperatures, microsporidian developmental stages were found earlier in silk glands, fat body and Malpighian tubules. Spore release through feces of infected larvae occurred seven days earlier at 24°C than at 18°C as well as two days earlier than at 21°C. Also the amount of released spores was significantly influenced by temperature. During the first six days of 5^{th} instar $2.7\times10^7 \pm 7.0\times10^6$ spores were released at 24°C, $8.9\times10^6 \pm 2.1\times10^6$ at 21°C and $3.1\times10^6 \pm 1.5\times10^6$ at 18°C, respectively. While control larva consumed more and grew faster with increasing temperatures, this effect was not significant in infected larvae. Overall, infected larvae consumed less and grew slower than uninfected controls. Infected larvae reared at 24°C died about 14 days earlier than those at 21° and 18°C. The faster disease progress is of importance for both pathways of horizontal transmission of *N. lymantriae* - spores are released with feces from living larvae and from cadavers after host death. Timing of the onset of spore release has an important effect on horizontal transmission success.

Keywords: development, growth, *Lymantria dispar*, Microsporidia, *Nosema lymantriae*, temperature

Contents

1 Introduction

As ectothermic animals, insects depend on their ambient temperature and, thus, temperature plays an important role for their metabolic processes, growth and development (Zaslavski, 1988). Insect activity increases with rising temperatures and thereby their metabolism. In the developmental stage, higher temperatures lead to accelerated growth (Wigglesworth, 1972). Many insects are parasitized by entomopathogenic microsporidia. Microsporidia are single-celled, obligate intracellular pathogens that depend on host cells to develop and reproduce. Environmentally resistant spores are the infective stage. These spores are orally ingested by the host, germinate in the midgut lumen and initiate the infection in the host (Solter & Becnel, 2000). Additionally, spores can be transmitted transovarial from parent to offspring (Becnel & Andreadis, 1999). Microsporidia that infect ectothermic hosts develop at temperatures that are typical temperature ranges of their hosts (Cali & Takvorian, 1999). Little data exist on how environmental temperature for the insect host affects entomopathogenic microsporidia.

In my study, the microsporidium *Nosema lymantriae* and its host, *Lymantria dispar*, were used as model organisms. *N. lymantriae* uses silk glands, fat body, gonads and Malpighian tubules of the host for mass proliferation and formation of environmental spores. Two main pathways have been described for horizontal transmission; spores are released from living larvae with feces and from cadavers after host death (Goertz & Hoch, 2008). Horizontal transmission success is affected by the timing of the onset of spore release. As previous studies regarding *N. lymantriae* were performed at one standardized temperature regime, we investigated in this study the impact of temperature on microsporidian development, spore release and developmental parameters of *L. dispar*. Moreover, the effect of temperature on the host-pathogen interaction was studied by analyzing growth and development of infected larvae.

1.1 The gypsy moth, *Lymantria dispar*

The gypsy moth, *Lymantria dispar* (Lep., Lymantriidae), is known as an important forest defoliator that reaches pest status during outbreaks. Its native range stretches from Europe to Japan. Around 1868, *L. dispar* was introduced into the United States, near Boston,

Massachusetts. It has been expanding from its original release site over northeastern U.S. and southeastern Canada and has become one of the most severe pests of urban and forest trees (McManus & McIntyre, 1981; Liebhold et al., 1992). Drought, warm spring temperatures and sunlight are primary factors that lead to outbreaks. In Europe this combination occurs mostly in south and south-eastern Europe. Consequently, outbreaks occur every 3-4 years in the Mediterranean and Balkan regions and every 7-10 years in Central Europe (Weiser 1987). Approximately every 10 years *L. dispar* larvae cause severe defoliation of oak forests in Eastern Austria (Hoch et al., 2001a; Hoch et al., 2006). Oak trees (*Quercus*) are preferred host plants of the gypsy moth, but larvae also feed and develop on hornbeam (*Carpinus*), beech (*Fagus*), chestnut (*Castanea*) or fruit trees like apple (Wellenstein & Schwenke, 1978). The flight period of *Lymantria dispar* occurs in Europe between July and beginning of September depending on weather and climate conditions. Adult insects show a strong sexual dimorphism. Females are brownish-white with black tibiae and antennae, 50-80 mm wing spread, plump body and – in the European race – lack capability of flight. Males are gray brown, with 35-50 mm wing spread and feathered antennae and are good flyers. Females attract males by a sexual pheromone. Eggs are laid on the bark of trees and covered with yellowish brown hairs. Larvae hatch in late April / early May and develop through five or six instars (Wellenstein & Schwenke, 1978).

Temperature, light and humidity are abiotic environmental factors that affect larval consumption and utilization of food in insects. Developmental time is highly influenced by temperature changes whereas food consumption and weight gain are less affected (Scriber & Slansky 1981). Insect performance may be also affected by an interaction of temperature and food quality (Lamb & Loschiavo, 1981). Lindroth et al. (1997) report that small changes in thermal regime and variation in dietary nitrogen content strongly affect gypsy moth performance. Larval consumption rates, growth rates, and developmental periods varied at different temperatures. Low temperatures reduced growth rates and prolonged developmental time. The quality and quantity of food, constitution, and natural enemies (parasites, predators and diseases) are limiting factors and lead to collapse of *L. dispar* outbreaks. Starvation as a consequence of complete defoliation of the host trees and a decline of food quality as a result of feeding on other plants than their favorites can cause high larval mortality (Wellenstein & Schwenke, 1978). Thompson (1946) lists 165 parasitic insect species of the gypsy moth in Europe. In Austria, more than 40 natural enemies are reported from the gypsy moth; particularly tachinids, such as *Parasetigena silvestris*, or braconids like *Glyptapanteles*

liparidis proved to be important mortality agents (Hoch et al., 2001a). Many other Hymenoptera, Diptera and nematodes act as egg, larval, and pupal parasitoids. Beetles, acarids, bugs and birds are among the basic predators in Europe (Wellenstein & Schwenke, 1978). *Calosoma sycophanta* (Coleoptera; Carabidae) is supposed to be very effective in high density populations in both, Central Europe and North America (Hoch et al., 2006; Weseloh, 1985). In North America, substantial predation occurs by mammals, especially rodents like *Peromyscus* spp. (Muridae) (Liebhold et al., 2005). In Austria, the mouse *Apodemus flavicollis* is considered to be an important predator of pupae (Gschwantner et al., 2002). Pathogens like viruses, bacteria, microsporidia and fungi can cause high mortality in *L. dispar* outbreaks. Wellenstein and Schwenke (1978) conclude that the most important mortality factor is the *L. dispar* nucleopolyhedrosis virus (NPV) that terminates practically all gypsy moth outbreaks. Also in North America, declines of gypsy moth populations are mainly found in company with NPV (Doane, 1970; Elkinton & Liebhold, 1990). Bacteria are likely to develop as secondary parasites (Novotny, 1988; Hoch et al., 2001a), but are of minor importance as primary mortality factors. Regarding fungi as pathogens, two strains of *Beauveria bassiana* appeared virulent (Wellenstein & Schwenke, 1978) and *Entomophaga maimaiga* is an important control agent in the U.S.A. that has a substantial impact on gypsy moth population dynamics (Hajek, 1999).

1.2 Microsporidia

Microspordia are seen as important natural enemies in European *L. dispar* populations (Maddox et al. 1999; Pilarska et al., 1998; Solter et al., 2000; Weiser, 1957). Zwölfer (1927) and Weiser (1957) give first descriptions of microsporidian diseases in *L. dispar* in Europe. Weiser and Novotny (1987) indicate that microsporidian infections appear in the period prior to gypsy moth outbreaks. During outbreaks, NPV becomes the dominant pathogen. There are no reports of microsporidia in North American gypsy moth populations (McManus et al., 1988). Microsporidia from Europe are therefore considered for introduction as classical biocontrol agents to North America (McManus & Solter, 2003). Canning (1982) suggests that microsporidia have little capability for use as true microbial insecticides. Solter & Becnel (2000), on the other hand, point out that microsporidia can be useful in classical biological control programs using inoculative release. Moreover, several studies suggest that

microsporidia may play a significant role in the population dynamics of gypsy moths (Purrini & Skatulla, 1978; Sidor & Jodal, 1983; Zelinskaya, 1980; Sierpinska, 2000).

Microsporidia are single-celled intracellular parasitic organisms that were classified among the Protozoa as Phylum Microspora (Sprague, 1977), later amended to the Phylum Microsporidia (Sprague et al., 1992; Sprague & Becnel, 1999). About ten years ago, the affiliation of microsporidia to fungi became evident by findings such as a mitochondrial heat-shock Hsp70 protein (Hirt et al., 1997). Also Van de Peer et al. (2000) and Weiss et al. (1999), ascertained the relationship to fungi. A recent classification places the Microsporidia among the kingdom of Fungi (Hibbet et al, 2007). Over 1200 microsporidian species have been described in various animal orders (Wittner, 1999); almost half of these have an insect as host (Becnel & Andreadis, 1999). In insects, microsporidian infections are rarely acute but mostly chronic. Microspordia develop in the cytoplasm of host cells and induce often sublethal effects on their hosts, such as loss of vigor, reduced fertility and shortened longevity (Brooks, 1988). Signs of infections manifest in changes of color, size and form, as well as abnormal development and behavior of the infected insect compared to healthy individuals. Microsporidia serve as natural enemies, but some also cause severe problems in colonies of beneficial insects like honeybees and silkworms or biological control agents such as parasitoids (Becnel & Andreadis, 1999).

Many entomopathogenic microsporidia use the gypsy moth as host. They infest different tissues within their host, such as the silk glands, midgut epithelium and associated muscle tissue, fat body, Malphigian tubules, nerve tissue and reproductive organs (McManus & Solter, 2003). Weiser (1957) listed seven microsporidian species in *L. dispar*, among them three species of *Nosema*. As ultrastructural and molecular information was not available to the author at that time, not all described species may actually represent a single species (Maddox & Sprenkel, 1978). McManus & Solter (2003) give an overview over all microsporidian species in gypsy moth and their systematic relationships among each other. All known microsporidia in *L. dispar* belong to the genera *Vairimorpha, Endoreticulatus* and *Nosema*.

1.3 *Nosema lymantriae*

Nosema lymantriae was isolated from *L. dispar* collected in former Czechoslovakia and described by Weiser (1957). It is a virulent pathogen causing high mortality in the larval stage of the host. It infects silk glands, fat body, gonads, and the Malpighian tubules. Infection begins with ingestion of environmental spores by larvae; these spores germinate in the midgut lumen and inject sporoplasms into midgut cells. Primary spores develop in the gut tissue and contribute to spread the infection to silk glands, fat body, gonads and Malpighian tubules where mass proliferation of the microsporidium takes place. Like in the closely related *Nosema portugal*, eventually environmental spores are formed in these tissues (Maddox et al., 1999). These spores are released with feces from living larvae and from cadavers after host death, thereby constituting the two main pathways of horizontal transmission of *N. lymantriae* (Goertz & Hoch, 2008) – other larvae may ingest the spores along with contaminated food and become infected.

In many studies the lifecycle, ultrastructure and taxonomic relationship of *Nosema* spp. (Maddox et al., 1999), the impact on its host, *L.dispar* (Goertz et al., 2004), competition of different microsporidia within their host (Solter et al., 2002; Pilarska et al., 2006) and transmission pathways (Goertz et al., 2007; Goertz & Hoch, 2008; Hoch et al., 2008) were analyzed. None of these studies discuss the impact of temperature on microsporidian development within its host. Analyses were carried out at standardized temperature conditions, either alternating between 24°C day / 18°C night (Goertz et al., 2004; Goertz et al., 2007; Goertz & Hoch, 2008), or constant at 21°C (Pilarska et al., 2006), 22°C (Maddox et al., 1999) or 24°C (Solter et al., 2002). In my study, three different temperatures (18°C, 21°C, 24°C) were selected to analyze their effect on the development of *N. lymantriae* and the disease progress in *L. dispar*. Dissection of infected larvae reared at different temperatures gave an overview of the infection progress of *N. lymantriae*. The influence of temperature on the development of *N. lymantriae* and the disease progress in *L. dispar* as well as combined the effects of temperature and infection on host growth and development was examined. Since Goertz & Hoch (2008) ascertained the important role of spores egested with feces in horizontal transmission, the influence of temperature on timing of spore release with feces was analyzed.

2 Material and Methods

2.1 Insects and Pathogens

2.1.1 Insects

Lymantria dispar larvae (New Jersey Standard Strain) were used as host insects. Larvae were obtained from egg masses provided by the USDA/APHIS Otis Method Development Center, Cape Cod, Massachusetts, USA. They were reared on wheat germ diet (Bell et al., 1981) in climate chambers with a photoperiod of 16 h light/8 h dark. Prior to inoculation, larvae were kept in groups in 250 ml plastic cups at 24°C ± 1°C. After inoculation, larvae were reared individually in glass Petri dishes of 9 cm diameter. Three different temperatures were used in the experiment: 18°C ± 1°C, 21°C ± 1°C and 24°C ± 1°C.

2.1.2 Pathogens

The microsporidium *Nosema lymantriae* (Isolate No. 1996-A), isolated from silk glands of *L. dispar* larvae, collected in 1996 near Levishte, Bulgaria, was used. *N. lymantriae* is stored in the microsporidia germ-plasma collection of the Illinois Natural History Survey (INHS), Urbana-Champaign, Illinois, USA, from which it was originally obtained for further reproduction and storage at our laboratory at BOKU Wien. For my experiments fresh spores were propagated in *L. dispar* larvae (following the method of Hoch et al., 2000). Therefore, larvae were infected with 1 µl of spore suspension in the concentration of 2×10^3 spores/µl at the beginning of third instar. Mature spores were harvested from infected silk glands during the fifth larval stage, approximately 14 days post infection (dpi), cleaned by filtration through cellulose tissue and centrifugation. The supernatant was discharged and the pellet, containing the spores, was suspended in distilled water. The spore suspension was mixed 1:1 with glycerol and stored in liquid nitrogen (Maddox & Solter, 1996), until used in the experiments. The maximum storage was four months.

2.1.3 Experimental Inoculation

When larvae reached premolt to third instar (indicated by slipped head capsule), they were collected into several 250 ml plastic cups without food. The following day, newly molted and starved third instar *L. dispar* larvae were inoculated with microsporidia following the methods of Bauer et al. (1998), as used by Hoch et al. (2000). Spore suspension was counted in a Neubauer hemacytometer and adjusted with distilled water to the concentration of 1×10^3 spores/µl. Diet cubes of 2 mm^3 were placed individually into the wells of 24 well tissue culture plates, 1 µl of spore suspension was applied to each cube. *L. dispar* larvae were placed individually into each well. Only larvae that consumed the entire diet cube within 24 h were used in my experiment. Control larvae were inoculated the same way but with distilled water instead of spore suspension.

2.2 Microsporidian development at different temperatures

To investigate the effect of temperature on *N. lymantriae* development in *L. dispar* larvae, newly inoculated larvae were separated in three treatment groups with following rearing temperatures: 18°C ± 1°C, 21°C ± 1 °C and 24°C ± 1 °C. The photoperiod was set at 16 h light / 8 h dark. Larvae were maintained in groups of five in glass Petri dishes of 9 cm diameter. Starting on the sixth day post inoculation (dpi), three larvae from each temperature group were randomly selected and dissected every second day. Larvae were opened ventrally and samples from the midgut, the fat body, the entire silk glands and 3 to 4 Malpighian tubules were prepared in insect saline. The fresh preparations of midgut, silk glands, fat body and Malpighian tubules were examined in a phase contrast microscope at 400× magnification for the presence of microsporidian life stages. The occurrence of primary spores, vegetative stages or mature environmental spores was recorded and their approximate abundance was estimated (1. spores or vegetative stages can be found after some search; 2. spores or vegetative stages are easily detected in most inspected microscope fields; 3. tissue is filled with spores). On the day, when all tissues, including the Malpighian tubules, were filled with mature spores, the dissections for this temperature group were terminated.

2.3 Influence of temperature and infection on larval development parameters

To determine the influence of temperature on *N. lymantriae*-infected *L. dispar* larvae, newly inoculated larvae were separated in three treatment groups with 30 infected and 30 control larvae per group. Larvae were kept under the same conditions as the larvae in the dissection experiments. Larvae were reared individually. The whole experiment was replicated in two trials. The following parameters were determined daily:

2.3.1 Growth, development and consumption

Determination of diet consumption

Larvae were put individually in glass Petri dishes of 9 cm diameter and fed with one fresh diet cube of 1.5cm × 1.5cm × 1cm 24 hours after inoculation. At that time larvae were separated in the three temperature groups. Every third day, the old diet cube was removed and replaced by a new one. Third and fourth instar received one single diet cube of 1.5cm × 1.5cm × 1cm. Beginning with the fifth instar larvae were fed with 2 or 3 diet cubes every third day. When larvae started its premolt, the diet cubes were removed. After molt, feeding was resumed.

Starting at the first day of fourth instar, fresh diet cubes were weighed before offering them to the larvae. Three days later, when food had to be renewed, the remaining diet pieces were collected and dried at 70°C until they showed a constant dry mass.

Additionally, the mass of control diet cubes was determined for each temperature every day: Five diet cubes were weighed and put individually in glass Petri dishes and placed in the climate cambers. The control cubes were removed three days later and dried at 70°C; dry mass was determined. The mean ratio of dry mass : fresh mass of control cubes was calculated for every days control cubes. Afterwards the dry mass of the diet cubes before feeding was determined after following formula:

Dry mass before feeding = mean ratio of control cubes × fresh mass of diet before feeding

For each 3-day feeding period, the diet consumption was calculated as follows:

Diet consumption = Dry mass of diet before feeding - Dry mass after feeding

To calculate the diet consumption for the whole instar, the results of all feeding periods within the instar were summed up.
The food consumption rate (CR) of every larvae for the entire fourth instar (L4) and three day periods of the fifth instar (L5) was computed. Unfinished feeding periods were excluded from further calculations.

CR L4 = Diet consumption of L4 / Duration of L4

CR first six days of L5 = Diet consumption of L5 / 6 days

L. dispar fresh mass (FM)
The fresh mass of larvae was measured on following days: on the first day post inoculation (dpi), first and last day of fourth instar (L4), first day of fifth instar (L5), every third day in fifth instar and mass of pupa two days after molt. Cadavers of larvae that succumbed to the infection were also weighed.

Determination of growth rate (GR)
The daily larval growth rate was estimated for the entire fourth instar and the first six days of fifth instar after following formulas:

GR L4 = (Fresh mass on last day in L4 - Fresh mass on 1st day in L4) / Duration of L4

GR first six days of L5 = (Fresh mass of 6th day in L5 - Fresh mass of 1st day in L5)/6 days

Growth rate and consumption rate were calculated only for the first six days of L5, because most of the infected larvae survived this period and comparisons between groups could be done. After these first six days of L5, mortality of infected larvae was high.

2.3.2 *Nosema lymantriae* spores released with feces

Excrements of larvae were removed every day. Feces of 16 infected larvae of each group were collected individually for each larva throughout the fourth instar and the first six days of fifth instar. The amount of spores in feces for fourth instar and the first six days of fifth instar of these larvae was determined following the method of Goertz & Hoch (2008): Feces were suspended in distilled water in a glass tube. The water-feces suspension was filtered through

cellulose tissue and centrifuged for 15 min. The supernatant was discharged and the spore-containing pellet was suspended in 2 ml distilled water. Samples of this spore suspension were counted in a Neubauer hemacytometer and the total amount of spores was calculated.
From the remaining inoculated larvae, two excremental pieces were examined every second day under phase contrast microscopy (400 × magnification) for the presence of spores, beginning seven days after inoculation. In case spores were present on two consecutive days, observation was stopped because continuous spore release can be assumed from than on (Goertz & Hoch, 2008).

2.3.3 Diagnosis of infections

At the end of the experiment, when infected larvae had died and control larvae pupated, all test insects were examined for the presence of *Nosema lymantriae* infection. Therefore, cross sections of the second abdominal segment were made with fine scissors. The sections, containing midgut, silk gland and fat body tissue, were inspected in a phase contrast microscope at 400× magnification.

2.3.4 Determination of sex of *L. dispar*

At the end of the experiments, the sex of the test insects was determined according to characteristics of pupae (Zwölfer, 1934) whenever possible. Infected insects that died as larvae were dissected and sex determined according the morphology of the gonads. For the first experiment the sex of dead larvae reared at 21°C and 24°C was not determined. Hence, wherever sex of larvae is important, data are only presented from the second trial.

2.4 Data analysis

Statistical analyses were carried out with SPSS 12.0.1. (SPSS Inc. 1989-2003). Sex of test insects had a highly significant impact on larval development and consumption. Therefore, data of male and female insects were analyzed separately. As sex was not determined for all dead larvae in trial 1, only trial 2 was analyzed for growth and development parameters.
When the variance of data was equal, a 2-way ANOVA was computed using the GLM procedure in SPSS to test the main effects of treatment (infection) and temperature on development, mortality and spore release. Furthermore, the interaction between treatment and

temperature was tested. Larval fresh mass on 1 dpi was used as covariate for the 2-way ANOVA to correct for any effect of larval fresh mass at the beginning of the experiment. Analysis of covariance is an alternative to conventional, ratio-based nutritional indices and allows less restrictive assumptions (Raubenheimer & Simpson, 1992).

When data did not meet the requirements for the computation of a 2-way ANOVA, treatment groups were compared by Kruskal-Wallis *H* test. Significant *H* tests were followed up by pairwise Mann-Whitney *U* Tests corrected for type I errors by the Bonferroni method. A 1-way ANOVA was computed to test the effect of temperature on the spore release with feces.

Spearman's Correlation Coefficient was used to test for correlations between fresh mass of larvae and their day of death, as well as correlations between the amount of spores released, consumption rate and growth rate.

3 Results

3.1 Microsporidian development at different temperatures

The progression of *Nosema lymantriae* infection varied with temperature and insect tissue. The infection started in midgut epithelium and muscularis with formation of primary spores and then spread to in silk glands and fat body. Eventually, Malpighian tubules and gonads were infected.

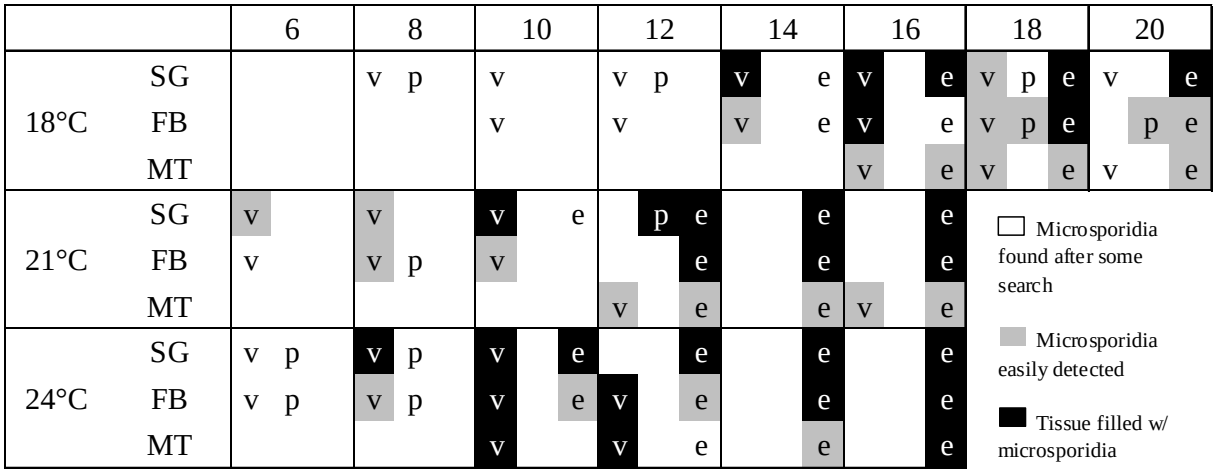


Fig. 1: Presence of various stages of *N. lymantriae* in silk glands (SG), fat body (FB) and Malpighian tubules (MT). Each box refers to combined results of microscopic examination of three larvae. Letters indicate microsporidian stage detected: v = vegetative stages (meronts and/or sporonts), p = primary spores, e = environmental spores. Larvae reared at 18°C completed fourth instar on 16 dpi, larvae reared at 21°C on 12 dpi and larvae at 24°C on 10 dpi.

In the midgut, mainly primary spores were found after some search, starting 6 dpi at 24°C and 8 dpi at 21°C and 18°C. Also vegetative stages were detected, but the total amount of microsporidian cells was not high and no spores were found after 12 dpi. On 6 dpi and 8 dpi, primary spores and vegetative stages were found in silk glands and fat body of larvae reared at 24°C and 21°C (Fig. 1). Starting 10 dpi, the amount of spores increased and first environmental spores were detected. Progression was slightly faster at 24°C than at 21°C. From 12 dpi on, silk glands and fat body were totally filled with spores. The infection progressed markedly slower at 18°C, where silk glands and fat body started to be filled with

spores beginning 14 and 16 dpi. Only few spores were detected in silk glands and fat body of 18°C reared larvae at earlier points in time. Overall, microsporidian development was about 4 days advanced at 24°C and 21°C compared to 18°C.

The microsporidian development in Malpighian tubules lagged behind silk glands and fat body. But it was also faster at 24°C and 21°C than at 18°C. Malpighian tubules started to be filled with vegetative stages 10 dpi at 24°C. First environmental spores were found two days later until Malpighian tubules were totally filled 14 and 16 dpi. 16 dpi first spores were found in Malpighian tubules at 18°C, the tissue was not totally filled with spores before 20 dpi.

Larvae remained in 4^{th} instar 3 to 4 days longer at 18°C than at 24°C and 21°C. All larvae were already in L5 12 dpi at 24°C, followed by larvae 14 and 16 dpi at 21°C. Larvae reared at 18°C entered L5 18 or 20 dpi.

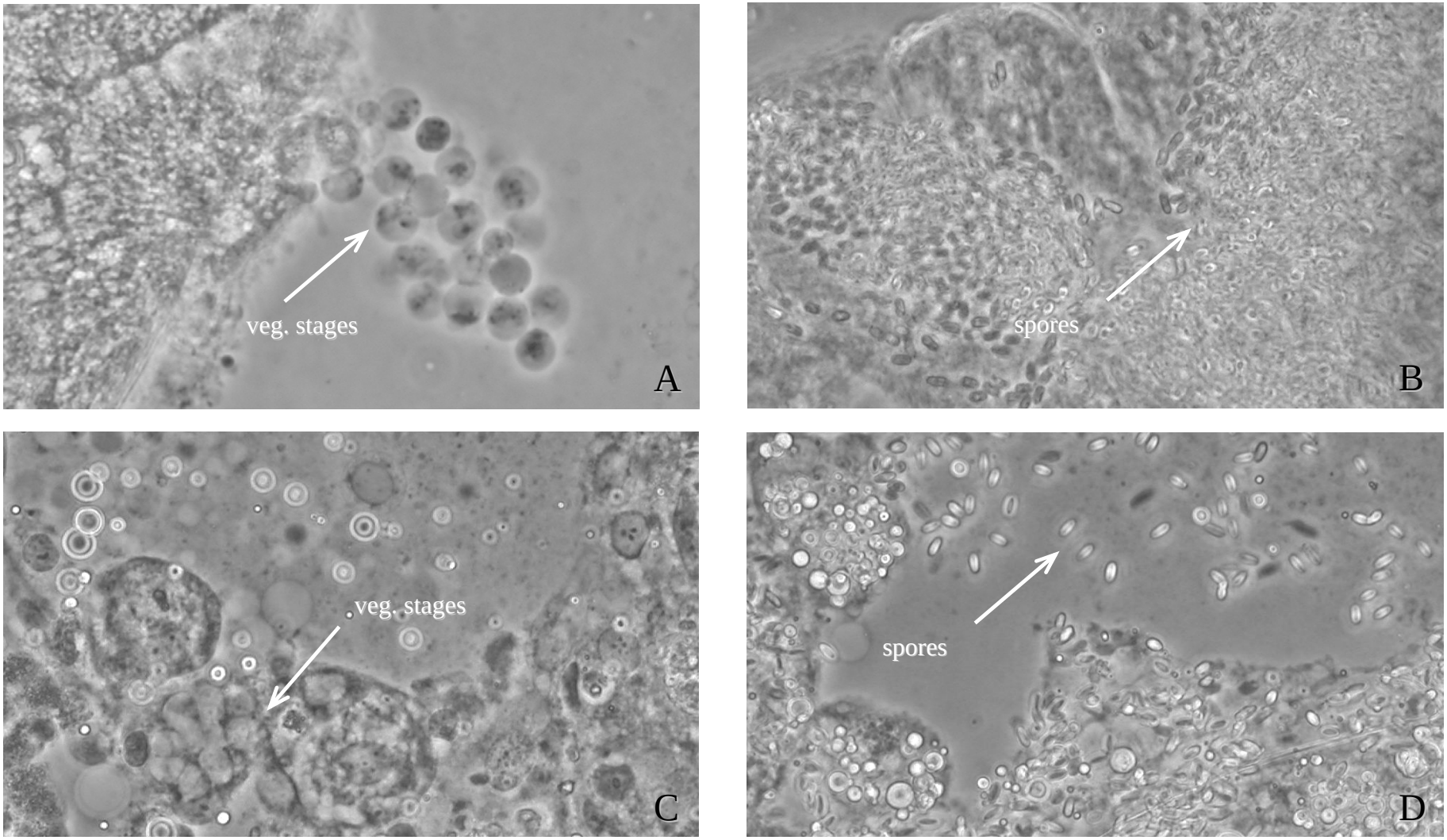


Fig. 2.: A - D. Light micrographs of the development of *N. lymantriae* in silk glands and fat body. A. Vegetative stages in silk glands, 6 dpi at 21˚C; B. Spores in silk glands, 8 dpi at 24˚C; C. Vegetative stages in fat body, 8 dpi at 24˚C; D. Spores in fat body, 12 dpi at 24˚C.

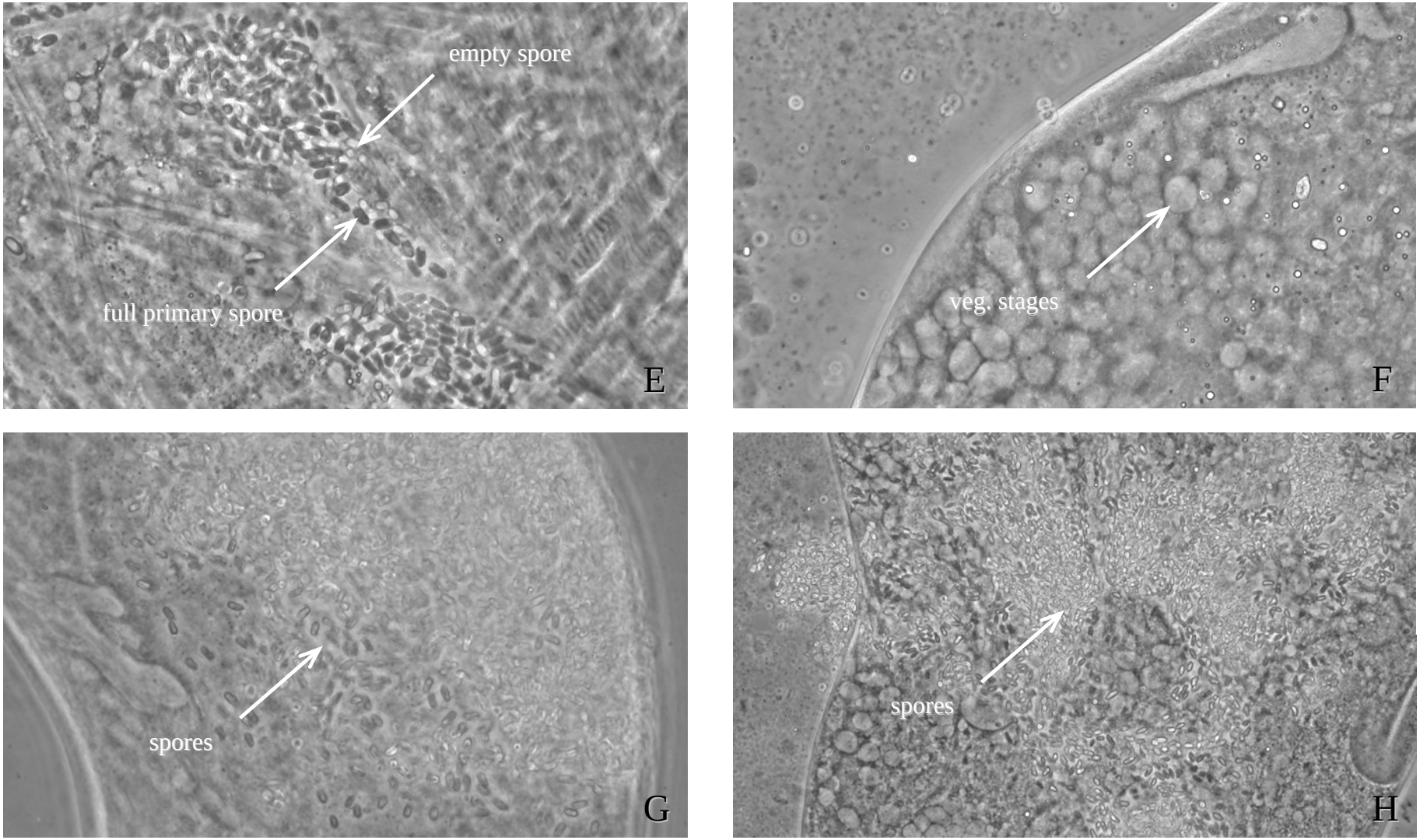


Fig. 3.: E - H. Light micrographs of the development of *N. lymantriae* in midgut and Malpighian tubules. E. Full and empty primary spores in midgut, 10 dpi at 21˚C; F. Vegetative stages in Malphigian tubules, 10 dpi at 24˚C; G. Spores in Malpighian tubules, 12 dpi at 21˚C; D. Spores and vegetative stages in Malpighian tubules, 14 dpi at 18˚C.

3.2 Mortality of microsporidia infected larvae

The *Nosema lymantria* infection caused 100% mortality during the larval stage of infected *L .dispar* (Fig. 4 & Fig. 5). Temperature had a significant effect on time of death. Larvae reared at 24°C died significantly earlier than those at 21°C and 18°C in both trials. The mean time of death was 41.3 ± 2.6 dpi at 18°C, 32.0 ± 1.6 dpi at 21°C and 23.5 ± 1.5 dpi at 24°C in trial 1 and 34.1 ± 2.7 dpi at 18°C, 34.0 ± 1.8 dpi at 21°C and 20.7 ± 1.2 dpi at 24°C in trial 2. Time of death was not significant different between 21°C and 18°C according to Mann-Whitney *U* Tests, corrected for type I errors by Bonferroni method (Trial 1: $p = 0.018$; Trial 2: $p = 0.673$; Bonferroni: $\alpha = 0.0167$).

50% mortality was reached on 22 dpi at 24°C, on 31 dpi at 21°C and on 42 dpi at 18°C in trial 1 (Fig. 4). In trial 2, half of the larvae had died on 20 dpi at 24°C, on 35 dpi at 21°C and on 28 dpi at 18°C (Fig. 5).

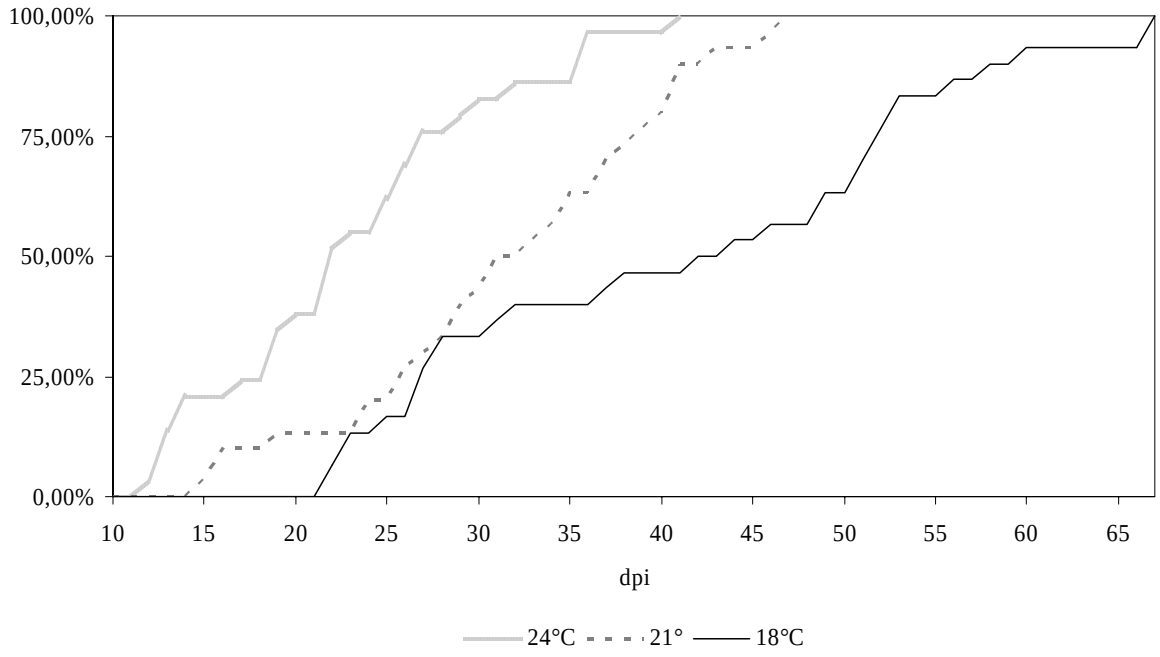


Fig. 4: Cumulative mortality of *N. lymantriae* infected larvae reared at 18°C, 21°C, and 24°C in trail 1. dpi = day post infection.

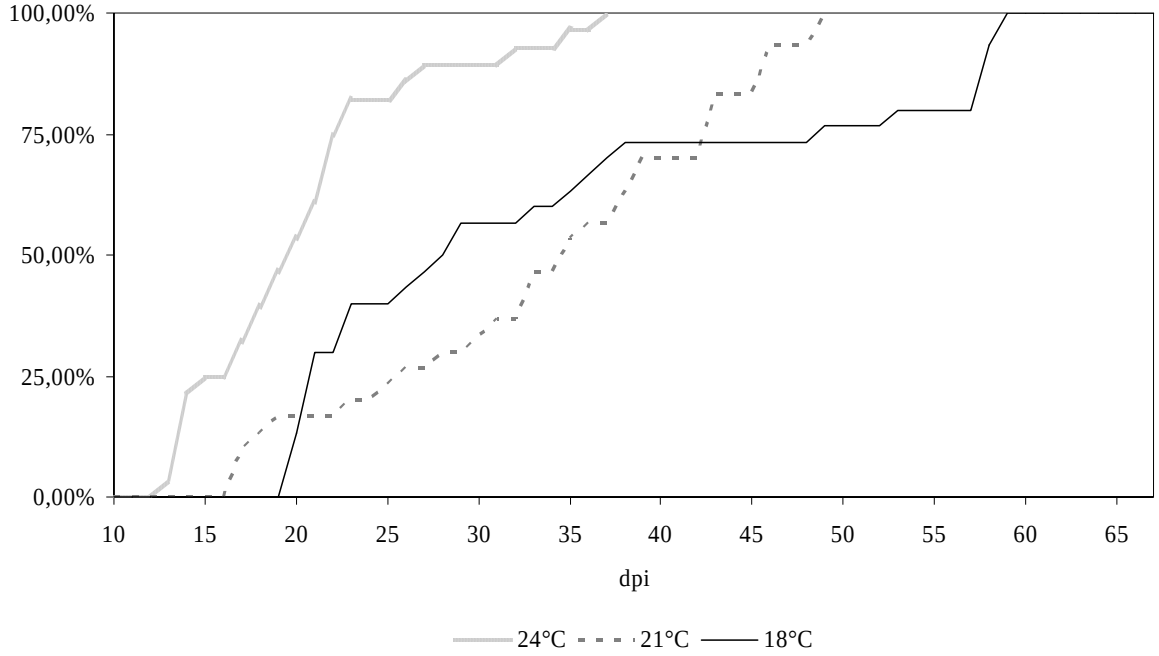


Fig. 5: Cumulative mortality of *N. lymantriae* infected larvae reared at 18°C, 21°C, and 24°C in trail 2. dpi = day post infection

Trial 1: Trial 2:

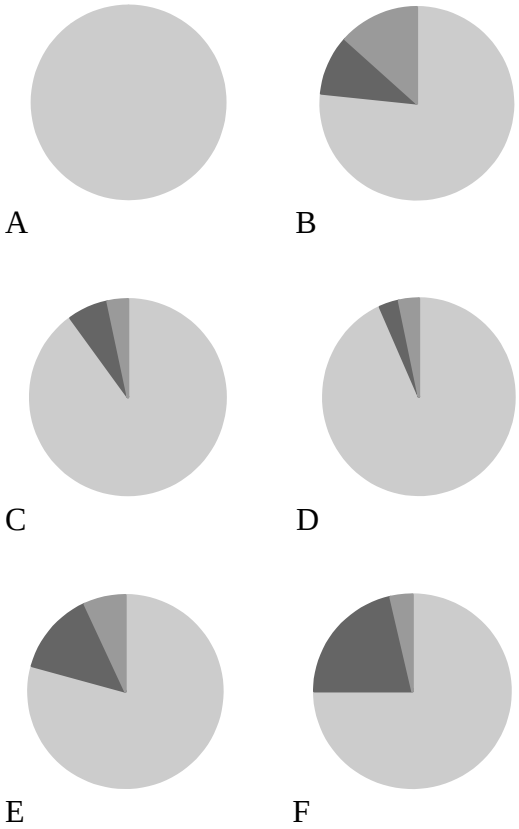


Fig. 6: Successful molts from L4 to L5 in % at 18°C (A & B), 21°C (C & D) and 24°C (E & F); Larvae molted normally; larvae died in premolt to L5; parts of old skin remained on larvae during molt and larvae died in consequence;

Infected larvae started to die 12 dpi at 24°C. At that time larvae molted to L5 and for some larvae molt caused death. Also at the other temperatures the molt from L4 to L5 was lethal for some infected larvae. First larvae died 15 dpi (trial 1) and 17 dpi (trial 2) at 21°C. On 18°C reared larvae started to die 22 dpi (trial 1) and 20 dpi (trial 2). In that period most larvae molted and some could not complete the molting process. Different timing of the molt from L4 to L5 in the two trials may at least partially explain differences in mortality. More larvae showed molting problems from L4 to L5 at 18°C in trial 2 than in trial 1 (Fig. 6), which is also reflected in faster mortality.

While *N. lymantriae* infection was lethal for 100% of tested larvae, most uninfected controls completed their larval stage. Only two larvae died because of physical injury during the experiment; they were excluded from further analyses.

3.3 *Nosema lymantriae* spores released with feces

Temperature had a significant effect on the time of first spore release in feces. In trial 2, spores were released earlier with higher temperatures. In trial 1, spores were released earlier at 24°C and 21°C than at 18°C (Table 1). Spore release started 13 dpi in trial 2 and 15 dpi in trial 1 at 24°C. While spore release occurred two days later in trial 2 and one day later in trial 1 at 21°C, spores were found 20 dpi in both trials at 18°C.

In trial 2, female larvae were also analyzed separately. Their spore release was significantly influenced by temperature (1-way ANOVA: $F = 179.567$, $p = 0.000$). In feces of female larvae reared at 24°C spores were found on 13.3 ± 0.2 dpi, at 21°C on 15.9 ± 0.2 dpi and at 18°C on 20.2 ± 0.3 dpi. The number of males in some treatment groups was too low for the computation of analyses regarding spore release.

Only three individuals released spores with feces in L4. All of these larvae died in L4 without even initiating premolt to L5.

The mean amount of spores released with feces in the first 6 days in L5 was significantly higher at 24°C than at 18°C in both trials (Table 1). The difference between 24°C and 21°C as well as 21°C and 18°C was not significant according to pairwise Mann-Whitney *U* tests.

Female larvae were analyzed separately in trial 2.

The results of the 1-way ANOVA (Temp.: F = 18.228, p = 0.000) as well as pairwise Mann-Whitney *U* tests show that the amount of released spores varied significantly at all temperatures (Fig. 7). Their amount of released spores was 3.9×10^7 at 24°C, 1.1×10^7 at 21°C, and 3.4×10^6 at 18°C. Because the total number of regarding males was lower than 4 individuals, the amount of spores in feces was not analyzed separately for males.

Table 1: First spore release and total amount of spores released in feces in first six days of female L5 (means ± S.E.)

	Trial	18°C	21°C	24°C
dpi 1st spores	1	20.07 ± 0.642 (14), *a*	16.18 ± 0.182 (11), *b*	15.44 ± 0.294 (9), *b*
	2	20.09 ± 0.436 (11), *a*	15.79 ± 0.187 (14), *b*	13.25 ± 0.164 (8), *c*
Number of spores in 1st six days in L5	1	$5.7\times10^6 \pm 2.4\times10^6$ (16), *a*	$7.0\times10^6 \pm 1.4\times10^6$ (16), *ab*	$1.6\times10^7 \pm 3.3\times10^6$ (14), *b*
	2	$3.1\times10^6 \pm 1.5\times10^6$ (11), *a*	$8.9\times10^6 \pm 2.1\times10^6$ (14), *ab*	$2.7\times10^7 \pm 7.0\times10^6$ (13), *b*

Number of larvae within a group is given in parentheses. Different letters within a row indicate significant differences between treatments based on pairwise Mann-Whitney *U* Tests (corrected for type I errors by Bonferroni method) following up significant *H* test.

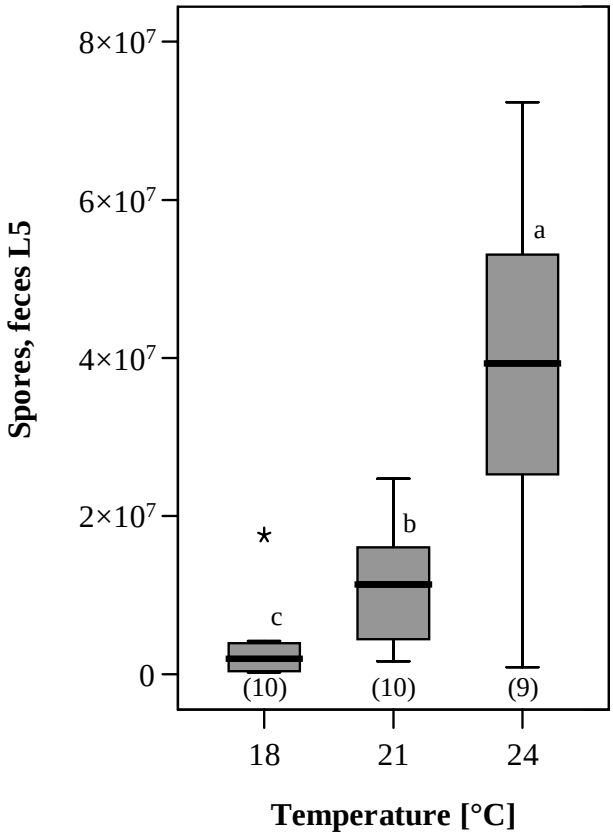


Fig. 7: Total amount of spores released in feces in first six days of L5 by female larvae in trial 2. Different letters indicate significant differences between treatments based on a pairwise Mann-Whitney *U* Test (corrected for type I errors by Bonferroni method) following up significant *H* test (χ^2 = 15.517, p = 0.000). Numbers of larvae within groups are given in parentheses.

Only at 24°C a significant correlation between the total amount of spores released in feces in the first six days of L5 and larval growth rate or consumption rate for this period could be ascertained using Spearman's ρ (Table 2).

Table 2: Correlation between the total amount of spores released in feces and consumption rate (CR) and larval growth rate (GR) in the first six days of L5 of female larvae

		18°C	21°C	24°C
CR	Spearman's ρ	-0.571	-0.286	0.786
	p	0.180	0.493	0.021
GR	Spearman's ρ	-0.167	-0.190	0.833
	p	0.688	0.651	0.010

3.4 Influence of temperature and infection on larval development parameters

3.4.1 Consumption

a) Fourth instar (L4)

Temperature and infection significantly influenced the consumption rate of male larvae in 4th instar (Temp.: $p = 0.000$, $\eta^2 = 0.638$; Inf.: $p = 0.004$, $\eta^2 = 0.267$; Table 3), also a significant interaction between temperature and infection was evident (Temp.*Inf.: $p = 0.027$, $\eta^2 = 0.234$; Table 3). The covariate, the larval fresh mass on the first dpi, had no significant influence on consumption in L4. The results of pairwise Mann-Whitney tests do not show significant differences between control- and infected larvae (Fig. 8). However, the numbers of male individuals per group were low (5 to 8).

The *U* tests indicated that the consumption rate of female larvae was significantly influenced by temperature, but not by *N. lymantriae* infection. Larvae fed more at higher temperatures, but no difference between infected and not infected larvae was shown in the *U* test (Fig. 9). But the effect of temperature was more pronounced in uninfected than in infected larvae, indicating an interaction between temperature and infection. A 2-way ANOVA was not computed for female larvae, because the variance of data was not equal.

Table 3: 2-way ANOVA for consumption rate (CR) of male larvae in L4

Factor	F	df	p	η^2
FM 1st dpi	2.817	1	0.492	0.018
Temperature	23.839	2	0.000	0.638
Infection	9.847	1	0.004	0.267
Temp. * Inf.	4.119	2	0.027	0.234

The fresh mass (FM) of larvae on the first day post infection (dpi) was taken as covariate.

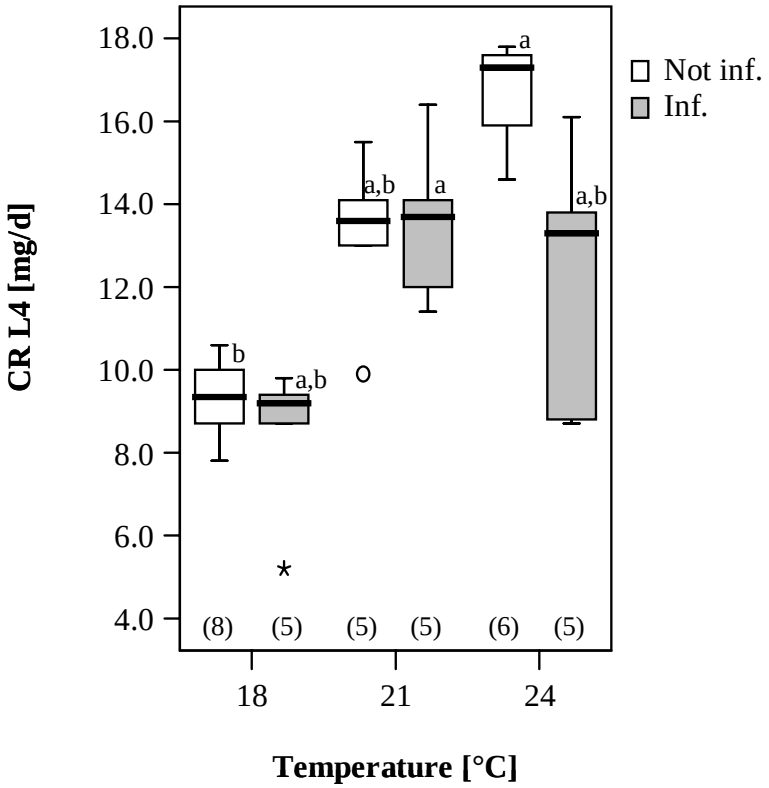


Fig. 8: Consumption rate (CR) of male larvae in L4. Different letters indicate significant differences between treatments based on pairwise Mann-Whitney *U* Tests (corrected for type I errors by Bonferroni method) following up significant *H* test (χ^2 = 22.923, p = 0.000). Numbers of larvae within groups are given in parentheses.

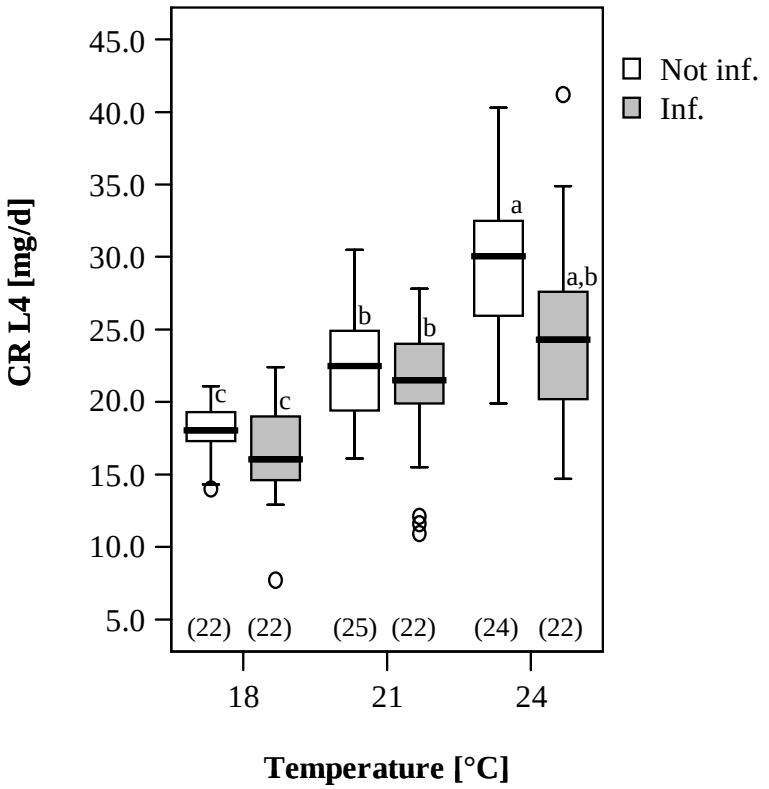


Fig. 9: Consumption rate (CR) of female larvae in L4. Different letters indicate significant differences between treatments based on pairwise Mann-Whitney *U* Tests (corrected for type I errors by Bonferroni method) following up significant *H* test (χ^2 = 66.576, p = 0.000). Numbers of larvae within groups are given in parentheses.

b) First six days of fifth instar (L5)

The effect of temperature on the consumption rate of male and female larvae was significant at the first six days of L5 (males: p = 0.000, η^2 = 0.714; females: p = 0.000, η^2 = 0.635). Female larvae were affected significantly by temperature and infection and an interaction between temperature and infection occurred (Table 5). Mean consumption rate of uninfected larvae varied significantly between 99.0 ± 2.7 mg/d at 18°C, 146.1 ± 4.1 mg/d at 21°C and 214.4 ± 5.3 mg/d at 24°C (Table 7). Infected larvae consumed 56 to 85mg/d and no significant variation caused by temperature was evident. Infection had no significant effect on consumption rate of males.

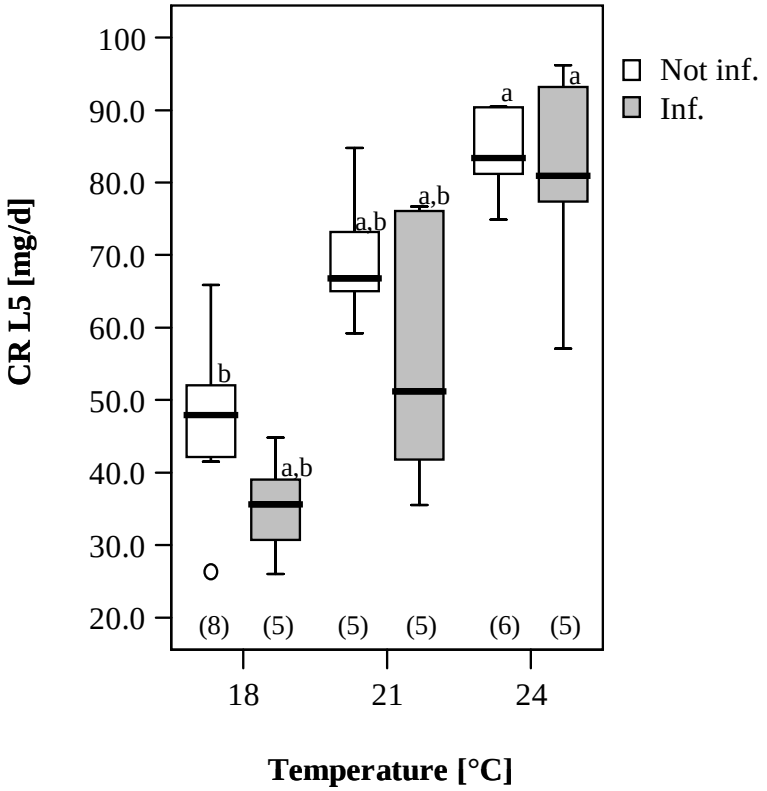


Fig. 10: Consumption rate (CR) of male larvae in the 1st six days of L5. Different letters indicate significant differences between treatments based on pairwise Mann-Whitney *U* Tests (corrected for type I errors by Bonferroni method) following up significant *H* test (χ^2 = 23.646, p = 0.000). Numbers of larvae within groups are given in parentheses.

Table 4: 2-way ANOVA for Consumption rate (CR) of male larvae at the 1st six days of L5

Factor	F	df	p	η^2
FM 1st dpi	0.987	1	0.492	0.018
Temperature	33.633	2	0.000	0.714
Infection	1.624	1	0.213	0.057
Temp. * Inf.	0.416	2	0.664	0.030

The fresh mass (FM) of larvae on the first day post infection (dpi) was taken as covariate.

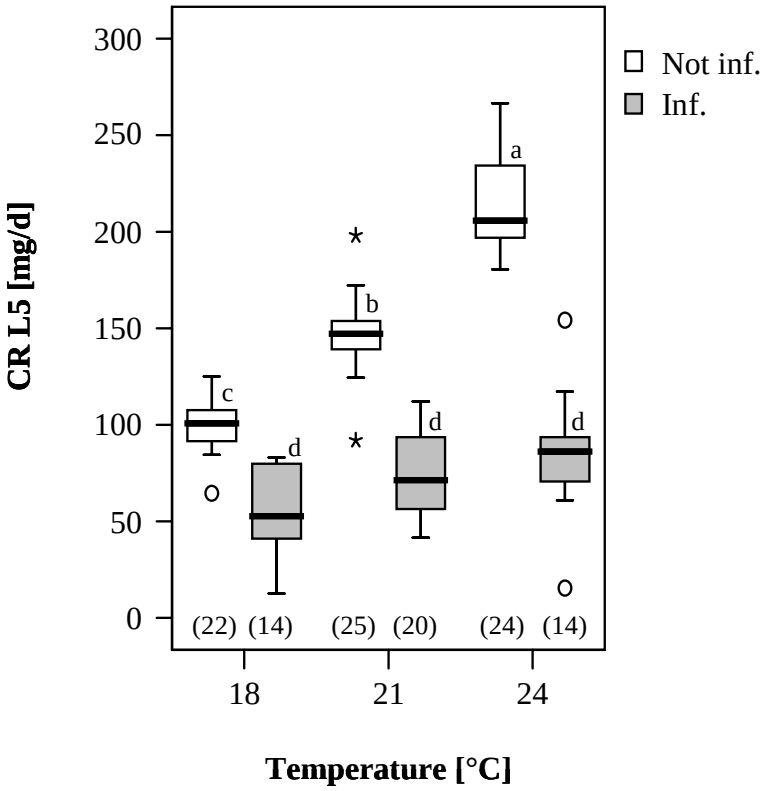


Fig. 11: Consumption rate (CR) of female larvae in the 1st six days of L5. Different letters indicate significant differences between treatments based on pairwise Mann-Whitney *U* Tests (corrected for type I errors by Bonferroni method) following up significant *H* test (χ^2 = 95.137, p = 0.000). Numbers of larvae within groups are given in parentheses.

Table 5: 2-way ANOVA for Consumption rate (CR) female larvae at the 1st six days of L5

Factor	F	df	p	η^2
FM 1st dpi	6.895	1	0.010	0.059
Temperature	95.817	2	0.000	0.635
Infection	369.734	1	0.000	0.771
Temp. * Inf.	36.241	2	0.000	0.397

The fresh mass (FM) of larvae on the first day post infection (dpi) was taken as covariate.

3.4.2 Larval growth

a) Fourth instar (L4)

Temperature had a significant impact on the growth rate of male larvae in L4 (Temp.: $p = 0.000$, $\eta^2 = 0.640$; Table 6). Control-larvae grew significantly faster at 21°C and 24°C than at 18°C (Fig. 12). This effect was not significant for infected larvae. The effect of infection was significant in the 2-way ANOVA, but with low η^2 (Inf.: $p = 0.049$, $\eta^2 = 0.136$; Table 6); non-parametric tests indicated no differences between infected and uninfected larvae within temperatures. No significant interaction between temperature and infection was shown; the effect of the covariate FM 1st dpi was not significant (Table 6).

Female control- and infected larvae grew significantly faster with increasing temperatures in L4, but infection had no significant effect on larvae (Fig. 13). The growth rate was equal for infected and non infected larvae within the same temperature. A 2-way ANOVA was not computed for female larvae, because the variance of data was not equal.

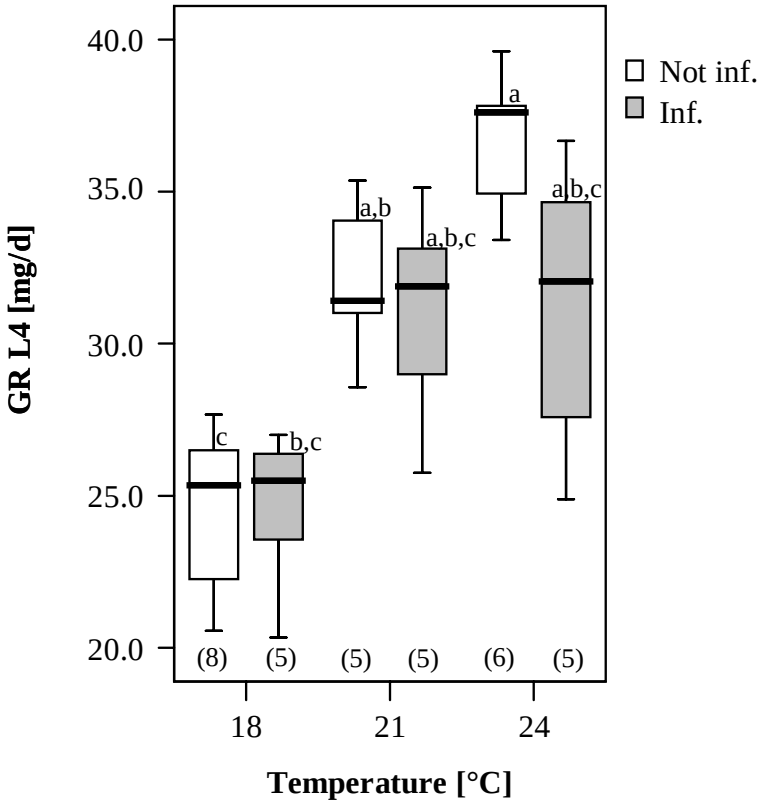


Fig. 12: Growth rate (GR) of male larvae in L4. Different letters indicate significant differences between treatments based on pairwise Mann-Whitney *U* Tests (corrected for type I errors by Bonferroni method) following up significant *H* test (χ^2 = 22.957, p = 0.000). Numbers of larvae within groups are given in parentheses.

Table 6: 2-way ANOVA for Growth rate (GR) of male larvae in L4

Factor	F	df	p	η^2
FM 1st dpi	0.485	1	0.492	0.018
Temperature	23.958	2	0.000	0.640
Infection	4.234	1	0.049	0.136
Temp. * Inf.	1.744	2	0.194	0.114

The fresh mass (FM) of larvae on the first day post infection (dpi) was taken as covariate.

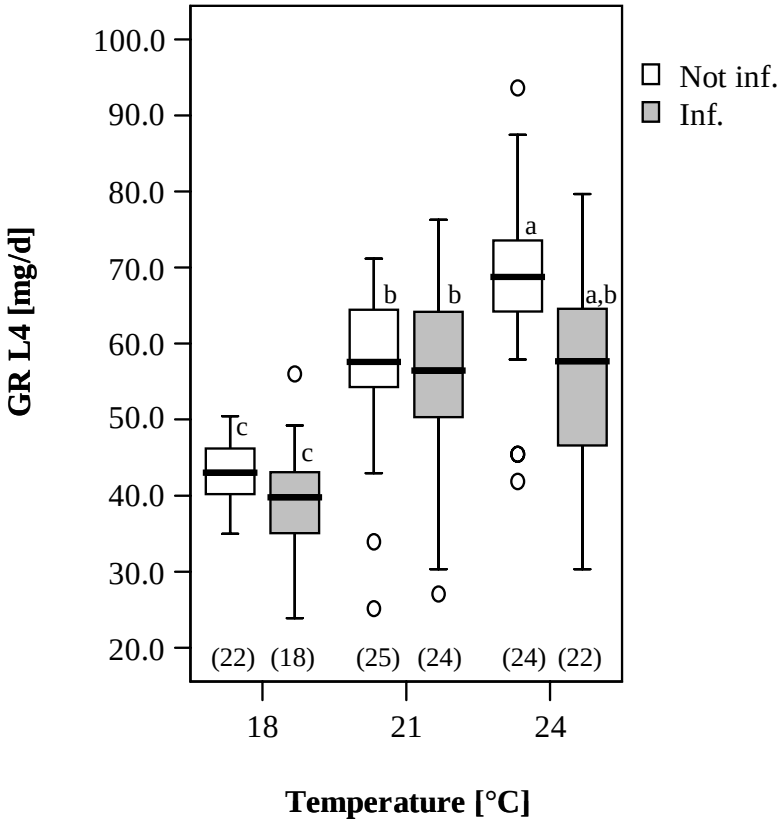


Fig. 13: Growth rate (GR) of female larvae in L4. Different letters indicate significant differences between treatments based on pairwise Mann-Whitney *U* Tests (corrected for type I errors by Bonferroni method) following up significant *H* test (χ^2 = 61.559, p = 0.000). Numbers of larvae within groups are given in parentheses.

b) First six days of fifth instar (L5)

Temperature and infection had no significant effect on the growth rate of male larvae in the first six days in L5 (Fig. 14). As the number of males was low (4 to 8 individuals per group), the data do not allow definitive conclusions. Temperature and *N. lymantriae* infection had a significant impact on the growth rate of *L. dispar* female larvae in the first six days of L5 (Fig. 15). A possible interaction between temperature and infection is indicated by the *U* tests (Fig.15) and is shown in a 2-way ANOVA (Temp.*Inf.: p = 0.000, F = 18.675, η^2 = 0.261; the ANOVA is problematic, however, because of unequal variances – Levene's test of equality of variances: p = 0.010); while control larva grew faster with increasing temperatures, this effect was not significant in infected larvae. Overall, infected larvae grew significantly slower than their uninfected controls at all three temperatures. The mean growth rate of uninfected larvae varied significantly between 218.5 ± 6.2 mg/d at 18°C, 295.1 ± 6.4 mg/d at 21°C and 388.7 ± 12.1 mg/d at 24°C (Table 7). Infected larvae gained 93 to 143 mg/d and no significant variation was caused by temperature (Table 7).

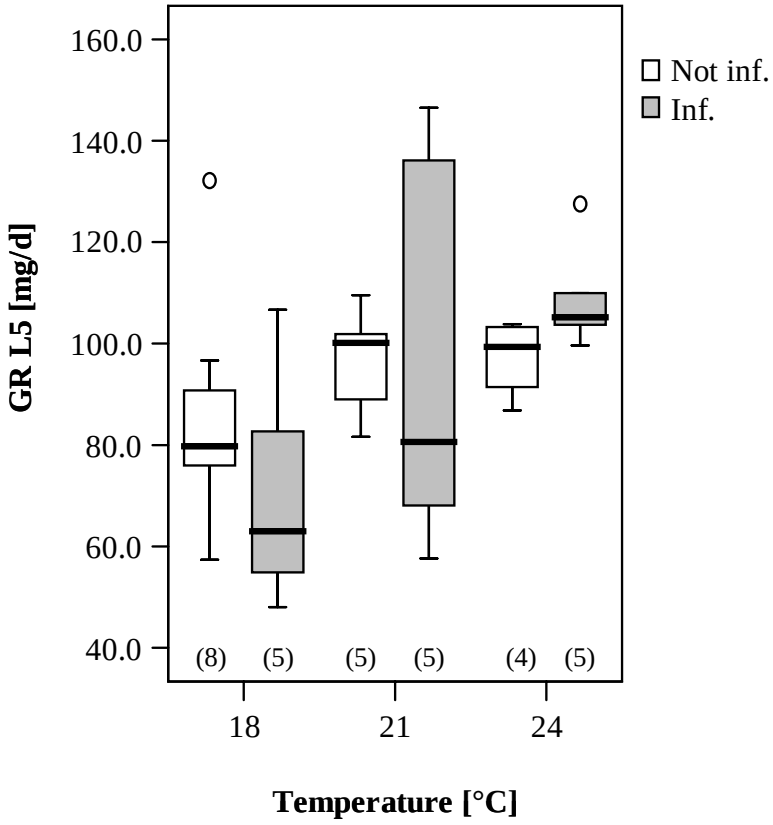


Fig. 14: Growth rate (GR) of male larvae at the 1st six days of L5. *H* test showed no significant differences between treatments (χ^2 = 9.154, p = 0.103). Numbers of larvae within groups are given in parentheses.

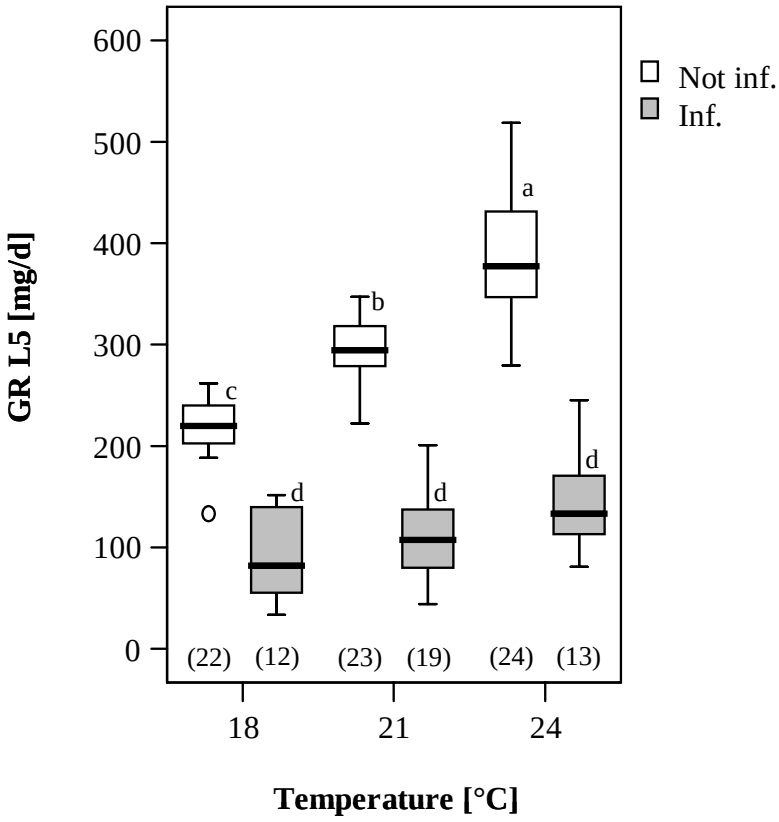


Fig. 15: Growth rate (GR) of female larvae at the 1st six days of L5. Different letters indicate significant differences between treatments based on pairwise Mann-Whitney *U* Tests (corrected for type I errors by Bonferroni method) following up significant *H* test (χ^2 = 99.097, p = 0.000). Numbers of larvae within groups are given in parentheses.

Table 7: Means ± S.E. of larval fresh mass (FM), growth rate (GR) and consumption rate (CR) in L4 and first six days of L5

Sex	Temp. [°C]	Treatment	FM 1 dpi [mg]	FM 1st d L4 [mg]	FM last d L4 [mg]	FM 1st d L5 [mg]	FM 6th d L5 [mg]	FM 2nd d P [mg]	FM death [mg]	GR L4 [mg/d]	GR L5 [mg/d]	CR L4 [mg/d]	CR L5 [mg/d]
Male	18	Not inf.	30.6 ± 1.6 (8)	73.8 ± 3.1 (8)	279.4 ± 10.1 (8)	263.3 ± 9.5 (8)	775.0 ± 53.5 (8)	713.0 ± 39.1 (8)	- -	24.6 ± 0.9 (8)	85.3 ± 7.7 (8)	9.3 ± 0.3 (8)	47.1 ± 4.0 (8)
		Inf.	22.8 ± 0.7 (6)	74.4 ± 1.6 (6)	279.6 ± 5.6 (5)	261.2 ± 5.8 (5)	687.4 ± 60.0 (5)	- -	551.2 ± 61.0 (6)	24.6 ± 1.2 (5)	71.0 ± 10.6 (5)	8.5 ± 0.8 (5)	35.2 ± 3.3 (5)
	21	Not inf.	26.5 ± 1.2 (5)	75.7 ± 4.2 (5)	280.1 ± 7.5 (5)	259.3 ± 7.1 (5)	838.0 ± 32.3 (5)	687.1 ± 28.6 (5)	- -	32.1 ± 1.2 (5)	96.5 ± 5.0 (5)	13.2 ± 0.9 (5)	69.8 ± 4.4 (5)
		Inf.	23.7 ± 1.5 (5)	74.9 ± 3.8 (5)	284.7 ± 11.0 (5)	264.3 ± 10.4 (5)	851.2 ± 114.7 (5)	- -	588.4 ±149.7 (5)	31.0 ± 1.6 (5)	97.8 ± 18.2 (5)	13.5 ± 0.9 (5)	56.3 ± 8.6 (5)
	24	Not inf.	25.7 ± 0.8 (6)	65.0 ± 3.9 (6)	249.1 ± 7.1 (6)	226.4 ± 6.9 (6)	803.5 ± 24.5 (4)	683.1 ± 18.2 (6)	- -	36.8 ± 0.9 (6)	97.3 ± 3.9 (4)	16.8 ± 0.5 (6)	84.0 ± 2.4 (6)
		Inf.	24.1 ± 1.3 (5)	66.7 ± 7.2 (5)	253.7 ± 18.2 (5)	229.8 ± 17.4 (5)	885.1 ± 37.1 (5)	- -	756.8 ± 41.2 (5)	31.2 ± 2.2 (5)	109.2 ± 4.9 (5)	12.1 ± 1.5 (5)	81.0 ± 6.9 (5)
Female	18	Not inf.	30.0 ± 1.1 (22)	106.2 ± 2.9 (22)	503.1 ± 24.0 (22)	492.6 ± 10.7 (22)	1803.5 ± 45.1 (22)	2007.9 ± 41.0 (22)	- -	40.9 ± 2.3 (22)	218.5 ± 6.2 (22)	18.0 ± 0.4 (22)	99.0 ± 2.7 (22)
		Inf.	28.4 ± 1.1 (24)	109.3 ± 4.0 (24)	505.4 ± 18.9 (18)	460.2 ± 18.2 (17)	1063.7 ± 84.0 (13)	- -	741.7 ± 69.9 (24)	39.4 ± 1.8 (18)	93.1 ± 12.6 (12)	16.4 ± 0.8 (22)	56.1 ± 6.1 (14)
	21	Not inf.	29.9 ± 1.5 (25)	99.7 ± 3.6 (25)	512.8 ± 12.3 (25)	480.1 ± 11.5 (25)	2250.9 ± 46.9 (23)	2111.7 ± 35.0 (25)	- -	58.7 ± 1.5 (25)	295.1 ± 6.4 (23)	22.8 ± 0.8 (25)	146.1 ± 4.1 (25)
		Inf.	27.5 ± 0.8 (25)	102.8 ± 3.5 (25)	524.3 ± 21.5 (24)	489.8 ± 19.7 (23)	1171.1 ± 76.8 (20)	- -	953.7 ± 94.2 (25)	55.4 ± 2.7 (24)	115.0 ± 10.7 (19)	21.1 ± 0.9 (25)	75.1 ± 5.1 (20)
	24	Not inf.	26.1 ± 0.9 (24)	101.2 ± 3.7 (24)	508.6 ± 16.8 (24)	469.4 ± 15.7 (24)	2801.6 ± 83.1 (24)	2321.7 ± 51.8 (24)	- -	68.6 ± 2.6 (24)	388.7 ± 12.1 (24)	29.6 ± 1.0 (24)	214.4 ± 5.3 (24)
		Inf.	25.8 ± 1.0 (23)	90.4 ± 5.2 (23)	479.1 ± 24.2 (21)	423.0 ± 23.7 (19)	1288.6 ± 94.4 (13)	- -	817.7 ± 92.6 (23)	57.7 ± 2.9 (21)	143.3 ± 12.8 (13)	24.7 ± 1.6 (22)	85.2 ± 8.5 (14)

FM 2nd d P = FM on second day of pupa; numbers of larvae within groups are given in parentheses.

3.4.3 Duration of larval stages

a) Fourth instar (L4)

Higher temperatures led to significantly shorter duration of the fourth instar in both, male (H test: $\chi^2 = 30.4$, $p < 0.001$) and female larvae (H test: $\chi^2 = 115.6$, $p < 0.001$). Pairwise Mann-Whitney *U* Test indicate that male larvae at 18°C remained significantly longer in L4 than larvae of other groups, which showed no significant difference in duration of L4 (Table 8). This effect may be due to the low number of male individuals. Temperature and infection significantly influenced the duration of L4 of female larvae. Control larvae and *N. lymantriae* infected larvae remained significantly longer in L4 at lower temperatures. The differences between infected larvae and control larvae were significant at 21°C and 24°C, but not at 18°C (Table 8). Overall, male larvae grew significantly faster than female larvae (2-way ANOVA: $F = 6.709$, $p = 0.010$, $\eta^2 = 0.040$).

b) Fifth instar (L5)

No microsporidian infected larvae completed its L5 and therefore duration of L5 could only be analyzed for control larvae. Temperature had a significant effect on the length of L5. Larvae finished their L5 significantly faster at higher temperatures (Table 8; $p = 0.000$, $\eta^2 = 0.799$). At 24°C, the instar was 2-3 days shorter than at 21°C and 7-8 days shorter than at 18°C in both, male and female larvae (Table 8). Female larvae remained significantly longer in L5 than males (2-way ANOVA: sex: $p = 0.000$, $\eta^2 = 0.152$) There was no significant interaction between sex and temperature (2-way ANOVA: Temp. * sex: $p = 0.540$, $\eta^2 = 0.015$). Males developed significantly faster than females.

Table 8: Duration of fourth (L4) and fifth (L5) instar of male and female larvae

	18°C		21°C		24°C	
Treatment	not inf.	inf.	not inf.	inf.	not inf.	inf.
♂ L4 [d]	8.4 ± 0.2 *a*	8.4 ± 0.2 *a,b*	6.4 ± 0.2 *b*	6.8 ± 0.2 *b*	5.0 ± 0 *b*	6.0 ± 0 *b*
♂ L5 [d]	17.5 ± 0.5 *a*		12.6 ± 0.2 *b*		9.7 ± 0.2 *c*	
n	8	5	5	5	6	5
♀ L4 [d]	9.7 ± 0.1 *a*	10.1 ± 0.2 *a*	7.0 ± 0.0 *b*	7.7 ± 0.1 *c*	6.0 ± 0.0 *d*	6.7 ± 0.2 *b*
♀ L5 [d]	18.5 ± 0.1 *a*		13.8 ± 0.4 *b*		11.5 ± 0.1 *c*	
n	22	18	23	24	24	21

Different letters within a row indicate significant differences between treatments based on pairwise Mann-Whitney *U* Tests (corrected for type I errors by Bonferroni method) following up significant *H* test.

3.4.4 Time of pupation and pupal fresh mass

Temperature significantly influenced the time of pupation in both trails. Larvae pupated significantly later at lower temperatures (H tests, Table 9). Sex also had a significant effect on time of pupation. Female pupated significantly later in trial 1 & 2 than male larvae (H tests: $\chi^2 = 64.063$, $p < 0.001$ and $\chi^2 = 81.280$, $p < 0.001$, respectively).

The pupal fresh mass of males was not significantly influenced by temperature in both trials (H tests: $\chi^2 = 4.462$, $p = 0.107$ and $\chi^2 = 0.083$, $p = 0.959$, respectively), but the effect of temperature was significant for female larvae in trial 1 (H tests: $\chi^2 = 18.412$, $p < 0.001$). Female larvae weighed more at higher temperatures (Table 9) and overall significantly more than male larvae at all temperatures in trial 1 & 2 (H tests: $\chi^2 = 58.352$, $p < 0.001$ and $\chi^2 = 55.480$, $p < 0.001$, respectively; post hoc U tests: $\alpha_{corr} < 0.0033$).

All infected insects died in the larval stage.

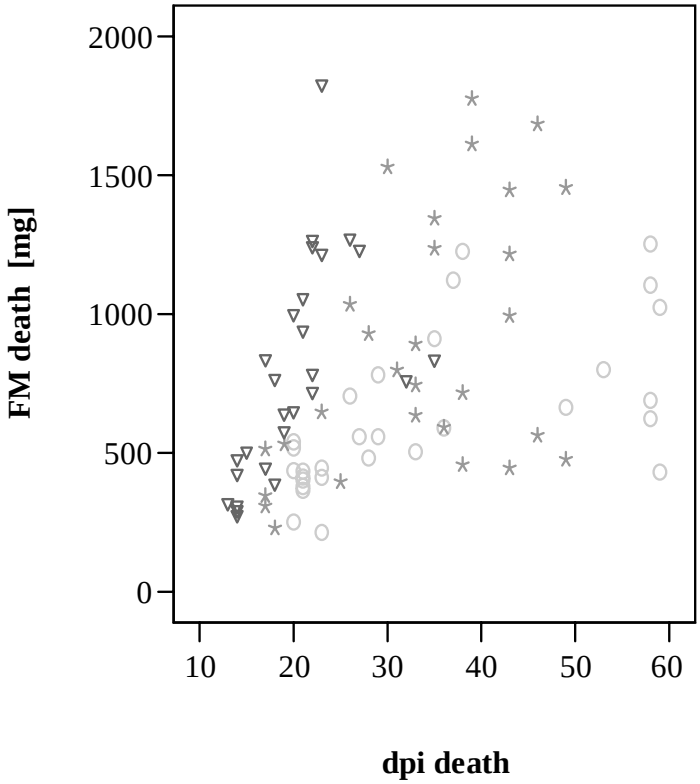
Table 9: Time of pupation (dpi = days post inoculation) and pupal fresh mass on second day of pupation of uninfected *L. dispar* in trial 1 and trial 2 (means ± S. E.)

	18°C	21°C	24°C
Trial 1:			
♂ dpi pupa	33.58 ± 0.69 *a*	24.78 ± 0.401 *b*	20.64 ± 0.338 *c*
♂ FM [mg]	1045.208 ± 186.457	655.889 ± 26.228	672.818 ± 21.957
n	12	9	11
♀ dpi pupa	36.88 ± 0.331 *a*	27.85 ± 0.182 *b*	24.64 ± 0.58 *c*
♀ FM [mg]	1904.441 ± 61.549 *a*	2152.100 ± 50.522 *b*	2440.929 ± 13.053 *b*
n	17	20	14
Trial 2:			
♂ dpi pupa	32.75 ± 0.62 *a*	25 ± 0.316 *b*	19.67 ± 0.211 *c*
♂ FM [mg]	713.0 ± 39.066	687.14 ± 28.635	683.133 ± 18.204
n	8	5	6
♀ dpi pupa	36.14 ± 0.151 *a*	27.22 ± 0.444 *b*	22.67 ± 0.177 *c*
♀ FM [mg]	2007.864 ± 40.961 *a*	2111.661 ± 34.998 *a*	2321.721 ± 51.763 *b*
n	22	23	24

Different letters within a row indicate significant differences between treatments based on pairwise Mann-Whitney *U* Tests (corrected for type I errors by Bonferroni method) following up significant *H* test. Fresh mass weighed on the second day after pupation. Larvae that developed into L6 were excluded.

3.4.5 Fresh mass of cadavers of larvae infected with *N. lymantriae*

The fresh mass of infected larvae after death was not significantly influenced by temperature (Table 7; *H* - Test: p = 0.086, χ^2 = 4.899). Sex had no effect on fresh mass of cadavers (*H* - Test: p = 0.133, χ^2 = 8.459). No significant correlation between day of death and FM of cadavers was evident in trial 1, but in trial 2 the FM of death and day of death were significantly correlated (Fig. 16).



	ρ	p
○ 18°C	0.690	0.000
✳ 21°C	0.459	0.012
▽ 24°C	0.804	0.000

Fig. 16: Fresh mass (FM) of cadavers and day of death (dpi = day post inoculation) in trial 2. Spearman's correlation coefficients ρ for fresh mass of cadaver and day of death is shown in the table.

4 Discussion

4.1 Effects of temperature on developmental parameters

Temperature significantly increased growth and consumption of infected and uninfected *Lymantria dispar* larvae in my experiments. This corresponds with Schedl (1936), who indicated that gypsy moth larvae develop faster at higher temperatures. Wigglesworth (1972) points out that higher temperatures in the developmental stages of insects lead to faster growth. Other studies of herbivorous insects confirm this (Leather & MacKenzie, 1994; Åsman, 2001; Stillwell & Fox, 2005). Åsman (2001) examined the effect of five constant temperatures between 12°C and 20°C on the development of the leek moth *Acrolepiopsis assectella* Zell. His studies reveal that leek moth larvae develop faster with increasing temperatures, twice as fast at 20°C than at 12°C. My results show that in fourth instar, male and female larvae grew faster and consumed more with increasing temperatures. In the first six days of fifth instar, female control larvae grew faster and consumed more with increasing temperatures. Effects in male larvae in fifth instar were similar but clearly less pronounced. Higher temperatures led to significantly shorter duration of fourth instar. Furthermore, uninfected larvae pupated significantly faster at higher temperatures. This agrees with studies of Williams et al. (2003) and Karolewski et al. (2007), who showed that the duration of larval gypsy moth development until pupation was significantly shortened by increasing temperatures. In the experiments of the latter authors, female larvae reared at 20°C showed the highest pupal mass, whereas the pupal mass of males was similar at 20°C and 25°C, but much lower at 15°C. On the other hand, in my experiments, the pupal mass of female and male larvae was significantly higher at increasing temperatures. At 24°C pupal mass was the highest. These apparently contradictory results may be a consequence of different diets. While Karolewski et al. (2007) fed larvae with leaves of *Quercus robur* – their quality may have deteriorated faster at higher temperatures – larvae in my experiments were reared on wheat germ diet. When reared on wheat germ diet, even 26°C rearing temperature leads to higher pupal mass than at 21°C (Kereselidze et al., 2009). Quite contrary to my studies are the studies of Lindroth et al. (1997), who reported that final pupal weights of *L. dispar* were not significantly affected by the direct effect of temperature (Thermal regimes: 19:16, 22:19, 25:22°C with 15:9h light-dark cycle). Reduced larval growth rates were outweighed by

prolonged developmental periods of gypsy moth larvae, leading to equal final mass. This was not the case in my study. According to Lindroth et al. (1997) reduced growth rates may result from decreased consumption rates and/or food utilization efficiencies. Reduced consumption rates and therewith reduced growth rates at lower temperatures in my studies confirm this opinion. In addition, females showed stronger growth response to increasing temperatures than males in the experiments of Lindroth et al. (1997). This applies to my studies as well, where the effects of temperature on growth and consumption were less obvious for male larvae, which on the other hand may also result from a low number of males in my experiments. It is evident that male and female larvae differ in consumption and utilization of food due to different numbers of instars and increased growth of female larvae. Female larvae tend to be heavier, as a result from increased food consumption (Scriber & Slansky, 1981).

4.2 Influence of *Nosema lymantriae* infection on developmental parameters

Nosema lymantriae proved to be a virulent microsporidium that significantly affected host development. All infected insects died during the larval stage in our experiments. Survival to the adult stage at percentages higher than 50% is only possible, when larvae become infected with *N. lymantriae* in their final instar (Goertz & Hoch, 2008). Negative effects on development become clearly noticeable prior to death. Infected *L. dispar* larvae grew less and consumed less than uninfected controls. The negative effects on host development are comparable to *Vairimorpha disparis* another microsporidium that also infects the fat body of *L. dispar* larvae (Hoch & Schopf, 2001). Apparently, infection of this important organ of metabolism has overall severe negative effects on physiology of the host larva. Boohene et al. (2003) interpret a reduced fecundity and longevity of adult microsporidia-infected *Muscidifurax raptor* females as a result of ovary and fat body infection by *Nosema muscidifuracis*. Females are dependent on protein reserves for egg maturation that are stored in the fat body that acts as a storage site for nutrients (Gaugler & Brooks, 1975). It is evident that endoparasitoids and -pathogens can influence the performance of host larvae to complete their own development. This may result in changed developmental time, consumption and food utilization of hosts, modified carbohydrate, lipid and protein metabolism, as well as diverse nitrogen utilization and excretion (Vinson & Iwantsch, 1980). Hoch et al. (2002) report significantly decreased carbohydrate and lipid levels in *L. dispar* larvae infected with *Vairimorpha* sp.

Microsporidia can also affect the development of parasitoids that use the gypsy moth as their host. Hoch et al. (2001b) show a significant longer developmental time, reduced pupation outcome, reduced eclosion, weight and lifespan of adults of the endoparasitic braconid *Glyptapanteles liparidis* when developing in a *Vairimorpha* sp. infected host larvae. As *G. liparidis* feeds on the hemolymph of *L. dispar* larvae only, the authors conclude that their results may be due to an inappropriate nutrition of the parasitoids, arising form a nutrient alternation by the microsporidia (Hoch et al., 2002). Rath et al. (2003) indicated that larval growth, food consumption and digestion of *Antheraea mylitta* were influenced by *Nosema* sp. infection. My study shows reduced growth rates and consumption rates of *Nosema* infected *L. dispar* larvae in fifth instar. Female larvae were significantly affected by microsporidian infection and showed reduced growth and consumption rates. The number of males was too low for definite conclusions. The documented reduced consumption rate of *N. lymantriae* infected larvae is of practical importance when considering this microsporidium as biological control agent. It shows that host plants get protection from defoliation already two weeks before the infected insect actually dies.

Moreover, the developmental time of infected female larvae was prolonged in fourth instar compared to control larvae. A longer developmental time due to a microsporidian infection was also described by Henn & Solter (2000). In their studies, gypsy moth larvae infected with the microspordium *Vairimorpha* sp. showed an extended development as a result of microsporidian influence on larval development, feeding activity, and conversion of food into body substance. Microsporidian nutrient requirements may eventually also differ at different developmental stages. Henn et al. (1998) discovered that the amino acid levels in infected mosquitoes varied with the different developmental stages of the microsporidium *Nosema algerae*. Also Sohi & Wilson (1975) concluded that the amino acid content within the host may change according to the various stages of protozoan development. As the inoculation of *N. lymantriae* was performed only at the beginning of the third instar, an earlier developmental stadium of the pathogen combined with different pathogen nutrition requirements may explain why the effect of microsporidian infection on larval development in fourth instar was not yet significant. On the other hand, the damage of the fat body caused by *N. lymantriae* was not high in fourth instar, which maybe a likely reason why the development rate did not differ between infected and control larvae. The dissection of infected larvae showed that in fourth instar, mostly vegetative stages of *N. lymantriae* were found in silk glands, fat body and Malphigian tubules of microsporidian larvae.

4.3 Influence of temperature and infection on developmental parameters

Temperature significantly affected host development as expected. *L. dispar* larvae developed faster, gained more weight, and consumed more at higher temperatures. Infection significantly interacted with temperature and the effects of temperature were outweighed by the microsporidian infection. Infected larvae did not show the same, significant positive response to higher temperature. Boohene et al. (2003) report that developmental times of microsporidia-infected *Muscidifurax raptor* were significantly longer than those of uninfected parasitoids and noticed an significant interaction of temperature and infection for males. In studies of Bruck et al. (2001) larvae of the European corn borer, *Ostrinia nubilalis*, were infected with *Nosema pyrausta* and incubated at 27°C until pupation was completed. After eclosion, half of the population was maintained at constant 16°C. The impact of this temperature on egg production was more profound on microsporidia-infected *O. nubilalis* than on uninfected controls, considering the egg production. While noninfected females produced only 5% fewer eggs at 16°C, egg production of *N. pyrausta* infected females declined 70%. Microsporidian infection may influence the temperature effect on the host. *N. lymantriae* infection leaded to a reduction of the positive effect of higher temperatures in my experiments.

4.4 Microsporidian development at different temperatures

Also the development of the pathogen was significantly affected by temperature. As obligate intracellular parasites, microsporidia depend on resources provided by the host. Hence, temperature may have an indirect host-mediated effect on *N. lymantriae* as well as a direct effect. Within the tested ranges, development of the microsporidium was faster, spores appeared earlier in feces, and infected hosts died earlier at higher temperature. This appears to be in contradiction to the findings of Solter et al. (1990) who concluded that the microsporidium *Nosema pyrausta* may develop more quickly at lower temperature relative to the host *O. nubilalis*. This conclusion, however, was based on developmental time of the host; the progress of infection or spore production was not examined. Likewise, Bruck et al. (2001) report that low temperatures interfered with oviposition of adult *O. nubilalis* while progression of *N. pyrausta* was not impaired. Both cited reports indicate that important

interactions exist between temperature and microsporidian infection in the effects on host development as were demonstrated in our system.

My results indicate that microsporidian development in *L. dispar* larvae is faster at higher temperatures. From 12 days post infection (dpi) on, silk glands and fat body of larvae reared at 24°C and 21°C were totally filled with environmental spores, while at 18°C, the infection progressed about four days slower. Malpighian tubules became filled with environmental spores 14 and 16 dpi at 21°C and 24°C, but this tissue was not filled with spores before 20 dpi at 18°C. Similar results are reported by Maddox (1968), who showed that the generation time of *Nosema necatrix* in the armyworm, *Pseudaletia unipuncta*, is shorter at higher temperatures (Temperature range: 15°C – 32°C). Studies on the effect of temperature on honeybee microsporidia reveal that the spore count of *Nosema ceranae* and *N. apis* found in adult honeybees (*Apis mellifera*) was the highest when bees were incubated at 33°C compared to 25°C and 37°C and microsporidian infected cells where detected earlier at 33°C than at 25°C and 37°C (Martín-Hernández et al., 2009). The highest temperature was the least favourable for both species. 37°C is considered as the upper temperature range for many microsporidia in insects (Becnel & Andreadis, 1999). Cultivations of *Nosema algerae* in mammalian cell culture at different temperatures illustrate that multiplication of this organism was severely diminished at elevated temperatures (36.0, 36.5, 37.0°C) compared to 29°C, that seems to be optimal for its development (Lowman et al, 2000). Most studies concerning the temperature effect on microsporidia discuss the impact of either very high or very low temperatures for microsporidan development. Examinations on the temperature dependence of *Edhazardia aedis* show that spores lost their infectivity within 24 hours when they were kept at 0, 5 and 40°C. At 10, 15 and 20°C infection levels remained the highest (Undeen et al., 1993). *Vairimorpha ephestiae*, a microsporidia of the wax moth *Galleria mellonella*, killed about 62% and 91% of infected wax moth larvae, when they were cultivated at 25°C and 21°C, but had no effect when larvae were reared at 30°C (Vorontsova et al., 2004). It is evident that microsporidia have an optimal temperature range in which viability is maintained (Maddox 1973) and concerning the temperature regime used in my studies, higher temperatures within the tested range were better for the development of *N. lymantriae* in gypsy moth larvae. Nevertheless, at a certain higher temperatures this positive correlation is likely to be lost.

4.5 Spore release and consequences for horizontal transmission

Successful horizontal transmission depends largely on the latent period, the delay between the initial infection and beginning of the infectious period and the number of released spores (Anderson and May, 1981). Goertz et al. (2004) show that a latent period of about 11 days is necessary until microsporidian infected larvae become infectious. In their experiments uninfected larvae were exposed to a *Nosema* sp. infected larvae. After 12 dpi the number of exposed larvae that became infected with *Nosema* increased significantly. At 9-12 dpi the transmission rate was lower. Goertz & Hoch (2008) report that first spore release with feces occurs only 12-19 dpi in *N. lymantriae*. My results indicate that temperature had a significant impact on the onset of spore release. At higher temperatures first spores were detected earlier in feces. Spore release of *N. lymantriae* started at 13 dpi at 24°C, 2 days later at 21°C and 7 days later at 18°C. Campbell et al. (2007) report similar results: Spores of *Nosema fumiferanae* were detected earlier in frass of spruce budworm larvae (incubated with 10^3 and 10^4 spores per larva), when larvae were reared at 24°C and 28°C compared to 20°C. Moreover, the total amount of spores released by *L. dispar* in feces was significantly affected by temperature in my experiments. Spore numbers in feces in the first six days of fifth instar differed between all treatments and was significantly higher at higher temperatures.

The dissection of infected larvae showed that microsporidian development was faster at higher temperatures. Spores were formed earlier in Malpighian tubules and consequently released earlier with feces at higher temperatures. At 24°C hosts died significantly earlier making high numbers of spores formed in the fat body available for release into the environment. Hence, horizontal transmission of *N. lymantriae* through cadavers to other *L. dispar* larvae begins earlier. Data from an experiment under field conditions indicated that it takes more than two weeks of exposure to infected larvae before susceptible larvae will acquire the infection. In the field, larvae need longer to encounter sufficient spores to initiate infection (Hoch et al. 2008). Hence, the faster progression of microsporidiosis at higher temperature has one ecologically important consequence by significantly reducing the duration of the latent period. A shortened latent period due to higher temperature will lead to a faster increase of the amount of inoculum in the environment of *L. dispar* larvae and consequently increase the likelihood of new infections in the population.

4.6 Conclusions

My studies show that temperature affects host development, which is faster at higher temperatures, where larvae gain more consume and weight more. Beyond that *N. lymantriae* infection influences larval development. Infected *L. dispar* larvae grow less and consume less than uninfected larvae. Moreover, the effects of temperature are outweighed by the microsporidian infection. *N. lymantriae* itself is significantly affected by temperature. Within the tested temperature ranges, development of the microsporidium is faster and infected hosts die earlier at higher temperatures. Spores appear earlier in feces at higher temperature. As one consequence the latent period, i.e. the time between acquiring infection and release of first spores, is shorter at higher temperatures. Hence, horizontal transmission of *N. lymantriae* transmission begins earlier.

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