PERIODICUM BIOLOGORUM
VOL. 119, No 4, 285–293, 2017
DOI: 10.18054/pb.v119i4.4998

UDC 57:61
CODEN PDBIAD
ISSN 0031-5362

original research article

Infection, course of disease and effects of *Canningia tomici* in *Tomicus piniperda* and *Tomicus minor* (Coleoptera: Curculionidae)

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Key words: pine shoot beetles, Microsporidia, transmission, survival, Scolytinae

Received February 14, 2017.
Revised November 27, 2017.
Accepted December 27, 2017.

Abstract

Background and purpose. *The pine shoot beetles* Tomicus piniperda *and* Tomicus minor *are secondary tree pests attacking weakened* Pinus sylvestris *and* Pinus nigra. *Outbreaks occasionally occur, causing considerable economic damage. The microsporidian pathogen* Canningia tomici *infects* T. piniperda *as its principal host. Bioassays were used to study the infectivity, vertical transmission, and effects on survival and fecundity of* C. tomici *on the two beetle species.*

Materials and methods. *Field collected beetles from several locations in Austria (Lower Austria, Burgenland and Styria), Finland (Hyytiälä), Poland (Sękocin), the Czech Republic (Stará Boleslav), and Croatia (Korenica) (3410* T. piniperda, *413* T. minor*) were dissected and evaluated for the presence of* C. tomici. *Uninfected beetles to be used for infection experiments were only collected from the Austrian sites.* Canningia tomici *spores were extracted from the infected organs of* T. piniperda *by dissection and tissues were homogenized in a glass tissue grinder. Microsporidian spores were suspended in water and were counted in a hemocytometer. Infection experiments began with* T. piniperda *and* T. minor *as soon as parental beetles were available in the field in spring, or filial beetles emerged from infested log sections in the laboratory. To test the effect of maturation feeding on infection success, filial beetles of both species were either inoculated immediately after emergence from a log section, or were allowed to feed on* P. sylvestris *twigs for several days before inoculation. Filial beetles were held in Petri dishes containing* P. sylvestris *twigs at 8 °C and long-day conditions (16L:8D) during the maturation feeding period. Afterwards, they were removed from the twigs, starved for 24 hours, and then inoculated with a 1-µl spore suspension or water. All beetles were checked daily until death, dissected and inspected for the presence of* C. tomici *spores. All data were analyzed with the software program R. Frequency data sets were compared using Chi-square analysis. Multiple comparisons were controlled for Type I errors using the Bonferroni method. The datasets of multiple dependent scale variables were analysed using the LM multivariate procedure, testing the effects of the following factors: year, temperature, inoculation, successful infection, maturation feeding, and sex.*

Results. *The overall prevalence of* C. tomici *in* T. piniperda *was 1.9%, with significantly more female* T. piniperda *infected. No infections were observed in* T. minor. *After feeding spore suspensions to parental and filial* T. piniperda *and* T. minor, *between 0% and 67% of the beetles were successfully infected, regardless of the incubation temperature or sex of the beetles. Survival time was significantly influenced by the incubation temperature and successful infection; in filial beetles the maturation feeding*

period was also an important factor. A lower incubation temperature and successful infection resulted in longer survival of individuals of both species.

Conclusion. *We conclude that when* C. tomici *infects the reproductive organs of its hosts, the lifespan of the host is extended, leading to increased reproduction, transmission, and survival of the pathogen in host populations.*

INTRODUCTION

In northern and central Europe, the pine shoot beetles *Tomicus piniperda* (L.) and *Tomicus minor* (Hartig) are tree pests that attack weakened *Pinus sylvestris* (L.) and *Pinus nigra* (Arnold) as their principal host species *(1, 2)*. *T. piniperda* occurs throughout Europe, from the northernmost regions of Norway, Finland, and Sweden, to the south of Central Europe. It has spread to China *(3)* and was accidentally introduced into the USA *(4)* and Canada *(5, 6)*. The related species *T. minor* occurs sympatrically with *T. piniperda*, but uses different stem regions on pine trees. It is widespread throughout Europe, from Scandinavia to the Mediterranean region *(2)*. Depending on the ambient temperature, overwintering adults of both species initiate flight activity in spring, with *T. minor* flying later than *T. piniperda*. Following mating, females tunnel mother galleries into the phloem and lay one clutch of eggs. Re-emergence of adult beetles occurs when breeding and oviposition is completed. Subsequently, they bore into shoots of mature pine trees for regeneration feeding, followed by the initiation of a sister brood in uninfested regions of the same tree or in uninfested logs. Both species may infest the same host tree; *T. piniperda* prefers the lower parts of trees with thick bark for breeding sites, while *T. minor* prefers trunk with thin bark. Both species bore/tunnel into one-year-old pine shoots for maturation feeding *(7, 8)*. Shoot feeding by young callow adults and older adult beetles causes the primary injuries to pine trees, resulting in twig death and weakening of the infested trees *(9, 10)*. However, both beetle species are known as secondary pests in pine stands during the endemic phase. Nonetheless outbreaks occur occasionally, during which the beetles are able to attack vigorous trees *(11)*. This causes considerable economic damage, particularly by *T. minor*. In addition to infesting living trees, both beetle species infest wind thrown, felled, and fire-damaged trees, as well as fresh logs *(7, 12-14)*. Moreover, these phloeophagous beetles introduce blue stain fungi (e.g. *Leptographium wingfieldii*, *Ophiostoma minus*), which interfere with water conduction, accelerate tree death, *(15-17)* and reduce the commercial value of timber when it develops blue stain.

Biological control has never been seriously attempted with bark beetles *(18, 19)* and should be reconsidered in light of the results of a study conducted by Kohlmayr et al. *(20)*, which reports the presence of the microsporidium *Canningia tomici* in cells of the midgut epithelium, adipose tissue, and ovariols of *T. piniperda*. Little has been reported about the pathogen-host interaction, effects of infection in *T. piniperda*, or the epidemiological consequences in populations. Additionally, because *T. piniperda* and *T. minor* can occur coincidentally in pine logs, transmission of pathogens from one species to the other one is theoretically possible.

The aim of this study was to investigate the effects of the microsporidium *C. tomici* on its host, *T. piniperda*, and on the congeneric species *T. minor*. Our research focused on the infectivity of *C. tomici* to both beetle species, as well as the effects of infection on the survival time of *T. piniperda* and *T. minor* parental and offspring beetles. We considered factors such as temperature and the influence of *C. tomici* infection on the fecundity of adult beetles and tested for vertical transmission of *C. tomici*.

MATERIAL AND METHODS

Host insects and pathogens

Adult beetles were collected in spring when *T. piniperda* (March to April) and *T. minor* (April to May) initiated swarming. Sampling was conducted over the entire 3-year period (2002–2004) at sampling sites with known populations of healthy, uninfected parental and filial beetles, and sites where *C. tomici*-infected beetles were known to occur and fresh spores could be obtained for experiments *(20)*. *T. piniperda* and *T. minor* were collected from several locations in Austria (Lower Austria, Burgenland and Styria), Finland (Hyytiälä), Poland (Sękocin), the Czech Republic (Stará Boleslav), and Croatia (Korenica). Uninfected beetles for infection experiments were only collected from the Austrian sites; infected *T. piniperda* were only found in samples from Finland, Poland, and the Czech Republic.

Parental beetles of both species were collected in the field by hand (either cut out of the bark from trap trees or beetle-infested log sections) and brought to the laboratory. These beetles were stored in a refrigerator (at 4°C) until inspection or in an incubator at 8°C and long-day rearing conditions (16L:8D) until they were to be used in experiments. Beetle-infested log sections were incubated in the insectary at the University of Natural Resources and Life Sciences (BOKU) in breeding cages at 21°C (± 1.5°C) and at long-day conditions (16L:8D). Emerging parental beetles and later filial beetles (recognized by their light brown color) were removed from breeding cages daily. A subsample thereof ($n \geq 50$; tables 2 and 3) was dissected to ensure that they were uninfected and could be used for infection experiments.

Canningia tomici spores were extracted from the infected organs of *T. piniperda* by dissection and homogenization of tissues (midgut epithelium, the gut muscules, Malpighian tubules, connective tissues, adipose tissues

Table 1. Canningia tomici *infection in field collected* Tomicus piniperda.

Year	Country	♂	♀	Total	$♂_{inf}$	$♀_{inf}$	Total inf
2002	Austria	216	222	438	0	0	0
	Czech Republic	100	125	225	0	1	1
	Finland	62	137	199	4	14	18
	Poland	75	77	152	0	2	2
2003	Austria	197	251	448	0	0	0
	Czech Republic	142	156	298	0	0	0
	Finland	26	190	216	1	24	25
	Poland	128	220	348	3	2	5
	Croatia	56	46	102	0	0	0
2004	Austria	119	140	259	1	0	1
	Czech Republic	152	191	343	1	1	2
	Finland	28	62	90	5	3	8
	Poland	94	127	221	2	1	3
	Croatia	26	45	71	0	1	1
	Total	1421	1989	3410	17	49	66

and the gonads) in distilled water, then purified by density gradient centrifugation *(21)*. The spores were suspended in water and counted in a Neubauer improved hemocytometer.

Inoculation of host insects and pathogen diagnosis

Inoculation of parental and filial *T. piniperda* and *T. minor* was conducted after the beetles had undergone a 24-hour starvation period by force feeding a 1-µl *C. tomici* spore suspension using a standard vaccinating eyelet. The spore concentration fed to the beetles ranged between 30 spores/µl and $1.28x10^4$ spores/µl, depending on the intensity of infection in the collected beetles (Table 1). The beetles in the control group were fed 1 µl water instead of the spore suspension. Beetles that fed on the spore suspension within two minutes were transferred into Petri dishes containing bark chips. The bark chips were changed every other day. The number of beetles per Petri dish varied from 1 to 50, depending on the number of beetles currently available. Following inoculation, and unless otherwise indicated, all beetles were stored under long-day conditions in incubators, either at 16°C (± 1°C) or 21°C (± 1°C), until death. Inoculated beetles were stored separately from control beetles. Both beetle species and parental and filial beetles were tested separately.

At the termination of each experiment, inoculated and control beetles were examined for the presence of *C. tomici* spores. Tissues were excised with fine scissors and fresh smears on microscope slides were inspected under bright field and phase contrast microscopy at 400x and 1000x magnification *(22)*.

Effects of *Canningia tomici* on the survival time of parental and filial *Tomicus piniperda* and parental and filial *Tomicus minor*

Infection experiments began with *T. piniperda* and *T. minor* as soon as parental beetles were available in the field in spring, or filial beetles emerged from infested log sections in the laboratory. Depending on the number of beetles that were collected or had emerged, varying numbers of parental or filial *T. piniperda* or *T. minor* were inoculated (Table 4 and 7).

To test the effect of maturation feeding on infection success, filial beetles of both species were either inocu-

Table 2. Canningia tomici *infection in field collected* Tomicus minor.

Year	Country	♂	♀	Total	$♂_{inf}$	$♀_{inf}$	Total inf
2002	Austria	135	157	292	0	0	0
	Czech Republic	6	8	14	0	0	0
2003	Austria	26	28	54	0	0	0
2004	Austria	14	39	53	0	0	0
	Total	181	232	413	0	0	0

Table 3. Canningia tomici *infection in* Tomicus minor *parental beetles. Number and percentages of inoculated and successfully infected beetles and the number of control beetles, reared either at 16°C or at 21°C, are displayed below.*

Temperature	16°C			21°C		
Sex	Female	Males	unknown	Female	Male	unknown
$N_{inoculated}$	111	41	6	110	66	30
$N_{infected}$	55	20	4	36	17	9
% infected	49.55	48.78	66.67	32.73	25.76	30.00
C	41	16	4	61	50	13
$C_{infected}$	1	0	0	0	0	0

Table 4. *Factors explaining the mean survival time of male and female* Tomicus minor *parental beetles. A linear model of survival time was calculated (ANOVA, F = 15.46, r^2 = 0.2839, df = 6 & 549, p < 2.2*10-16). Asterisks show the significance level: p < 0.05 *, p < 0.01 **, p < 0.001 ****

Factor	Mean Sq	F	p	
Year	5377	9.787	0.0019	**
Temperature	77051	140.241	< 2.2*10-16	***
Inoculation	144	0.262	0.6092	
Successful infection	23219	42.260	1.842*10-8	***
Sex	231	0.4207	0.5173	
Isolate / Number of spores	2135	3.887	4.13*10-5	***

lated immediately after emergence from a log section, or were allowed to feed on *P. sylvestris* twigs for several days prior to inoculation. Filial beetles were held in Petri dishes containing *P. sylvestris* twigs at 8°C and long-day conditions (16L:8D) during the maturation feeding period. Afterwards, they were removed from the twigs, starved for 24 hours, and then inoculated with a 1-µl spore suspension or water. All beetles (infected or in the control group) were held in Petri dishes as described above and checked daily until death. Afterwards, they were dissected and inspected for the presence of *C. tomici* spores.

Vertical transmission of *Canningia tomici* and its effects on the fecundity of *Tomicus piniperda* and *Tomicus minor*

In order to examine the consequences of a *C. tomici* infection on the fecundity of *T. piniperda* and *T. minor*, parental females and male beetles were inoculated with a 1-µl spore suspension, using the same mode of inoculation as described above. Individual pairs of *T. piniperda* or *T. minor* were placed onto fresh log sections of *P. sylvestris* respectively and caged using capsule pits *(23)*. Caging breeding pairs on fresh log sections forced them to initiate their galleries at a selected point in the bark. This proce-

Table 5. *Survival time (mean ± SD) of* Tomicus minor *parental beetles in the control group, of beetles successfully infected with* Canningia tomici *and of beetles inoculated but not successfully infected with* C. tomici. *All beetles were incubated either at 16°C or at 21°C.*

Temperature	Sex	Control	Sucessfully infected	Inoculated
16°C	♂	41.8 ± 38.7	54.6 ± 21.8	62.0 ± 42.9
21°C	♂	23.8 ± 19.9	30.4 ± 10.5	15.4 ± 11.8
16°C	♀	49.7 ± 41.1	49.2 ± 15.8	30.0 ± 34.7
21°C	♀	27.6 ± 18.2	32.9 ± 12.4	10.6 ± 10.2

Table 6. Canningia tomici *infection in* Tomicus minor *filial beetles. The number and percentages of inoculated and successfully infected beetles and the number of control beetles, reared either at 16°C or at 21°C, are displayed below.*

Temperature	16°C					21°C				
Sex	Female		Males		unknown	Female		Male		unknown
mat. feeding	Yes	No	Yes	No	-	Yes	No	Yes	No	–
$N_{inoculated}$	63	16	37	18	43	77	2	35	3	57
$N_{infected}$	22	3	11	2	16	11	0	10	0	8
% infected	34.92	18.75	29.73	11.11	37.21	13.58	0.00	8.57	0.00	14.03
C	25		18		7	27		20		15
$C_{infected}$	0		0		0	0		0		0

Table 7. *Factors explaining the mean survival time of male and female* T. minor *filial beetles. A linear model of survival time was calculated (ANOVA, F = 12.61, r² = 0.345, df = 20 & 439, p < 2.2*10-16). Asterisks show the significance level: p < 0.05 *, p < 0.01 **, p < 0.001 ****

Factor	Mean Sq	F	p	
Year	687	3.252	0.072	
Temperature	11970	56.662	2.964*10-13	***
Isolate/Number of spores	24995	7.888	8.318*10-16	***
Maturation feeding	1799	8.515	0.0037	**
Successful infection	13393	63.398	1.460*10-14	***
Sex	443	2.097	0.1483	

Table 8. *Survival time (mean ± SD) of* Tomicus minor *filial beetles of the control group, of beetles successfully infected with* Canningia tomici *and of beetles inoculated but not successfully infected with* C. tomici. *All beetles were incubated either at 16°C or at 21°C.*

Temperature	Sex	Control	Sucessfully infected	Inoculated
16°C	♂	17.8 ± 2.9	31.8 ± 10.2	21.2 ± 18.6
21°C	♂	5.3 ± 4.6	28.0 ± 12.6	10.3 ± 12.5
16°C	♀	23.4 ± 22.7	31.3 ± 10.7	25.1 ± 24.4
21°C	♀	14.9 ± 11.1	28.1 ± 11.3	15.6 ± 15.1

dure made it possible to follow the galleries of infected parental beetles and their offspring. In order to prevent dehydration of the *Pinus* log sections, logs were sprayed with tap water once a week. Log sections were incubated in the laboratory at 21°C (± 1.5°C) and galleries were opened after 7–8 weeks. Parental beetles were recollected from the capsule pits after death or re-emergence and examined for microsporidian infection. All eggs, larvae, pupae, and callow adults were removed, counted, dissected, and inspected under bright field and phase contrast microscopy.

Analysis of data

All data were analyzed with the software program R *(24)*. Frequency data sets were compared using Chi-square analysis. Multiple comparisons were controlled for Type I errors using the Bonferroni method. The datasets of multiple dependent scale variables were analysed using the LM multivariate procedure, testing the effects of the following factors: year, temperature, inoculation, successful infection, maturation feeding, and sex.

RESULTS

Prevalence of Canningia tomici in Tomicus piniperda and Tomicus minor

Different numbers of female (45 to 251) and male (26 to 216) *T. piniperda* were collected from field sites in four (2002) or five (2003 and 2004) countries. In total, 3410 beetles were collected and dissected (Table 1). A total of 66 *T. piniperda* were found to be infected with *C. tomici*. Significantly more females (2.5%) were infected with *C. tomici* than males (1.2%) (Chi-square test, $\chi^2 = 97.6$, df = 1, $p < 2.2*10^{-6}$). Additionally, a significantly higher proportion of *T. piniperda* from sites in Finland and Poland were infected with *C. tomici* (Chi-square test, $\chi^2 = 212.3$, df = 4, $p < 2.2*10^{-6}$), as compared to the other countries.

A total of 413 *T. minor* beetles were field-collected from two countries (2002: Austria and Czech Republic) and one country (Austria: 2003 and 2004) (Table 2). None of the *T. minor* individuals were found to be infected with *C. tomici*. The percentage of female beetles was 56.2%. During the second and third year of the study period, fewer beetles were dissected and checked for a *C. tomici* infection.

Infectivity and effects of Canningia tomici on Tomicus piniperda

Parental generation

Inoculation of 132 *T. piniperda* parental beetles (67 females, 54 males and 11 beetles of unknown sex) and incubation at 16 °C induced a *C. tomici* infection in one female (0.76 %) that survived 113 days post-inoculation (dpi). None of the male beetles were successfully infected with *C. tomici*. When 165 *T. piniperda* parental beetles (75 females, 73 males and 17 beetles of unknown sex) were inoculated with *C. tomici* and incubated at 21 °C, slightly more beetles (3.03 %, n=5) were successfully infected, as compared to beetles incubated at 16°C (n=1). One infected female and four infected males died 10 dpi and 15 dpi, respectively.

None of the control beetles (16 °C: 39 females, 36 males and 7 beetles of unknown sex; 21 °C: 48 females, 40 males and 4 beetles of unknown sex) were infected with *C. tomici*.

T. piniperda filial generation

Compared to the parental generation, a higher proportion (10.82%) of inoculated *T. piniperda* filial beetles incubated at 16°C were successfully infected with *C. tomici*. There were 194 beetles in total (58 females with maturation feeding and 22 females without; 57 males with maturation feeding and 17 males without; 11 beetles of unknown sex with maturation feeding and 40 beetles without). Seven females (five with maturation feeding and two without) and eight males (six with maturation feeding and two without) were successfully infected with *C. tomici*. Of 307 inoculated *T. piniperda* filial beetles (68 females with maturation feeding and 43 without; 66 males with maturation feeding and 43 without; 83 beetles of unknown sex) incubated at 21°C, 6.19 % were successfully infected with *C. tomici*. Five females and five males with maturation feeding were successfully infected. No female and three males without maturation feeding were infected. Neither the sex nor the incubation temperature significantly influenced the proportion of successfully infected beetles; however, a slightly higher percentage of males were infected with *C. tomici* than females (Chi-square test, $\tau = 4$, df = 1, $\alpha > 0.05$; 16°C: $\chi^2 = 0.5$, 21°C: $\chi^2 = 0.7$, female: $\chi^2 = 0.1$, male: $\chi^2 = 0.3$).

None of the control beetles (16° C: 53 females, 57 males and 18 beetles of unknown sex, 21° C: 72 females, 77 males and 54 beetles of unknown sex) were infected with *C. tomici*.

Infectivity and effects of Canningia tomici *on* Tomicus minor

Parental generation

Incubating of 158 inoculated *T. minor* parental beetles at 16 °C, resulted in almost 50% infection with *C. tomici* (Table 3). Of 206 inoculated *T. minor* incubated at 21 °C, 30.1% became infected. Neither sex nor the incubation temperature influenced the proportion of *T. minor* parental beetles successfully infected with *C. tomici* (Chi-square test, $\tau = 4$, df = 1, $\alpha > 0.05$, 16°C: $\chi^2 = 0.01$; 21°C: $\chi^2 = 0.952$; female: $\chi^2 = 6.4$; male: $\chi^2 = 5.9$, Table 4).

The mean survival time of filial *T. minor* beetles was significantly influenced by the incubation temperature (ANOVA, $p < 2.2*10^{-16}$, table 5), the isolate and/or spore concentration used (ANOVA, p = 4.13*10-5), and the successful establishment of *C. tomici* in *T. minor* (ANOVA, p = 1.842*10-8). Mean survival time was not influenced by sex of the beetles. Beetles incubated at 16°C lived 18-47 days longer than beetles incubated at 21°C (Table 5). Infected parental *T. minor* beetles lived up to 12 days longer than beetles in the control group.

One female in the control group incubated at 16°C was infected with *C. tomici*.

T. minor filial generation

When 177 *T. minor* filial beetles were incubated at 16°C and 174 inoculated at 21°C, 30.51 % and 16.67%, respectively, were successfully infected with *C. tomici* (Table 6). A lower but not significant proportion of males were successfully infected with *C. tomici* compared to females (Chi-square test, $\tau = 4$, df = 1, $\alpha > 0.05$, 16°C: $\chi^2 = 1.024$, 21°C: $\chi^2 = 2.8$, female: $\chi^2 = 8.2$, male: $\chi^2 = 0.01$). Furthermore, we did not observe any effect of the incubation temperature on the proportion of beetles successfully infected with *C. tomici*.

The mean survival time of filial *T. minor* beetles was significantly influenced by the incubation temperature (ANOVA, p = 2.964*10-13, Table 7), the duration of the maturation feeding (p = 0.0037), successful infection with *C. tomici* (ANOVA, p = 1.460*10-14), the isolate and/or spore concentration used (ANOVA, p = 8.318*10-16) . Mean survival time of beetles was not influenced by sex and the sucessful inoculation with *C. tomici* spores. Beetles incubated at 16°C lived 3-13 days longer than beetles incubated at 21°C (Table 8). Beetles successfully infected with *C. tomici* lived up to 23 days longer than beetles in the control group.

Vertical transmission of Canningia tomici

Tomicus piniperda

In total, 35 pairs of *T. piniperda* parental beetles were inoculated with a spore suspension of *C. tomici* and 15 pairs were inoculated with water. None of beetles inoculated with the spore suspension were infected with *C. tomici*.

Tomicus minor

In total, 60 pairs of *T. minor* parental beetles were inoculated with a spore suspension of *C. tomici* and 37 pairs were inoculated with water. Inoculation with *C. tomici* was successful in 20 *T. minor* pairs. None of the control beetles were infected with *C. tomici*. No infection was found in progeny of the infected parental *T. minor*. Comparing the breeding systems of pairs inoculated with *C. tomici* with systems of the control group, no significant differences were found in the total length of the mother galleries (Wilcoxon rank sum test, W = 324, p = 0.6412), the total number of progeny produced (Wilcoxon rank sum test, W = 328, p = 0.5809), nor the progeny/cm mother gallery (Wilcoxon rank sum test, W = 238, p = 0.2169). The total length of the mother gallery produced by infected pairs was 7.0 ± 4.0 cm, with a length of 2.4 ± 3.2 cm for the left arm and 4.7 ± 4.3 cm for the right arm. The total length of the mother gallery produced by beetle

pairs in the control group was 9.0 ± 8.2 cm; the length of the left arm was 4.3 ± 6.5 cm and the length of the right arm was 4.7 ± 6.3 cm. On average, an infected female laid 27.4 ± 18.4 in each mother gallery; a female in the control group produced an average of 32.2 ± 31.5 in each mother gallery.

DISCUSSION

Pine shoot beetles are reported to be a serious problem in Scandinavian forestry, since they damage infested pine trees by feeding in pine shoots, causing growth loss and crown damage; by introducing bluestain fungi, degrading timber quality; and by killing weakened trees *(25)*. Several microsporidian species, a diverse group of obligate intracellular pathogens infecting arthropods and fish *(26, 27)*, belong to the natural enemy complex of bark beetles *(19)*. In some cases, they are believed to have the potential for biological control in other host-pathogen systems *(28)*.

T. piniperda and *T. minor* are widely distributed species in the Palearctic region *(29)*; their current distribution correlates with the occurrence of its host species of the genus *Pinus (30, 31)*. As a cold-tolerant species *(31)*, *T. piniperda* prefers mean temperatures of approximately 17°C during the summer and 0°C during the winter months; it is absent in regions with higher mean temperatures during the warmest or coldest seasons of the year *(30)*. Furthermore, *T. piniperda* and *T. minor* are, in contrast to *Tomicus destruens*, unable to develop in warm and dry climates *(32)*. This clear preference might also be reflected in our data. The mean survival time of both beetle generations, i.e. parental and filial beetles, as well as both sexes, was significantly influenced by incubation temperature. Parental *T. minor* incubated at 16°C lived up to 4 times longer than beetles incubated at 21°C. The survival time of filial *T. minor* was prolonged between 3 and 8 days when incubated at the lower temperature. Additionally, this might be supported by the higher prevalence of beetles both infected with *C. tomici* and collected in countries north of Austria.

Another interpretation of these data might conclude that beetles incubated at lower temperatures died later due to their reduced metabolism and the better food quality of the bark chips, which did not decay as quickly.

The mean survival time of *Tomicus minor* was also influenced by the successful establishment of a *C. tomici* infection. The results of our data indicate that a successful infection of *T. minor* did not negatively influence the mean survival time of the tested beetles; surprisingly, it resulted in a prolonged survival time of the infected *T. minor*. Such behavior reported also from other microsporidia infected insect species e.g. gypsy moth, *Lymantria dispar* L. *(33)*. To our knowledge, most of the studies on the effects of microsporidia in insects report a reduced survival time and higher mortality rate in infected insects. Only few studies report no negative effects of a microsporidian parasite on its native host; although the infection of two lady beetles resulted in prolonged larval development and no higher mortality was recorded *(34–36)*. We suggest that the prolonged survival time of infected *T. minor* might increase the possibility of infecting organs other than the gut, such as the ovaries that make vertical transmission of the microsporidian pathogen possible.

The life cycle of *T. minor* or *T. piniperda* under the bark of infested trees suggests that vertical transmission might be the main transmission pathway of *C. tomici*. If horizontal transmission does occur, it would be expected to occur during the period of maturation feeding of the beetles and/or the following mating and tunneling of the egg galleries in the breeding chambers. Our results further indicate that the potential for horizontal transmission before maturation feeding period is low; we were never able to infect 100% of the test beetles and successful infection of filial beetles increased with a maturation feeding period. Though the infection of gonads and the vertical transmission of *C. tomici* has been reported for *T. piniperda (20)*, we were not able to provide any further evidence for the vertical transmission of *C. tomici* in both tested beetle species. Our results show that the successful infection of *T. piniperda* and *T. minor* offspring did not depend on the rearing temperature or sex of the beetles but was influenced by the duration of maturation feeding. There are probably several reasons for our low success in orally infecting *T. piniperda* and *T. minor*. First, we were unable to store *C. tomici* spores in liquid nitrogen, which runs counter to reported storage conditions for several microsporidian species (ref), and *C. tomici* spores were only viable for few weeks when stored in a refrigerator at 7°C (unpublished data). Additionally, the process of inoculation might be suboptimal, as *Tomicus* spp. feed in the phloem of trees without drinking liquid water, and were force-fed 1µl of a liquid spore suspension. Additionally, physiological conditions inside the gut may change during the lifecycle of the beetles. Parental beetles and callow adult beetles that had just emerged from the pupal chamber were less susceptible to infection with *C. tomici* than filial beetles with at least two weeks of maturation feeding.

We recorded a higher prevalence of infected *T. piniperda* collected in Finland and Poland. This result may reflect the relatively higher abundance of both *Pinus* ssp. and these two pine shoot beetle species in these countries *(11)*. Permanent food sources may be conducive to a permanent bark beetle population and further enhance endemism of pathogens in its host population. Also, our results indicate a longer survival time of both beetle species at lower temperatures and the successful infection with *C. tomici* also resulted in a prolonged survival time of both species. We conclude that the lower mean temperatures in more northern habitats may favor the devel-

opment and successful transmission of *C. tomici* in *T. piniperda* beetles.

One individual *T. minor* beetle in the control group was infected with *C. tomici*. We do not have a conclusive explanation for this. It is unclear as to whether or not this infection occurred before the beetle was collected in the field, or if the infection was due to contamination or mistakenly assigning the beetle to the control group in the laboratory. We would prefer to exclude the possibility of a natural infection for two reasons: 1) no microsporidian species have been reported to infect *T. minor* and 2) we only used beetles for all our experiments collected at field sites without any record of an infection with *C. tomici* in *T. piniperda* and *T. minor*.

C. tomici was originally isolated from *T. piniperda* but was also found to be infective for *T. minor* in the laboratory. Infected *T. minor* were found to be infected to a higher degree than *T. piniperda*. Malformed spores were not observed. These results indicate that *C. tomici* is also a pathogen for *T. minor*, although *C. tomici* has not been reported in field-collected *T. minor* beetles, and we were also unable to show that *C. tomici* is vertically transmitted to its progeny. A broader ecological host range of *C. tomici* that includes both *T. piniperda* and *T. minor* would imply that horizontal transmission of *C. tomici* might be difficult to achieve for several reasons. In principle, both species occur in the same habitat and are also observed to infest the same host trees; therefore, at least food-related gut conditions may be nearly identical in both species. Nevertheless, the species exhibit some degree of niche separation, thereby decreasing the potential of horizontal transmission of *C. tomici* between species. *T. piniperda* usually infests the lower trunk of host trees with thicker bark, and swarms and infests weakened trees earlier. *T. minor* prefers to infest the upper parts of tree trunks with thinner bark, initiates the swarming period later, and infests weakened trees often already colonized by *T. piniperda*. This separation in space and time of the two beetle species limits the opportunity for horizontal transmission of *C. tomici*. Although we were unable to provide evidence of pathogen transfer from parental beetles to offspring, results of an earlier study suggest that *C. tomici* is transmitted vertically in *T. piniperda (20).*

Acknowledgements

We present this paper in memory of Prof. Dr. Jaroslav Weiser (Czech Republic).

We thank the Austrian Science Fund (Project: P 14994 – B06), the OEAD Ernst Mach scholarship, Prof. Dr. Kari Heliövaara and Dr. Päivi Lyytikäinen-Saarenmaa (Institute of Applied Zoology, University of Helsinki, Finland), Dr. Jacek Hilszczański (IBL Forest Research Institute, Sękocin Stary, Poland), Dr Miloš Knížek (Forestry and Game Management Research Institute, Jílovitště, Czech Republic), foresters and forest owners in Austria (Ing. Wolfgang Meissl, Ing. Peter Dorner, Maria and Karl Wosika), and Roman Wanjek and Andrea Stradner from BOKU University.

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