Influence of *Helicosporidium* spp. (Chlorophyta: Trebouxiophyceae) Infection on the Development and Survival of Three Noctuid Species

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Environ. Entomol. 33(1): 54–61 (2004)

ABSTRACT A new *Helicosporidium* spp. isolate recently purified from an aquatic weevil, *Cyrtobagus salviniae* Calder & Sands, was capable of infecting and reproducing in three heterologous noctuid hosts. Regardless of host species, oral treatment of *Heliocoverpa zea* (Boddie), *Spodoptera exigua* (Hübner), or *Trichoplusia ni* (Hübner) early instars with cyst preparations of *Helicosporidium* spp. resulted in ≈50% infection of the challenged larvae. The sex ratio did not differ between infected and control groups, suggesting the existence of a natural, nonsex-related resistance to the disease. Injection of *Helicosporidium* spp. into the hemocoels of late instars resulted in virtually 100% infection, indicating that resistance is related to the ingestion of the pathogen and therefore affiliated with midgut-mediated barriers. The pathogen's development was not interrupted by metamorphosis; likewise, the infection did not necessarily interrupt the insects' development. When treated as early instars, 50–90% of the infected larvae formed pupae, of which 20–30% emerged as adults. However, a high proportion of the infected adults (62–86%) had malformed wings, and their longevity was reduced compared with that of healthy adults. Infected *S. exigua* adults that seemed to be morphologically healthy were able to mate and produce viable offspring. The disease was detected in five of 12 groups of progeny produced by infected adults. However, the relative infection rate in the filial generation was low (2–5%). To our knowledge, this is the first evidence for a vertical transmission of helicosporidial infection.

KEY WORDS entomopathogenic algae, *Helicosporidium*, *Heliocoverpa zea*, *Spodoptera exigua*, *Trichoplusia ni*

THE HELICOSPORIDIA ARE A UNIQUE, unclassified group of pathogens isolated from a small and diverse group of invertebrates. These organisms were at one time considered to be either protozoa or fungi but have been unclassified since 1931 with only one named species, *Helicosporidium parasiticum* Keilin. Significantly, Helicosporidia have been reported to be pathogenic to various invertebrate hosts, including insects, mites, cladocerans, and trematodes (Sayre and Clark 1978, Purrini 1984, Avery and Undeen 1987a, Pekkarinen 1993). Recently, we have characterized two genetically distinct *Helicosporidium* spp. isolates from the black fly *Simulium jonesi* Stone & Snoddy (Diptera: Simuliidae) and from the weevil *Cyrtobagus salviniae* Calder & Sands (Coleoptera: Curculionidae), found in Gainesville, FL (Boucias et al. 2001; Tartar et al. 2002, 2003). Microscopic examination has shown that these organisms possess a cell phenotype not found in any known protist. Microscopic observation of the vegetative growth of *Helicosporidium* spp. under in vivo and in vitro conditions led Boucias et al. (2001) to associate this protist with green algae, particularly the unicellular, nonphotosynthetic, and pathogenic algae belonging to the genus *Prototheca.* Phylogenetic analyses of several *Helicosporidium* spp. genes (rDNA, actin, β-tubulin, including 18S rDNA) all identified this organism as a member of the green algae clade (Chlorophyta). Moreover, nuclear phylogeny focused on Chlorophyta depicted *Helicosporidium* spp. as a close relative of *Prototheca wickerhamii* and *Prototheca zopfii* within the class Trebouxiophyceae (Tartar et al. 2002, 2003).

In vivo studies have shown that the Helicosporidia are transmitted per os to both dipteran and lepidopteran hosts (Kellen and Lindegren 1974, Fukuda et al. 1976, Boucias et al. 2001). Light microscopic examination of midgut preparations demonstrated that the cyst stage binds to the peritrophic matrix and dehisces. Upon activation, the pellicles rupture and release filamentous cells that penetrate the peritrophic matrix. Adhesion of intact cysts to the peritrophic matrix suggests that mechanical pressure expressed during dehiscence of the compressed cyst may mediate ingress though the midgut barriers (peritrophic matrix and/or the microvillar membranes). Potentially, the filamentous cells penetrate the midgut columnar epithelium and differentiate into an intermediate stage that gains ingress through this tissue and enters the hemocoel. Within the hemocoel, vegetative cells replicate.

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0046-225X/04/0054–0061$04.00/0

Within 10–14 d, infected insects contain massive numbers of mature cysts in the cream-colored hemolymph.

Importantly, both the black fly and the weevil isolates have been shown to infect and to replicate in insect hosts that can be readily maintained under laboratory conditions. Currently, the original hosts from which these algae were isolated are not available for detailed pathogen–host laboratory studies. In this study, we examined the impact of infection with the *Helicosporidium* spp. weevil isolate on the survival and development (selected fitness parameters) of different noctuid species. The infection data gathered with per os challenge assays were compared with bioassays involving the hemocoelic injection of helicosporidial cells. Then, a series of mating experiments examined 1) the ability of this transstadial disease to affect the reproductive fitness of both female and male adults, and 2) whether this pathogenic alga can be vertically transmitted from infected *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) adults to filial generations.

Materials and Methods

Preparation of *Helicosporidium*. The *Helicosporidium* spp. used in this study originated from an infected aquatic weevil, *C. salviniae*, collected in Australia and maintained under quarantine in Gainesville, FL. Cysts were propagated in orally infected *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larvae and purified from homogenates of late instars and pupae by high-speed centrifugation (16,000 × *g*, 30 min) on a 20–80% linear gradient of Ludox HS40 (PerkinElmer Life Sciences, Boston, MA). The band containing the cysts was collected, diluted in sterile water, and subjected to several cycles of low-speed centrifugation (4,000 × *g*, 10 min) to remove residual gradient material. After suspension in a cryoprotectant (10% dimethyl sulfoxide plus 10% glycerol), cysts were counted using a hemacytometer and aliquots containing 1×10^8 cysts/ml were stored at −70°C. Before each experiment, cyst preparations were thawed quickly, washed twice in sterile water (2,000 × *g*, 10 min), and resuspended either in 0.1% aqueous Nile Blue A or in sterile water.

Insects and Infection Experiments. Larvae of *H. zea* and eggs of *S. exigua* and *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae) were obtained from established colonies at the Center for Medical, Agricultural, and Veterinary Entomology, USDA–ARS, Gainesville, FL. Hatching neonates and larvae were provided with artificial insect diet and maintained at constant conditions (27 ± 1°C, 70 ± 5% RH, photoperiod of 12:12 [L:D] h). Third instars with an average weight of 5.8 ± 3.8 mg ($n = 545$) were used for oral treatments; injection treatments were conducted using fifth and sixth instars (153.4 ± 37.2 mg; $n = 222$).

Third instars were placed in individual wells of 24-well microtiter plates, starved for 16 h, and thereafter presented 2-μl droplets of a Nile Blue cyst suspension at a known concentration: 0 (for control), 1×10^3, 1×10^4, 5×10^4, 1×10^5, or 2×10^5 cysts per larva. After 4–7 h, larvae that consumed the droplets were weighed and transferred to single 1-oz cups with artificial insect diet. Treated larvae were incubated at constant conditions (27 ± 1°C, 70 ± 5% RH, photoperiod of 12:12 [L:D] h) and checked for mortality daily. To determine infection rates, control and test larvae were bled by needle puncture within 3–8 d after oral treatment (larval weight ≥100 mg), and the presence of *Helicosporidium* spp. in the hemolymph of test larvae was recorded using differential interference contrast (DIC) optics (Leica DM R, Leica Microsystems Wetzlar GmbH, Wetzlar, Germany). Insects diagnosed as not infected were checked again 1–3 d after pupation.

For injection treatments, 5 μl of aqueous cyst suspension was injected into the hemocoels of late fifth (*S. exigua* and *T. ni*) or sixth instars (*H. zea*) at a concentration of 2×10^5 cysts per larva. Control insects were injected with sterile water. Larvae were weighed, transferred to single 1-oz cups containing artificial diet, and incubated at constant conditions (27 ± 1°C, 70 ± 5% RH, photoperiod of 12:12 [L:D] h). Five to eight days after injection, the presence of cysts in the hemolymph was recorded as described for the oral infection experiments. By this time, all larvae except for three had pupated.

In all experiments, pupae were weighed and sexed, and insect development and mortality were followed throughout adult emergence. Three to five replicates with differing numbers of larvae ($n = 7$–28) were conducted for each control and treatment.

Mating Experiments. To examine the effect of *Helicosporidium* spp. infection on adult fecundity and to elucidate the ability of the pathogen to be vertically transmitted to F_1 progeny, a series of mating experiments were conducted with infected and control *S. exigua* adults that were obtained from injection treatments. One female and one male were transferred into a 16-oz paper cup. A piece of tissue towel along the edge served as egg-laying substrate and a Kimwipe (Kimberly-Clark Corporation, Roswell, GA) soaked in a 10% sucrose solution in a small petri dish (60 mm in diameter) provided nutrient to the adults. Each cup was held in a separate plastic box to prevent pheromone interference between different mating trials. Insects were incubated at constant conditions (27 ± 1°C, 70 ± 5% RH, photoperiod of 12:12 [L:D] h). When eggs were laid, pieces of artificial diet were added to the cup, and humidity was increased by covering the bottom of the plastic box with wet paper towels. The numbers of deposited eggs were counted daily using a stereomicroscope. Dead adults were removed from the cup, and their infection status was confirmed by examination of tissue smears for the presence of Helicosporidia by using DIC optics. Two to five days after egg hatch, 50 second instars were transferred to a clean paper cup with artificial diet and reared at constant conditions (27 ± 1°C, 70 ± 5% RH, photoperiod of 12:12 [L:D] h). During the course of larval development, smears of 10 neonate larvae and hemolymph samples of at least 20 fourth or fifth instars were examined for the presence of Helicosporidia.

Table 1. Percentage of infection (mean ± SD) in three noctuid species after treatment with weevil *Helicosporidium* sp. cyst preparations

Treatment	Concentration (cysts per larva)	*H. zea*	*S. exigua*	*T. ni*
Oral challenge	0 (control)	0 ± 0a (5)	0 ± 0a (3)	0 ± 0a (5)
	1×10^3	0 ± 0a (3)	Not tested	Not tested
	1×10^4	50 ± 21b (3)	Not tested	Not tested
	5×10^4	60 ± 31b (3)	0 ± 0a (3)	Not tested
	1×10^5	57 ± 16b (3)	Not tested	26 ± 18b (3)
	2×10^5	58 ± 17b (3)	39 ± 37b (3)	55 ± 24c (3)
Injection	0 (control)	0 ± 0a (3)	0 ± 0a (3)	0 ± 0a (3)
	2×10^5	100 ± 0c (3)	95 ± 4c (3)	100 ± 0d (3)

Means followed by different letters within a column are significantly different ($P \leq 0.05$; SAS genmod procedure and lsmeans statement). Means are based on three to five replicate bioassays; the numbers of replicates are given in parentheses. Seven to 28 individuals were tested per replicate.

Upon pupation, the infection status of at least 20 pupae was determined as described for the infection bioassays.

Five to nine replicates were conducted for each of the following four mating combinations: 1) control females mated with control males, 2) infected females mated with control males, 3) control females mated with infected males, and 4) infected females mated with infected males.

Statistical Analyses. All statistical analyses were conducted using The SAS System for Windows (SAS Institute 1999). For each lepidopteran species, comparisons of infection rates, mortality rates, sex ratios, and adult malformation were done by logistic regression with the genmod procedure (Neter et al. 1990). When significant differences were observed, means were separated using the least-square means (lsmeans) statement of SAS. Pupal weights, developmental times, adult longevity, and egg numbers were subjected to analysis of variance (ANOVA) by using the procedure for mixed linear models (Köhler et al. 1992); means were separated using the lsmeans statement. Throughout the article, mean values are accompanied by the standard deviation. Data sets obtained from infection experiments were pooled and presented according to treatment (regardless of applied concentration) and infection status of the insects. This resulted in the comparison of three groups: 1) control, 2) challenged and uninfected, and 3) challenged and infected. To calculate and to analyze the total numbers of eggs deposited per female, only mating trials that produced viable offspring were included.

Results

Each of the three species tested were infected by per os challenge with the cyst preparations. The infection rates varied considerably among replicates, as indicated by the high standard deviations in Table 1. For *H. zea*, the threshold concentration initiating infection per os was between 10^3 and 10^4 cysts per larva. Significantly, no dose response was observed in *H. zea:* regardless of a 20-fold increase in the applied concentration, oral treatment infected an average 50–60% ($P > 0.05$, df = 1) of the treated larvae. There was a higher threshold for infectivity in both *S. exigua* (between 5×10^4 and 2×10^5 cysts per larva) and *T. ni* ($<10^5$ cysts per larva). In *T. ni*, the infection rate at 10^5 cysts per larva was significantly lower than at 2×10^5 cysts per larva ($P = 0.0011$, df = 1, $\chi^2 = 10.67$). Unlike these results, hemocoelic injection of this pathogen into older larvae resulted in a consistently high (95–100%) infection rate (Table 1). Within 5 d after injection, numerous vegetative cells, empty pellicles, and some cysts were detected in the hemolymph of these insects. After 5 d, cysts circulated freely in the hemolymph, and insects could be diagnosed reliably as infected. Two injected *S. exigua* were diagnosed as uninfected, which can be explained as follows: one larva died 4 d after treatment, which was too early to show a manifest infection of the hemolymph; one pupa succumbed to a secondary bacterial infection of the hemolymph.

After oral challenge of early instars, the total mortality in all three species was significantly higher in infected insects compared with control insects and with challenged but uninfected insects ($P \leq 0.001$, df = 1, $\chi^2 = 13.16$–56.21) (Table 2). In addition, the mortality rate of challenged but uninfected *T. ni* was

Table 2. Percentage of mortality in three noctuid species after treatment with weevil *Helicosporidium* sp. cysts

Treatment	Species	Group	n^a	Dying larvae	Dying pupae	Total mortality
Oral challenge	*H. zea*	Control	66	5a	33a	36a
		Uninfected	113	4a	22a	25a
		Infected	85	48b	68b	84b
	S. exigua	Control	38	0a	11a	11a
		Uninfected	84	4a	14a	17a
		Infected	29	10a	50b	55b
	T. ni	Control	52	8a	8a	15a
		Uninfected	64	14ab	33b	42b
		Infected	39	26b	79c	85c
Injection	*H. zea*	Control	35	0a	11a	11a
		Infected	45	0a	78b	78b
	S. exigua	Control	36	6a	23a	28a
		Infected	43	0b	37a	37a
	T. ni	Control	40	0a	25a	25a
		Infected	47	0a	83b	83b

Mortality rates followed by different letters within a species and treatment are significantly different ($P \leq 0.05$, df = 1, SAS genmod procedure and lsmeans statement).

[a] Total numbers of insects per group.

Table 3. Development of three noctuid species after oral treatment with weevil *Helicosporidium* sp. cysts

Species	Group	Pupal weight (mg)	Pupation[a]	Eclosion[b]	Adult longevity (d)	Malformed adults[c] (%)
H. zea	Control	440 ± 78a	10.6 ± 1.9a	19.9 ± 2.1a	4.3 ± 1.7a	14a (42)
	Uninfected	456 ± 66a	10.0 ± 1.5b	19.1 ± 1.5b	5.1 ± 1.6b	22a (85)
	Infected	392 ± 77b	11.3 ± 1.3c	19.6 ± 1.1ab	1.3 ± 0.5c	86b (14)
S. exigua	Control	133 ± 16a	6.3 ± 0.8a	12.1 ± 0.5a	4.7 ± 1.6a	9a (34)
	Uninfected	141 ± 22b	6.4 ± 0.7a	12.2 ± 0.6a	5.4 ± 1.9a	3a (70)
	Infected	127 ± 17a	6.7 ± 0.7b	12.8 ± 0.8b	2.5 ± 1.4b	62b (13)
T. ni	Control	265 ± 35a	8.7 ± 0.9a	14.0 ± 1.0a	4.2 ± 1.7a	5a (44)
	Uninfected	238 ± 54b	8.8 ± 1.5a	14.2 ± 1.5a	4.0 ± 1.3a	16a (37)
	Infected	253 ± 29ab	9.0 ± 1.1a	15.0 ± 1.7a	3.4 ± 1.5a	83b (6)

Means (±SD) followed by different letters within a species are significantly different ($P \leq 0.05$, df = 73–213; SAS mixed procedure and lsmeans statement).

[a] Pupation is the time (d) to pupa formation from the time of larval treatment.

[b] Eclosion is the time (d) to adult emergence from the time of larval treatment.

[c] Malformation rates followed by different letters within a species are significantly different ($P \leq 0.05$, df = 1, SAS genmod procedure and lsmeans statement). The total numbers of adults per group are given in parentheses.

significantly higher than in the corresponding control group ($P = 0.0025$, df = 1, $\chi^2 = 9.12$). In infected *H. zea*, 58% of the deaths occurred during the larval stage, whereas in infected *S. exigua* and *T. ni*, a higher proportion of insects died after pupation (81 and 70%, respectively). In all species tested, a significant proportion of orally infected adults (62–86%) emerged with malformed wings, compared with 3–22% malformation in the control and the uninfected orally challenged adults ($P \leq 0.006$, df = 1, $\chi^2 = 7.56$–19.02) (Table 3).

After injection of late instars, almost all insects formed pupae. However, in *H. zea* and *T. ni*, 78 and 83% of the infected pupae died (Table 2) and 90 and 88% of the emerging adults were malformed, respectively (Table 4). In the corresponding control groups of *H. zea* and *T. ni*, pupal mortality (11 and 25%, respectively), and rates of adult malformation (19 and 20%, respectively) were significantly lower (mortality: $P < 0.0001$, df = 1, $\chi^2 = 26.51$ and 25.34, respectively; malformation: $P = 0.0016$ and 0.0041; df = 1, $\chi^2 = 9.97$ and 8.22, respectively). Pupal and total mortality as well as the occurrence of malformed adults did not differ significantly between control and infected *S. exigua* derived from injected late instars ($P > 0.05$, df = 1) (Tables 2 and 4).

Four fitness parameters were selected to evaluate the influence of helicosporidial infection on host development: 1) pupal weight, 2) time after treatment needed for pupation, 3) time after treatment needed for adult eclosion, and 4) longevity of the adults. Pupal weight was significantly reduced in orally infected *H. zea* compared with their controls ($P = 0.0007$, df = 213, $t = 3.44$). However, the weights of orally infected *S. exigua* and *T. ni* pupae did not differ from the corresponding control groups ($P > 0.05$, df = 128 and 129, respectively) (Table 3). Pupation was delayed slightly in orally infected *H. zea* and *S. exigua* compared with control insects ($P = 0.0286$, df = 213, $t = 2.2$; and $P = 0.0106$, df = 142, $t = 2.59$; respectively), whereas in orally infected *T. ni*, there was no significant delay in pupa formation ($P > 0.05$, df = 129) (Table 3). Compared with the time after treatment needed for adult emergence in control insects, eclosion was only delayed in orally infected *S. exigua* ($P = 0.0002$, df = 114, $t = 3.8$), but not in the other two species ($P > 0.05$; *H. zea* df = 138, *T. ni* df = 84) (Table 3). Adult longevity was significantly reduced in orally infected *H. zea* and *S. exigua* ($P < 0.0001$, df = 86, $t = 4.85$; and $P = 0.0178$, df = 77, $t = 2.42$, respectively) but not in orally infected *T. ni* ($P > 0.05$, df = 73) (Table 3). When *Helicosporidium* spp. were injected into late instars of all tested species, the resulting infection had no influence on pupal weight or on the time needed for pupation and eclosion after the treatment ($P > 0.05$, df = 36–85) (Table 4). The life span of infected adults,

Table 4. Development of three noctuid species after injection with weevil *Helicosporidium* spp. cysts

Species	Group	Pupal weight (mg)	Pupation[a]	Eclosion[b]	Adult longevity (d)	Malformed adults[c] (%)
H. zea	Control	445 ± 59a	6.2 ± 0.8a	15.4 ± 1.0a	4.6 ± 1.5a	19a (31)
	Infected	447 ± 85a	6.2 ± 0.8a	15.3 ± 1.1a	1.9 ± 0.9b	90b (10)
S. exigua	Control	113 ± 21a	3.1 ± 0.5a	9.3 ± 0.8a	5.1 ± 1.6a	0a (26)
	Infected	113 ± 16a	3.3 ± 0.5a	8.9 ± 0.9a	3.1 ± 2.0b	11a (27)
T. ni	Control	226 ± 30a	3.0 ± 0.0a	9.5 ± 0.7a	3.4 ± 1.4a	20a (30)
	Infected	232 ± 30a	3.0 ± 0.1a	9.9 ± 0.4a	1.5 ± 0.8b	88b (8)

Means (±SD) followed by different letters within a species are significantly different ($P \leq 0.05$, df = 21–85; SAS mixed procedure and lsmeans statement).

[a] Pupation is the time (d) to pupa formation from the time of larval treatment.

[b] Eclosion is the time (d) to adult emergence from the time of larval treatment.

[c] Malformation rates followed by different letters within a species are significantly different ($P \leq 0.05$, df = 1, SAS genmod procedure and lsmeans statement). The total numbers of adults per group are given in parentheses.

Table 5. Sex ratio (female:male) in three noctuid species after oral treatment or injection with weevil *Helicosporidium* sp. cysts

Species	Group	Oral Challenge		Injection	
		Pupae	Adults	Pupae	Adults
H. zea	Control	36:27a	22:20a	16:19a	14:17a
	Uninfected	53:56a	42:43a	No pupae	No adults
	Infected	19:25a	9:5a	23:22a	4:6a
S. exigua	Control	20:18a	18:16a	14:20a	9:17a
	Uninfected	38:43a	29:41a	One pupa	No adults
	Infected	12:14a	6:7a	21:22a	12:15a
T. ni	Control	20:28a	19:25a	21:19a	17:13a
	Uninfected	26:29a	16:21a	No pupae	No adults
	Infected	15:14a	2:4a	29:18a	5:3a

Ratios followed by the same letter within a species are not significantly different ($P > 0.05$, df = 1; SAS genmod procedure).

however, was significantly reduced for each species (*H. zea:* $P < 0.0001$, df = 39, $t = 5.34$; *S. exigua:* $P < 0.0134$, df = 21, $t = 2.7$; *T. ni:* $P < 0.0013$, df = 36, $t = 3.49$) (Table 4). In the three tested species, the sex ratio did not differ between control, uninfected, and infected groups ($P > 0.05$, df = 1) (Table 5).

Microscopic examination of hemolymph samples revealed the presence of detectable vegetative cells and characteristic pellicles in the hemolymph within 5 d after challenge. Hemocytes were commonly observed to support vegetative cell development (Fig. 1A). Vegetative cells were released to the hemolymph and continuously multiplied throughout the development of the insect (Fig. 1B and C). Massive numbers of freely circulating vegetative cells and cysts were observed within 8 d after treatment in both pupal and adult stages (Fig. 2).

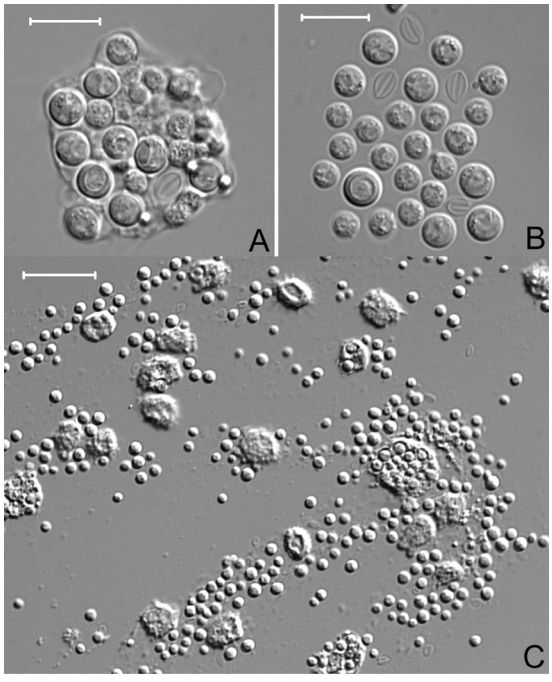

Fig. 1. *Helicosporidium* spp. in the hemolymph of *S. exigua.* (A) Multiplication of vegetative cells within a larval hemocyte. Bar, 10 μm. (B) Released vegetative cells, cysts, and pellicles in pupal hemolymph. Bar, 10 μm. (C) Larval hemolymph sample 5 d after oral infection showing several intact and infected hemocytes and high numbers of freely circulating helicosporidial cells. Bar, 50 μm.

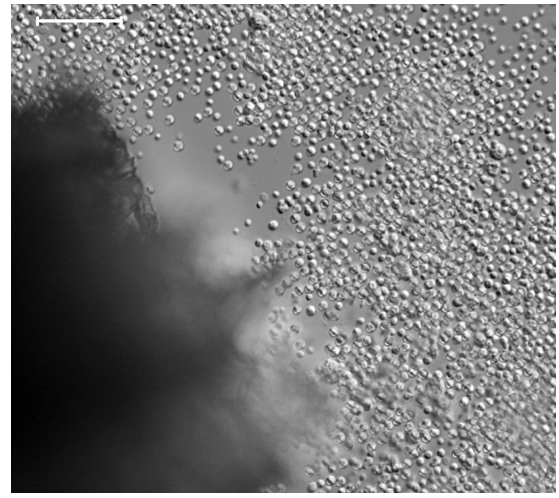

Fig. 2. Massive numbers of cysts released from a dissected leg of an infected adult. Bar, 50 μm.

Mating experiments were conducted with *S. exigua* adults that looked morphologically normal and were obtained from hemocoelic *Helicosporidium* spp. injections. In these experiments, the average adult longevity did not differ between control and infected females (8 ± 3 and 6 ± 1 d, respectively; $P > 0.05$, df = 24, $t = 1.5$) or between control and infected males (7 ± 2 and 6 ± 1 d, respectively; $P > 0.05$, df = 24, $t = 1.56$). Offspring were obtained from all mating combinations. However, in two of five pairings between control female and infected male and in four of nine pairings between infected female and infected male, no egg deposition occurred. In one of the latter pairings, the female laid eggs that did not hatch, indicating that these eggs were not fertilized. When control or infected females were mated with control males, they deposited viable eggs in all experiments ($n = 6$ for each combination). All pairings that did not yield viable offspring were excluded from the calculation of total egg numbers deposited per female. Infected females deposited significantly fewer eggs than control females (601 ± 207 and 896 ± 217 eggs per female, respectively; $P = 0.0075$, df = 17, $t = 3.03$). Significantly, both infected females and males were capable of transmitting the disease to their progeny; each of the two mating combinations with one infected and one control adult resulted in a 33% chance of vertical transmission of the *Helicosporidium* spp. weevil isolate (Table 6). When both parents were infected, the chance of disease transmission increased to 67%. Infection was not detected in newly hatching F_1 larvae but in 4% of the examined late instars and pupae ($n = 638$). Infection rates in the filial generations did not differ between the different parental combinations

Table 6. Transmission of weevil *Helicosporidium* spp. from infected *S. exigua* adults to F1 progeny

Female X Male	Number of pairings with disease transmission	Percent infection in F1[a]	
		Neonates	Late instars and pupae
Control X Infected	1/3	0 (30)	2a (173)
Infected X Control	2/6	0 (60)	5a (348)
Infected X Infected	2/3	0 (30)	5a (117)

[a] Infection rates followed by the same letter are not significantly different ($P > 0.05$; df = 1; SAS genmod procedure and Ismeans statement). The total number of examined insects are given in brackets.

($P > 0.05$, df = 1) (Table 6). All of the infected F_1 progeny died as pupae.

Discussion

Until recently, the genus *Helicosporidium* was considered to be either a protozoan (Kudo 1931) or a fungus (Weiser 1964, 1970), but since its first description by Keilin (1921), its taxonomic position has remained uncertain (Kellen and Lindegren 1974, Tartar et al. 2002). Based on their phylogenetic analyses, which identified *Helicosporidium* spp. as closely related to the achlorophylous green algal genus *Prototheca*, Tartar et al. (2002, 2003) proposed the transfer of the genus *Helicosporidium* to Chlorophyta, Trebouxiophyceae. These findings characterized *Helicosporidium* spp. as the first described invertebrate pathogenic alga. Its closest relatives, *P. zopfii* and *P. wickerhamii*, are the causal agents of bovine mastitis and of malignant skin lesions in humans and mammalians, respectively; these chronic diseases are also referred to as protothecosis (Iacoviello et al. 1992, Jánosi et al. 2001a, Chao et al. 2002). The ubiquitous, saprophytic Prototheca propagate excessively in humid environments and have been isolated from various sites such as streams, lakes, wet soil, cow's milk, sewage, and animal wastes (Jánosi et al. 2001b; Chao et al. 2002). Helicosporidia have been detected in diverse groups of invertebrates, the majority of which have at least one aquatic life stage. These include the orders Insecta, Acarina, Cladocera, and Trematoda (Kellen and Lindegren 1973, Sayre and Clark 1978, Purrini 1984, Pekkarinen 1993, Boucias et al. 2001). Helicosporidia also have been isolated from ditch water (Avery and Undeen 1987a,b). These pathogenic algae cause lethal or chronic infections in various invertebrate hosts. Whereas the in vivo development of different *Helicosporidium* spp. isolates has been investigated in a few studies, the influence of helicosporidial infection on the host's development is poorly documented. Keilin (1921) was the first to describe the life cycle of *H. parasiticum* in a dipteran host, *Dasyhelea obscura* Winnertz (Diptera: Ceratopogonidae). Histological examination of heavily infected larvae revealed freely circulating parasitic "corpuscles" present in all tagmata. To date, several *Helicosporidium* spp. isolates have been documented to infect and replicate in different invertebrate hosts, and all isolates exhibited a heavy infection of the hemolymph with freely circulating cysts or spores (Weiser 1970; Kellen and Lindegren 1973, 1974; Fukuda et al. 1976; Lindegren and Hoffmann 1976; Hembree 1981; Boucias et al. 2001; Seif and Rifaat 2001). Boucias et al. (2001) demonstrated infectivity of a *Helicosporidium* spp. isolated from the black fly *S. jonesi*, toward heterologous lepidopteran and dipteran hosts. They recorded lethal infections in *H. zea* and *Manduca sexta* (L.) (Lepidoptera: Sphingidae) within 14 d after oral challenge with 10^5 cysts per insect. *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) larvae, injected with 10^5 cysts per insect, all succumbed to the disease and died at the larval pupal molt. In agreement with the findings reported herein, microscopic examination revealed that development and replication of the black fly *Helicosporidium* spp. occurred mainly in the host hemolymph. Among the Diptera challenged with the black fly isolate, infectivity varied, but all susceptible species, *Musca domestica* L. (Muscidae), *Aedes taeniorhynchus* Wiedemann, *Anopheles albimanus* Wiedemann, and *Anopheles quadrimaculatus* Say (Culicidae), supported multiplication of the pathogen in their hemolymph.

Our results clearly demonstrate the capability of *Helicosporidium* spp. isolated from an aquatic weevil, *C. salviniae*, to replicate in three heterologous lepidopteran species. Hemocytes of infected larvae were observed to support vegetative cell development of the *Helicosporidium* spp. Potentially, the vegetative cells were resistant to the phagocytic process and continued to grow and divide within the phagocytic vacuole. The ability of the infected larvae to undergo metamorphosis and produce fully formed, infected adults provides indirect evidence that this pathogen did not invade the undifferentiated imaginal discs.

The most significant aspect of helicosporidial infection was the chronic character of the disease. Infection was trans-stadially maintained and did not necessarily interrupt metamorphosis in the host. The ability of *Helicosporidium* spp. to be trans-stadially transmitted has been reported in various insect hosts. Avery and Undeen (1987a) and Seif and Rifaat (2001) determined that many of *H. zea* and *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae) larvae orally treated with a mosquito *Helicosporidium* spp. emerged as heavily infected adults. Larvae of two mosquito species, *Culex salinarius* Coquillett and *Culex pipiens* L. (Diptera: Culicidae), also have been reported to survive helicosporidial infection and to develop into normally formed adults (Kim and Avery 1986, Seif and Rifaat 2001). In the current study, between 15 and 45% of the insects that were infected with weevil *Helicosporidium* spp. as larvae emerged as adults (Table 2), of which 10–89% seemed to be morphologically healthy (Tables 3 and 4); they did not show malformations or any exterior signs of disease, although their hemocoels contained helicosporidial cells at extremely high densities. Additionally, these heavily infected *S. exigua* adults were able to mate and produce viable offspring.

The variability in infection rates after oral application observed in this study seems to be the status quo for *Helicosporidium*. Fukuda et al. (1976), for example, successfully transmitted a mosquito isolate per os to 14 of 17 tested mosquito species, but infection rates were modest and varied considerably between species and among replicates within each species. In the current study, oral treatment of *H. zea*, *S. exigua*, and *T. ni* early instars with a high dosage of the pathogen (2×10^5 cysts per larva) yielded average infection rates of 58, 39, and 55%, respectively, with high variability between replicates (Table 1). The variable response was circumvented by injection of the pathogen into the hemocoel of late instar larvae, which resulted in virtually 100% infection in all three species. The results of per os bioassays, namely, low and variable infection rates and a lack of dose response, suggest that these insects possess an inherent resistance to helicosporidial infection. Comparison of susceptibility to oral versus injection treatments (variability versus constancy, modest versus high infection rates) indicates that resistance barriers against *Helicosporidium* spp. are affiliated with the ingress of the pathogen through the midgut. In an in vitro study with the black fly *Helicosporidium* spp. isolate, Boucias et al. (2001) demonstrated that fluids extracted from the midgut lumen of *H. zea* initiate dehiscence of the cyst and release of the filamentous cell. They also showed that, in vivo, ingested cysts bind to the peritrophic matrix, dehisce, and release filamentous cells, which penetrate the peritrophic matrix and the midgut epithelium to invade the hemocoel of the host. In this study, it is most likely that midgut-mediated barriers were responsible for the moderate infection rates in *H. zea*, *S. exigua*, and *T. ni* after ingestion of the pathogen. Because sex-specific pathogenic efficacy can be excluded, the overall susceptibility of only 40–60% of orally challenged larvae also suggests that the resistance to the disease is probably genetically determined. Per os treatment experiments with F1 progeny obtained from orally challenged, uninfected *S. exigua* could help to elucidate this hypothesis.

In their original culicid hosts, two different mosquito *Helicosporidium* spp. isolates have been shown to exhibit dose-dependent infection rates that were directly proportional to the applied spore concentration. A 4-h, per os exposure to concentrations ranging from 5×10^2 to 8×10^3 spores/ml (16-fold increase) of an Egyptian *Helicosporidium* spp. isolate from *C. pipiens* yielded infection rates in third instar *C. pipiens* increasing from 10 to 100% (Seif and Rifaat 2001). A similar treatment of *Aedes aegypti* (L.) larvae with a *Helicosporidium* spp. from Thailand (isolated from *A. aegypti*) at concentrations ranging from 5×10^2 to 5×10^4 spores/ml (100-fold increase) resulted in infection rates increasing from 4 to 100% (Hembree 1981). As opposed to these findings, no dose response was seen herein after oral exposure of a heterologous host, *H. zea*, to the weevil *Helicosporidium* spp. isolate. Regardless of a 20-fold increase in the ingested dosage (ranging from 10^4 to 2×10^5 cysts per insect), only 50–60% of the challenged larvae showed manifestation of the disease. This evidence again suggests that these heterologous insect hosts possess an inherent resistance to helicosporidial infection.

Infection significantly reduced adult longevity, but the reduction of adult life span was generally affiliated with wing malformation. As demonstrated in the mating experiments with *S. exigua*, infected adults that seemed normal were able to mate and reproduce. The number of eggs deposited per female, however, was significantly reduced in infected females. Failure in reproduction can be attributed to male infection. Potentially, infected males did not copulate. The disease was transmitted to the filial generation by both genders. Preliminary histological examinations did not detect any helicosporidial life stages inside eggs or invading female or male reproductive tissue (V.-U.B., unpublished data). When Kim and Avery (1986) investigated effects of another *Helicosporidium* spp. isolate on adult fecundity of the mosquito *C. salinarius*, the progeny of infected adults did not carry the disease. In addition, microscopic examination of adult tissue showed that ovaries of infected *C. salinarius* females were free of the pathogen. The mechanism of disease transmission in *S. exigua* remains to be elucidated. To our knowledge, this is the first evidence of vertical transmission of a helicosporidial infection.

Acknowledgments

We thank Kelly Sims and Hannah Snyder for technical assistance; Nancy Lowman and Susan White (USDA–ARS, Gainesville, FL) for providing insect eggs and larvae; Yongsung Joo (Statistical Consulting Unit, Department of Statistics, University of Florida, Institute of Food and Agricultural Sciences, Gainesville, FL) for statistical advice; and James Becnel, Bonifacio Magalhaes, Sasha Shapiro, Aurelien Tartar, and two anonymous reviewers for their critical comments on earlier drafts of the manuscript. The study was partially financed by the National Science Foundation Cell Biology Program 7214847-12 "C." Florida Agricultural Experiment Station Journal Series No. R-09594.

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Received for publication 24 June 2003; accepted 10 September 2003.