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## INFECTIVITY OF TWO ISOLATES OF *HELICOSPORIDIUM* SPP. (CHLOROPHYTA: TREBOUXIOPHYCEAE) IN HETEROLOGOUS HOST INSECTS

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### ABSTRACT

Members of the genus *Helicosporidium* are the first described algal insect pathogens. They have a close affinity to the non-photosynthetic algae of the genus *Prototheca*, and have a wide host range, infecting many species of aquatic and terrestrial insects. In this study the infectivity of two *Helicosporidium* spp. isolates, originating from a black fly (SjHe) and an aquatic weevil (CsHe), was tested against a weevil *Diaprepes abbreviatus* (L.) and three mosquito species *Anopheles quadrimaculatus* Say, *Culex quinquefasciatus* Say, and *Aedes aegypti* (L.). The weevil constitutes a new experimental host record for helicosporidia. The CsHe isolate was more virulent than the SjHe isolate in *D. abbreviatus*. *Anopheles quadrimaculatus* was the most susceptible mosquito species measured by infection rate and mortality. The infectivity and virulence of SjHe and CsHe isolates did not differ in any of the mosquito species.

**Key Words:** *Helicosporidium* spp., entomopathogenic alga, mosquitoes, citrus root weevil, bioassays

### RESUMEN

Los miembros del género *Helicosporidium* son los primeros patógenos algáceos descritos para insectos. Estos patógenos tienen una afinidad cercana a las algas no fotosintéticas del género *Prototheca*, y tienen un rango amplio de hospederos, infectando muchas especies de insectos acuáticos y terrestres. En este estudio la capacidad de infección de dos aislamientos de *Helicosporidium* spp., obtenidos de una mosca negra (SjHe) y un gorgojo acuático (CsHe), fue probada contra el gorgojo *Diaprepes abbreviatus* (L.) y tres especies de mosquitos *Anopheles quadrimaculatus* Say, *Culex quinquefasciatus* Say, y *Aedes aegypti* (L.). El gorgojo es considerado un nuevo registro de un hospedero experimental para helicosporidia. El aislamiento de CsHe fue mas virulento que el aislamiento de SjHe en *D. abbreviatus*. *Anopheles quadrimaculatus* fue la especie de mosquito mas susceptible medida por la tasa de infección y de mortalidad. La infectividad y virulencia de los aislamientos de SjHe y CsHe no fue diferente dentro de las especies de mosquitos.

Since the first description by Keilin (1921), invertebrate pathogens in the genus *Helicosporidium* have been detected worldwide in diverse groups of arthropods, including several orders of insects, mites, crustaceans, and trematodes (Sayre & Clark 1978; Purrini 1984; Avery & Undeen 1987; Pekkarinen 1993). Until recently, their taxonomic position remained unclear. Keilin (1921) first described *Helicosporidium parasiticum* as a protozoan, but Weiser (1970) proposed that the helicosporidia are best placed among the lower fungi. Most recently, Boucias et al. (2001) suggested that the vegetative development of helicosporidia was similar to the autosporegenic growth of unicellular algae. Significantly, genetic analysis has defined the genus *Helicosporidium* as a member of the green algal class Trebouxiophyceae (Chlorophyta) and, as such, it represents a novel clade of invertebrate pathogens (Boucias et al. 2001; Tartar et al. 2002, 2003; Tartar & Boucias 2004). The treboux-

iophyte green algae are generally photosynthetic and free-living. However, the closest relatives to helicosporidia, the genus *Prototheca*, are achlorophyllous algae that have evolved a heterotrophic life style, infecting vertebrates.

The infectious cyst, the stage that defines the genus *Helicosporidium*, is comprised of three ovoid cells and a coiled, elongate filamentous cell enclosed within an outer pellicle. The cyst dehisces when ingested by the insect host; within the midgut lumen the pellicle ruptures, releasing the filamentous cell and the three ovoid cells. The invasive filamentous cells pass through the midgut epithelial layer and gain ingress to the hemocoel. Within the hemocoel, the filamentous cells differentiate into vegetative cells, which undergo autosporegenic cell divisions (Bläske-Lietze et al., unpublished). Vegetative cells have been observed to replicate within the phagocytic hemocytes and to develop extracellularly in the hemolymph. Af-

ter multiple 2-4 cell autosporogenic cell divisions, a portion of the hemolymph-borne vegetative cells differentiate into cysts.

Unlike many insect pathogens, the helicosporidia lack specificity and readily infect a broad range of insects. For example, Fukuda et al. (1976) isolated helicosporidia from *Culex nigripalpus* Theobald and they were able to infect 14 mosquito species and one non-mosquito species of Diptera, three species of Coleoptera, and two species of Lepidoptera with this isolate. More recently, Seif & Rifaat (2001) isolated helicosporidia from *Culex pipiens* L. in Egypt and cross-infected *Aedes caspius* (Pallas), *Culex antennatus* (Becker), *Culiseta longiareolata* (Macquart), and *Culex perexiguus* Theobald. Hembree (1979, 1981) isolated *Helicosporidium* sp. from both *Aedes aegypti* (L.) and *Culex quinquefasciatus* Say in Thailand, and later evaluated this *Helicosporidium* as a microbial control agent. However, Hembree (1981) dismissed the use of helicosporidia as biological control agents due to its infectivity to the predatory larvae of *Toxorhynchites splendens* (Wiedemann). Sayre & Clark (1978) further demonstrated the broad host range of this group, reporting that a *Helicosporidium* sp. from the cladoceran *Daphnia magna* (Straus) was infectious to *Ae. aegypti* larvae. Avery & Undeen (1987) found that helicosporidia, isolated from pond water and amplified in *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae), were infectious to *Anopheles quadrimaculatus* Say, *Culex salinarius* Coquillett, and *Ae. aegypti*. In total, helicosporidia have been isolated from five species of mosquitoes, and these mosquito isolates have been transmitted to 21 additional species of mosquitoes and seven non-mosquito hosts in the laboratory (Chapman et al. 1967; Fukuda et al. 1976; Hembree 1979, 1981; Seif & Rifaat 2001).

To date, four coleopteran isolates of helicosporidia have been reported in the literature. In a broad species screen, a *Helicosporidium* sp. isolate from the nitidulid *Carpophilus mutilatus* (Erichson) in Texas was shown to cross-infect six heterologous nitidulid species, the dermestid *Trogoderma variabile* Ballion, the cucujid *Oryzaephilus surinamensis* (L.), and the anobiid *Lasioderma serricorne* (F.), as well as five pyralid lepidopterans, one culicid dipteran, and three mite species (Kellen & Lindegren 1973; Kellen & Lindegren 1974; Lindegren & Hoffmann 1976). Purrini (1985) identified *Helicosporidium* sp. cysts in field-collected adults of the scarabaeid *Oryctes monoceros* Ol. in Tanzania. A weevil isolate originating from the curculionid *Cyrtobagus salviniae* Calder & Sands in Florida infected and replicated in three noctuid hosts (Bläske & Boucias 2004). Most recently, *Helicosporidium* sp. infections were found in German bark beetle populations of *Dendroctonus micans* (Coleoptera: Scolytidae) (Yaman & Radek, 2005).

In this study, a comparative analysis of two *Helicosporidium* spp. isolates was conducted, the first originating from a local population of larval *Simulium jonesi* Stone & Snoddy (Diptera: Simuliidae) and the second isolated from a quarantined lab population of an aquatic weevil *C. salviniae* that was imported from Australia and colonized for release as a insect biological control against *Salvinia molesta* (Mitchell). To date, the only morphological characteristic that separates these two isolates is the length of the filamentous cell (Bläske-Lietze, unpublished), a potential adaptation related to pathogen ingress. It was speculated that these two isolates, originating from insects in different orders and from geographically distinct regions, might represent pathotypes and therefore display different host range properties. Therefore in-depth bioassays of these two helicosporidial isolates were conducted against four insect species: the citrus root weevil, *Diaeprepes abbreviatus* (L.) (Coleoptera: Curculionidae), and three species of mosquitoes, *An. quadrimaculatus*, *Cx. quinquefasciatus*, and *Ae. aegypti* (Diptera: Culicidae).

## MATERIALS AND METHODS

### Preparation of *Helicosporidium*

Two *Helicosporidium* spp. isolates used in this study were the black fly isolate (SjHe) from the black fly, *S. jonesi*, collected in 2002 and 2003 (Alachua County, Florida), and the weevil isolate (CsHe) from *C. salviniae* collected from a colony in Alachua County. Both isolates were amplified in *H. zea* and extracted on a continuous gradient of Ludox HS40 (Perkin Elmer Life Sciences, Boston MA) (Bläske & Boucias 2004). The cyst-containing band was subjected to several cycles of low-speed centrifugation to remove residual gradient material and stored at 4°C. The number of cysts in each preparation was determined with a hemacytometer.

### Infection Assays with Weevils

Four-week-old *D. abbreviatus* larvae were starved for 16 h in wells of a 96-well tissue culture dish. Individual starved larvae ( $15 \pm 4$  mg) were provided with a carrot cube ( $1 \text{ mm}^3$ ) treated with either 1  $\mu\text{l}$  of water (control) or cyst suspensions (SjHe or CsHe) at  $2.5 \times 10^5$  cysts/ $\mu\text{l}$  and incubated at constant conditions ( $26 \pm 1^\circ\text{C}$ ,  $70 \pm 5\%$  RH, darkness). Larvae that had completely consumed the carrot after 96 h were transferred to diet cups containing artificial diet. Three weeks after the treatment, control and treated larvae were weighed and examined for helicosporidial infection. Hemolymph samples were collected by needle puncture and the presence of helicosporidial life stages (vegetative cells, cysts, pellicles) was

recorded with differential interference contrast (DIC) optics. After initial diagnosis at 3 weeks, all larvae were transferred to fresh diet cups, incubated for an additional 3 weeks, then re-examined. Throughout the experiments, survival was recorded every other day, and dead individuals were weighed and diagnosed for infection as described above. In three to five replicate assays, a total of 67, 38, and 44 larvae were used for control, SjHe, and CsHe treatments, respectively.

In a second assay, 5-week-old *D. abbreviatus* adults were starved for 16 h and then force-fed 1- $\mu$ l droplets of either a 20%-sucrose solution or a cyst suspension ( $2.5 \times 10^5$  cysts/ $\mu$ l) in 20% sucrose. Droplets were delivered into the adults' esophagus with a 1-ml syringe fitted with a blunt-tip 30G 1/2 needle mounted onto a microapplicator. After an additional 24-h starvation period, the adult weevils were placed in cups containing a citrus leaf and moist cotton and incubated at  $26 \pm 1^\circ\text{C}$ . Insects were examined daily and leaves and cotton exchanged twice a week. After 3 weeks, hemolymph samples were subjected to microscopic examination. Ten adults were tested per control and isolate. The experiment was repeated three times.

#### Infection Assays with Mosquitoes

Bioassays assessed the activity of SjHe and CsHe against three mosquito species, *An. quadrimaculatus*, *Ae. aegypti*, and *Cx. quinquefasciatus*. For each bioassay, 100 first-instars were placed in a petri dish with 98 ml of deionized water amended with a 1-ml dose of helicosporidia (treatment) or deionized water (control) and a 1-ml volume of 2% alfalfa and potbelly pig chow mixture (2:1) as a nutritional source. Final test concentrations of helicosporidia in the petri dishes ranged from  $10^2$  to  $10^6$  cysts/ml. Larvae were incubated at constant conditions ( $26 \pm 1^\circ\text{C}$ , 12:12 photoperiod) for 24 h then transferred to enamel pans, and water was added to make 500 ml final volume. After 7 d, the surviving larvae were counted in each pan and a sub-sample of 12 randomly examined for infection under phase-contrast optics. Larvae containing live helicosporidial cells (vegetative or cyst stage) and those containing melanized helicosporidial cells were considered infected. The development of control and treated *An. quadrimaculatus* and *Ae. aegypti* at six d post-treatment was assessed by head capsule width.

#### Statistical Analyses

Statistical analyses were done with the SAS System for Windows (SAS Institute 1999). Percent infection, percent survival/mortality data, head capsule measurements, and weight gains were subjected to analysis of variance by the procedure for general linear models (glm) in bal-

anced designs and the procedure for mixed linear models (mixed) in unbalanced designs (Neter et al. 1990; Rao 1998; Younger 1998). The means were separated by the least square means statement (ls means). To investigate the relationship between the probability of larval mortality and the weight at the time of needle puncture in weevils, a correlation analysis was conducted with the logistic regression procedure (logistic) and the regression procedure (reg) (Rao 1998; Younger 1998). Mean values ( $\pm$  SD) are presented.

## RESULTS

#### Infection Assays with Weevils

Both *Helicosporidium* spp. isolates were able to infect and reproduce in *D. abbreviatus* larvae and adults. Infection rates in larvae were high ( $>85\%$ , Table 1) and did not differ between isolates after 3 weeks ( $df = 1$ ,  $P = 0.1138$ ,  $F = 4.07$ ) or after 6 weeks ( $df = 1$ ,  $P = 0.7627$ ,  $F = 0.10$ ). Four SjHe-treated larvae that did not show any symptoms of the disease after 3 weeks were diagnosed as infected after 6 weeks, which is indicated by the higher cumulative infection rate after 6 weeks in Table 1. After 3 weeks, larval weight was reduced in CsHe-infected *D. abbreviatus* ( $105 \pm 78$  mg) compared with uninfected larvae ( $228 \pm 75$  mg) ( $df = 144$ ,  $P < 0.0001$ ,  $t = -7.94$ ) (Table 2). The weight of SjHe-infected larvae ( $191 \pm 82$  mg) was lower than in control larvae ( $df = 144$ ,  $P = 0.0258$ ,  $t = -2.25$ ) but higher than that of CsHe-infected larvae ( $df = 144$ ,  $P < 0.0001$ ,  $t = 4.68$ ). After 6 weeks, all uninfected larvae (from control and treated groups) had reached an average weight of  $478 \pm 144$  mg ( $n = 54$ ), approximately twice as much as the weight of surviving SjHe- and CsHe-infected larvae ( $252 \pm 115$  mg,  $n = 27$ ). An apparent symptom of infected larvae was the cream-colored hemolymph clearly visible through the integument (Fig. 1). No mortality occurred within 3 weeks post-treatment of the larvae. The percentage of larvae that died between 3 and 6 weeks (after the first bleeding for diagnostics) was higher in CsHe-infected larvae than in control and SjHe-infected larvae ( $df = 2$ ,  $P = 0.0062$ ,  $F = 10.28$ ) (Ta-

TABLE 1. INFECTION RATES IN *DIAPREPES ABBREVIATUS* LARVAE AFTER ORAL TREATMENT WITH *HELICOSPORIDIUM* SPP. CYSTS AT  $2.5 \times 10^5$  CYSTS PER LARVA.

Isolate	Cumulative infection (%) after	
	3 wk	6 wk
Controls	$0 \pm 0$	$0 \pm 0$
SjHe	$76 \pm 3$	$86 \pm 8$
CsHe	$88 \pm 10$	$88 \pm 10$

TABLE 2. LARVAL WEIGHT GAIN IN *DIAPREPES ABBREVIATUS* AFTER ORAL TREATMENT WITH *HELICOSPORIDIUM* SPP. CYSTS AT  $2.5 \times 10^5$  CYSTS PER LARVA.

Isolate	Infection	N	Weight (mg) <sup>a</sup>			Percent mortality between 3-6 wk
			Initial	After 3 wk	After 6 wk	
Controls	No	67	15 ± 4 a	228 ± 75 a	480 ± 149 a	25 a
SjHe	Yes	32	15 ± 4 a	191 ± 82 b	251 ± 112 b	38 a
	No	6	16 ± 3 a	192 ± 85 ab	441 ± 21 ab	N/A
CsHe	Yes	39	15 ± 4 a	105 ± 78 c	255 ± 130 b	80 b
	No	5	13 ± 3 a	229 ± 55 ab	472 ± 86 a	N/A

<sup>a</sup>Means followed by a different letter are significantly different ( $P \leq 0.05$ ; SAS mixed procedure and ls means statement).

ble 2). There was a strong negative correlation between mortality and larval weight at the time of diagnostic needle puncture (3 weeks after treatment) ( $df = 1$ ,  $P < 0.0001$ ,  $\chi^2 = 24.9177$ , logistic procedure;  $r = -0.7575$ , reg procedure) (Fig. 2). Microscopic observations revealed a slower development of the SjHe-isolate in the host hemolymph (Fig. 3A, B). Approximately 50% of SjHe-infected larvae showed vegetative cell development but no cyst differentiation within 3 weeks of the treatment, whereas cysts were found in all CsHe-infected larvae at this time (Fig. 3B).

Oral force treatment of *D. abbreviatus* adults with either isolate resulted in 100% infection after 3 weeks of challenge. No mortality occurred within this time frame. The CsHe isolate developed faster in the host than the SjHe isolate (Fig. 3C, D). Within 3 weeks of oral challenge, many cysts and vegetative cells were observed in the hemolymph of CsHe-treated weevils (Fig. 3D). In SjHe-treated weevils, the majority of helicosporidial life stages were contained within hemocytes (Fig. 3C), and only a few vegetative cells could be seen circulating freely in the hemolymph.

Infection Assays with Mosquitoes

All three of the tested mosquito species were susceptible to helicosporidial infection. *Anopheles quadrimaculatus* was the most susceptible of the three species, experiencing higher mortality ( $df = 4$ ,  $P = 0.0229$ ,  $F = 3.14$ ) (Fig. 4) and infection rates ( $df = 4$ ,  $P < 0.0001$ ,  $F = 48.91$ ) (Fig. 5) at 7 d post-treatment than the other two species tested. Mortality in treated *Cx. quinquefasciatus* and *Ae. aegypti* did not differ from mortality in any of the control groups (Fig. 4). The SjHe isolate did not cause any infection in *Cx. quinquefasciatus* at  $10^5$  cysts/ml (Fig. 5).

For both isolates, the hemolymph of infected mosquito larvae was filled with vegetative stages 7 d after exposure. Cyst production was also seen at this time. Melanized helicosporidial cells were observed in the head and thorax region of mosquitoes infected with the CsHe isolate. The SjHe isolate did not cause this melanization response unless vegetative stages were present in the hemolymph.

*An. quadrimaculatus* larvae exhibited a clear dosage response to both the SjHe and CsHe iso-

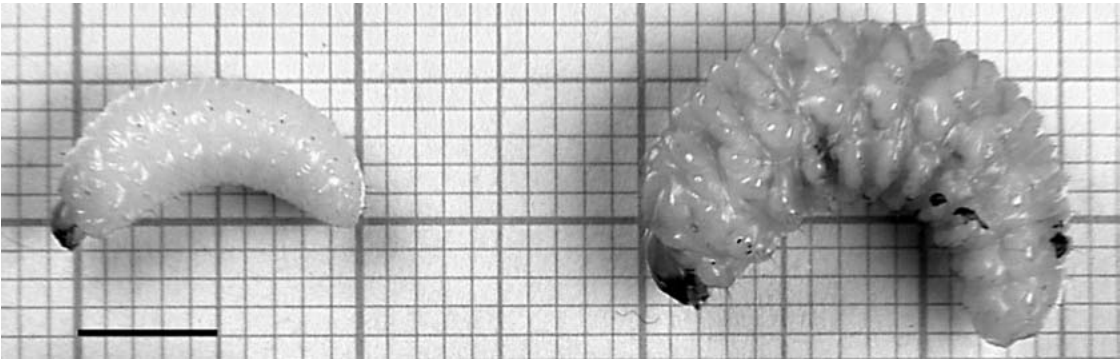


Fig. 1. Infected (left) and control (right) *Diaprepes abbreviatus* larvae 6 weeks after oral challenge with the CsHe *Helicosporidium* sp. isolate. Bar: 5 mm.

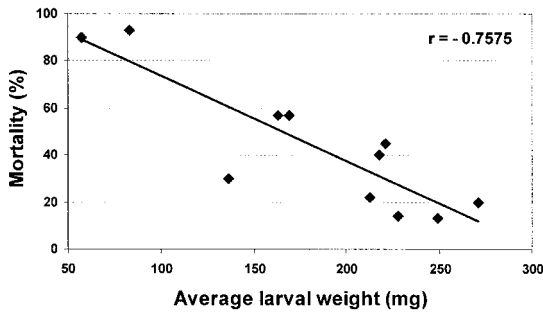


Fig. 2. Probability of mortality as a function of weight of *Diaprepes abbreviatus* larvae at the time of needle puncture (SAS reg procedure). Data are extrapolated from five replicate control assays and three replicate treatment assays with each isolate of *Helicosporidium* sp.

late of *Helicosporidium* sp. Mortality and infection rates increased with increasing dosages of cysts (Table 3). These increases in mortality and

infectivity were statistically significant for both isolates except for the mortality due to SjHe. Over a dosage range from  $10^2$  to  $10^5$  cysts/ml, mortality increased from  $20 \pm 26$  to  $59 \pm 36\%$  ( $df = 5$ ,  $P = 0.134$ ,  $F = 1.91$ ) and from  $10 \pm 9$  to  $93 \pm 2\%$  ( $df = 5$ ,  $P = < 0.0001$ ,  $F = 69.08$ ) after exposure to the SjHe and CsHe isolates, respectively. Treatments with the SjHe and CsHe isolate over a dose range from  $10^2$  to  $10^5$  cysts/ml resulted in increasing infection rates from  $2 \pm 4$  to  $64 \pm 48\%$  ( $df = 4$ ,  $P = < 0.0001$ ,  $F = 13.72$ ) and from  $14 \pm 24$  to  $71 \pm 51\%$  ( $df = 5$ ,  $P = 0.0007$ ,  $F = 6.24$ ), respectively. On average, 35% of the 7-day mortality of *An. quadrimaculatus* was expressed within the first 24 h after exposure to  $10^5$  cysts/ml (Fig. 6). This pronounced 24-h mortality was not seen in the other two species of mosquito. When exposed to  $10^5$  cysts/ml, the mortality of *An. quadrimaculatus* at 24 h was higher than the 24-h mortality of *Cx. quinquefasciatus* and *Ae. aegypti* ( $df = 8$ ,  $P = 0.0015$ ,  $F = 3.82$ ) (Fig. 6).

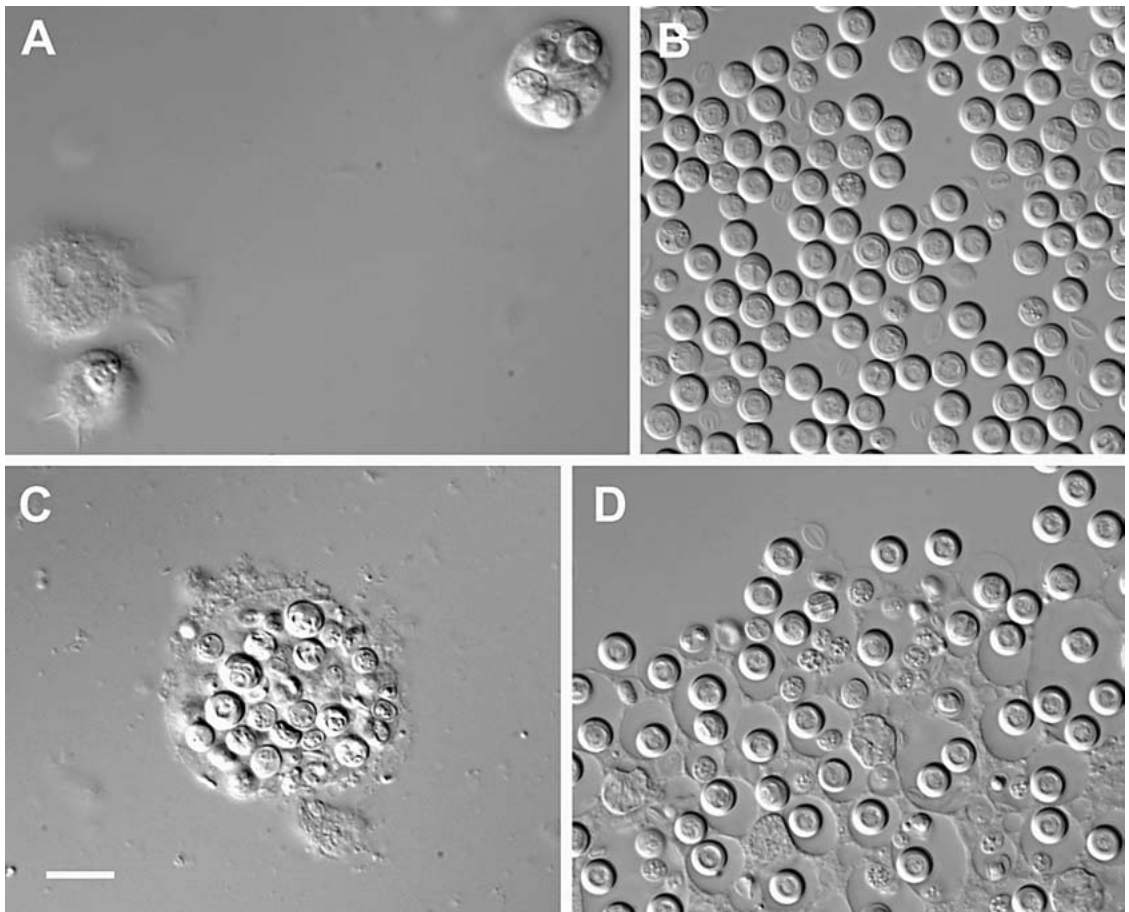


Fig. 3. Hemolymph samples from infected *Diaprepes abbreviatus* larvae (A, B) and adults (C, D) three weeks after oral challenge with *Helicosporidium* sp. A, C: SjHe isolate. B, D: CsHe isolate. Bars: 10  $\mu$ m.

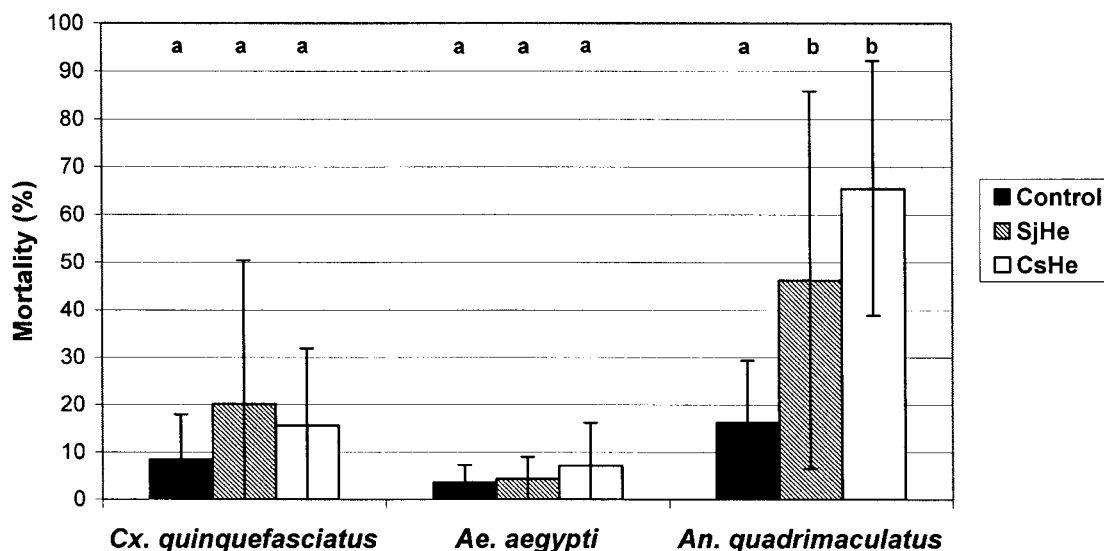


Fig. 4. Seven-day mean ( $\pm$  SD) percent mortality of *Culex quinquefasciatus*, *Aedes aegypti*, and *Anopheles quadrimaculatus* at a dosage of  $10^5$  cysts/ml. Different letters indicate significant differences ( $P \leq 0.05$ ; SAS glm procedure and ls means statement).

Infection did not have an effect on the development of *An. quadrimaculatus* larvae. At 6 d post-treatment, head capsule size of *An. quadrimaculatus* exposed to SjHe or CsHe helicosporidia did not differ from control head capsule size ( $df = 2$ ,  $P = 0.2999$ ,  $F = 1.27$ ) (Fig. 7). In *Ae. aegypti*, infection with the SjHe isolate did affect larval development. The head capsule size of larvae exposed to SjHe was smaller than control head capsule sizes ( $df = 1$ ,  $P = 0.0185$ ,  $F = 6.88$ ).

#### DISCUSSION

Our ability to infect a new coleopteran host, *D. abbreviatus*, and three mosquito species with the same two isolates of helicosporidia supports the wide host range of helicosporidia. In addition, these two isolates infect three species of noctuid Lepidoptera (Bläske & Boucias 2004; Bläske-Lietze & Boucias 2005) and the SjHe isolate also infects three other dipteran hosts (Boucias et al. 2001). However, in the present study, suitability for helicosporidial development varied among different host and isolate systems. Of the mosquitoes tested, *An. quadrimaculatus* was the most susceptible, whereas *Cx. quinquefasciatus* and *Ae. aegypti* were less suitable hosts. Similar results were obtained with a *Helicosporidium* sp. isolated from *Cx. nigripalpus* (Fukuda et al. 1976). The developmental cycle of the pathogen appeared to be prolonged in the weevil host. In contrast to the known susceptible lepidopteran and dipteran species (Boucias et al. 2001; Bläske & Boucias 2004; Bläske-Lietze & Boucias 2005), *D. abbreviatus*

has a long life cycle with an extended larval period. When reared on artificial diet under controlled conditions, the larval stages of the weevil *D. abbreviatus*, the noctuid *Spodoptera exigua* (Hübner), and the mosquito *An. quadrimaculatus* last 106-125 d (Lapointe 2000), 13-15 d, and 7-9 d, respectively. After three weeks, four of the weevil larvae exposed to SjHe appeared uninfected, but at six weeks, these larvae were diagnosed as infected, indicating that the infection progressed slowly. Lepidopteran larvae have been found infected with this isolate within 2 d post-treatment (Bläske-Lietze & Boucias 2005), while mosquito larvae have been found infected with this isolate within 3-4 d post-treatment (Boucias et al. 2001). Also, cyst differentiation in the hemolymph appears to be adjusted to the speed of development of the host insect. Whereas the SjHe isolate formed mature cysts within 6-7 d in *S. exigua* (Bläske-Lietze & Boucias 2005), and within 7 d in different mosquito hosts, in approximately 50% of SjHe-infected weevil larvae and in 100% of SjHe-infected weevil adults, no cysts had differentiated within 3 weeks. Mortality of *D. abbreviatus* larvae was highest between 3 and 6 weeks post-treatment, while the majority of infected *S. exigua* larvae died at pupation (10 d post-treatment), and Fukuda et al. (1976) and Hembree (1981) reported that mosquito mortality occurred in the fourth instar (5-7 d post-treatment).

The interaction between host development and helicosporidia development is complex, and while the host life span may influence the helicosporidia development, the helicosporidia also influence the

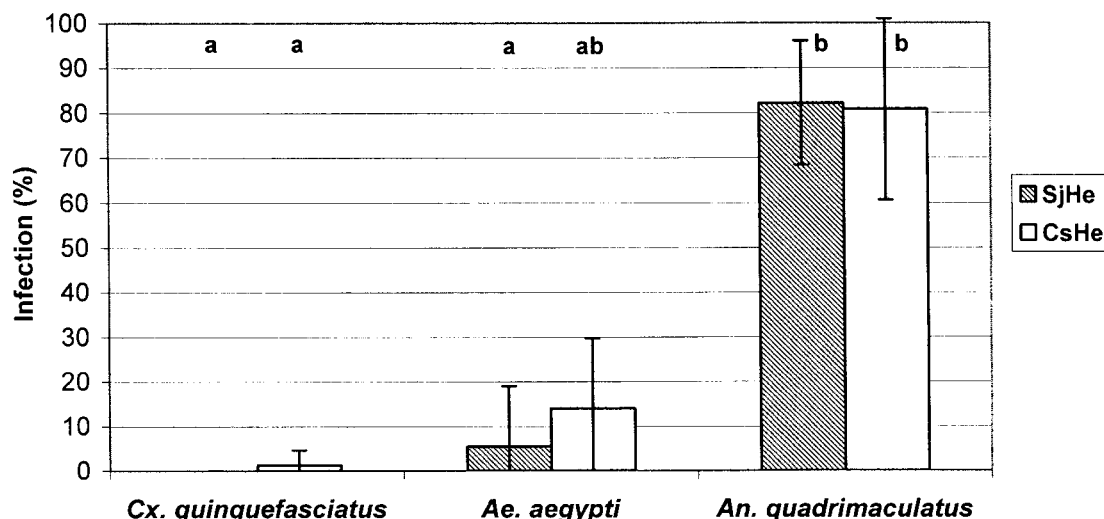


Fig. 5. Seven-day mean ( $\pm$  SD) percent infection of *Culex quinquefasciatus*, *Aedes aegypti*, and *Anopheles quadrimaculatus* at a dosage of  $10^6$  cysts/ml. Different letters indicate significant differences ( $P \leq 0.05$ ; SAS glm procedure and ls means statement).

host's development, as seen in the larval weight gain and head capsule data. It appears that helicosporidia cause a variety of developmental responses in its many hosts. Recently, Bläske & Boucias (2004) reported that helicosporidia infection did not affect larval weight gain in three noctuid hosts. However, infection reduced adult longevity and produced deformities in adults, indicating that development of adults was affected though larval weight gain was not. Larval weight gain in *D. abbreviatus* was severely affected by infection with helicosporidia, though what effect this infection would have on the larvae as they pupate and emerge as adults remains to be seen. Unlike the *D. abbreviatus*, *An. quadrimaculatus* did not experience a delay in development due to infection with helicosporidia. This might be explained by the short developmental time of mosquito larvae compared to the weevil. However, exposed *Ae. aegypti* larvae did experience a developmental delay. It is difficult to say what would account for the differences in the two mosquito species. Mosquitoes are known to exhibit a variety of developmental responses to pathogen infection. Giblin & Platzer (1985) found that mosquito larvae parasitized by a mermithid nematode exhibited a reduced feeding rate, and developed more slowly than uninfected larvae. Alternatively, Agnew et al. (1999) found that larval females infected with a microsporidian parasite developed more quickly than uninfected females and emerged as smaller adults. The high 24-h mortality of *An. quadrimaculatus* observed in the present study may have had an effect on the ap-

parent development rate, as the mosquito larvae are sensitive to density, and develop more quickly under low-density conditions than high-density conditions (Christophers 1960).

While both isolates infected the experimental hosts, the two isolates did exhibit differences. Within 3 weeks, the lower virulence of the SjHe isolate was evident in orally challenged *D. abbreviatus*. The SjHe isolate had less of an effect on body weights of infected larvae and developed more slowly in the hemolymph of both larvae and adults of the citrus root weevil. Despite high infection rates caused by both isolates (76 and 88% by SjHe and CsHe isolate, respectively), no larval mortality occurred within 3 weeks. Between 3 and 6 weeks, however, a high number of CsHe-infected larvae died (80%), whereas of the SjHe-infected and control larvae, the majority survived (62 and 75%, respectively). Mortality was negatively correlated to larval weight at the time of the first needle puncture (3 weeks post-treatment), which accounts for the similar weights of CsHe- and SjHe-infected weevil larvae recorded after 6 weeks. The CsHe isolate has also been shown to be very virulent in susceptible noctuid hosts; following oral challenge with  $2 \times 10^5$  cysts per larva, 55% of infected *S. exigua* died within 13 d and 85% of infected *Trichoplusia ni* (Hübner) died within 15 d (Bläske & Boucias 2004).

In the mosquito system, the two isolates showed no difference in their influence on the development of *An. quadrimaculatus* or infection and mortality of the three tested species of mos-



TABLE 3. MEAN ( $\pm$  SD) MORTALITY AND INFECTION RATES IN *ANOPHELES QUADRIMACULATUS* 7 D AFTER EXPOSURE TO SJHE AND CSHE AT DIFFERENT DOSAGES.

Dose (cysts/ml)	Isolate	N	Percent Mortality <sup>a</sup>	Percent Infection <sup>a</sup>
0	SjHe	6	15 $\pm$ 10 a	0 $\pm$ 0 a
10 <sup>2</sup>	SjHe	5	20 $\pm$ 26 a	2 $\pm$ 4 a
10 <sup>3</sup>	SjHe	6	22 $\pm$ 34 a	7 $\pm$ 6 a
10 <sup>4</sup>	SjHe	6	28 $\pm$ 31 a	44 $\pm$ 22 b
10 <sup>5</sup>	SjHe	3	59 $\pm$ 36 a	64 $\pm$ 48 b
0	CsHe	6	11 $\pm$ 9 a	0 $\pm$ 0 a
10 <sup>2</sup>	CsHe	3	10 $\pm$ 9 a	14 $\pm$ 24 a
10 <sup>3</sup>	CsHe	3	11 $\pm$ 3 a	27 $\pm$ 25 b
10 <sup>4</sup>	CsHe	6	23 $\pm$ 10 b	65 $\pm$ 26 c
10 <sup>5</sup>	CsHe	3	93 $\pm$ 2 c	71 $\pm$ 51 c

<sup>a</sup>Means followed by a different letter are significantly different ( $P \leq 0.05$ ; SAS glm procedure and ls means statement).

quitoes. While there was no statistical difference between mortality and infection rates with the CsHe and SjHe isolates, the observed melanization of the CsHe isolate indicates that mosquitoes are less suitable hosts for the CsHe isolate as opposed to the SjHe isolate. Several other researchers have observed melanization of coleopteran and other isolates of *Helicosporidium* sp. in mosquitoes. Fukuda et al. (1976) reported that melanization occurred with mosquitoes exposed to helicosporidia isolated from the nitidulid beetle *C. mutilatus*. Avery & Undeen (1987) reported similar melanization in mosquito larvae infected with a pond-water isolate and suggested that the melanization may be an indication that mosquitoes are abnormal hosts for helicosporidia. The phylogenetic affinity of mosquitoes to the original sim-

ulid host may explain the lack of melanization in individuals infected with the SjHe isolate.

The results of this study demonstrate that both the SjHe and the CsHe *Helicosporidium* sp. isolates caused dosage-dependent infection rates in *An. quadrimaculatus* that were directly proportional to the applied cyst concentration. Dosage-dependent mortality was confined to the CsHe isolate. A dose response to helicosporidial challenge has been shown in other studies conducted with different host insects and different *Helicosporidium* sp. isolates. For example, treatment of *Ae. aegypti* larvae with a mosquito isolate from Thailand (isolated from *Ae. aegypti*) at concentrations ranging from  $5 \times 10^2$  to  $5 \times 10^4$  cysts per milliliter (100-fold increase) resulted in infection rates increasing from 4 to 100% (Hembree,

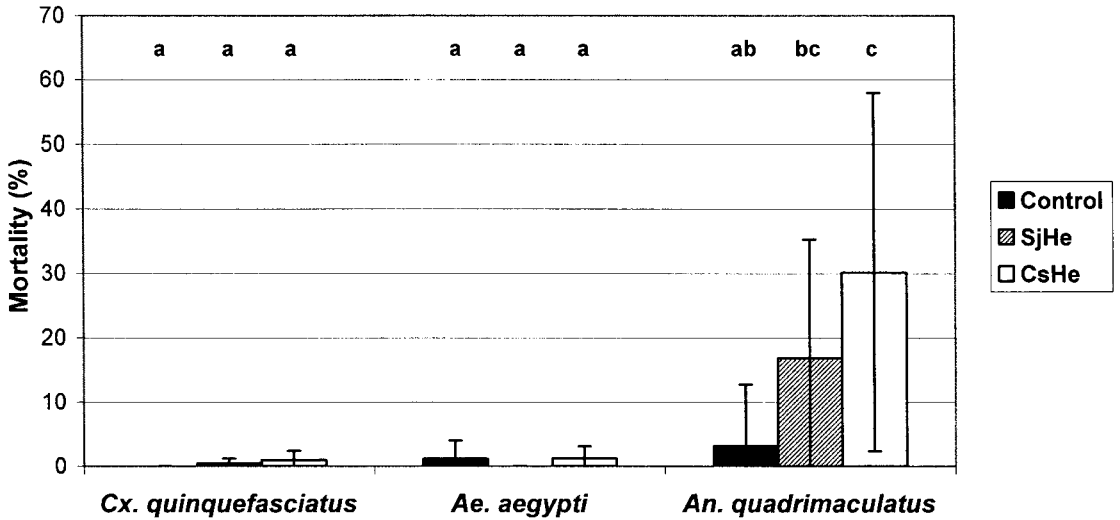


Fig. 6. Mean percent ( $\pm$  SD) mortality of *Culex quinquefasciatus*, *Aedes aegypti*, and *Anopheles quadrimaculatus* recorded 24 h post-treatment with  $10^5$  cysts/ml. Different letters indicate significant differences ( $P \leq 0.05$ ; SAS glm procedure and ls means statement).

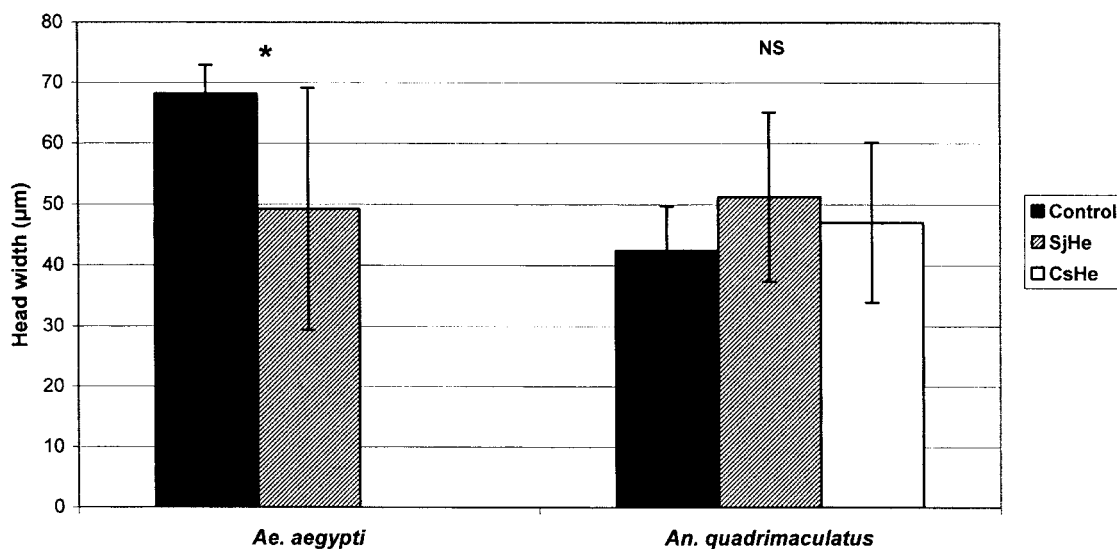


Fig. 7. Mean ( $\pm$  SD) head capsule width of *Aedes aegypti* and *Anopheles quadrimaculatus* 7 d post-treatment. An asterisk (\*) indicates a significant difference from the control ( $P \leq 0.05$ ); NS indicates no significant difference from the control ( $P > 0.05$ ) (SAS glm procedure and ls means statement).

1981). In contrast to these findings, no dosage response was seen in a study conducted with the CsHe isolate and larvae of the noctuid *H. zea* (Bläske & Boucias 2004). Regardless of a 20-fold increase in the administered dosage (ranging from  $10^4$  to  $2 \times 10^6$  cysts per insect), only 50 to 60% of the challenged *H. zea* showed manifestation of the disease.

In the present study, helicosporidia also had a pronounced effect on the 24-h mortality of *An. quadrimaculatus*. This early pulse of mortality was likely the result of septicemia facilitated by the ingestion of helicosporidia. Dead first-instar larvae of *An. quadrimaculatus* exposed to SjHe *Helicosporidium* sp. at  $10^6$  cysts/ml contained numerous helicosporidial filamentous cells piercing the midgut epithelium; the resulting damage to the midgut barriers potentially allowed for the ingress of opportunistic bacteria. Factors that influenced the filamentous cell's penetration of the gut—the number of cysts ingested, dehiscence rate of cysts in the midgut, the strength of the midgut wall, or the resident gut microflora—thus may have dictated the early larval mortality observed with *An. quadrimaculatus*. Avery & Undeen (1987) reported a similar effect of high 72-h mortality in *An. quadrimaculatus*, *Cx. salinarius*, and *Ae. aegypti* exposed to helicosporidia isolated from pond water.

The helicosporidia remain cryptic pathogens. This study has demonstrated both the wide host range and the discrete species effects of two isolates. While these two isolates are capable of infecting a similar range of species, there are defi-

nite isolate effects in the timing, intensity, and symptoms of infection. Further research is needed to understand the evolutionary relationship between these two isolates and their hosts.

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