

Transmission of Ascovirus from *Heliothis virescens* (Lepidoptera: Noctuidae) by Three Parasitoids and Effects of Virus on Survival of Parasitoid *Cardiochiles nigriceps* (Hymenoptera: Braconidae)

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ABSTRACT In field crops in the southeastern United States, larvae of *Heliothis virescens* (F.) are often infected with ascoviruses, especially toward the end of the growing season. Ascoviruses are unusual in that they are difficult to transmit *per os*, and several studies have provided data indicating that these viruses are vectored mechanically by parasitic wasps during oviposition. In Georgia, three parasitoids commonly parasitize *H. virescens* larvae: *Cardiochiles nigriceps* Viereck, *Campoletis sonorensis* (Cameron), and *Microplitis croceipes* (Cresson). In the current study, we investigated the transmission of ascovirus by these parasitoids by using females that were collected in the field or reared in the laboratory. After a single oviposition by *C. nigriceps* in an *H. virescens* larva with a 4-d-old ascovirus infection, all subsequent healthy larvae parasitized by this female developed ascovirus infection. After oviposition in an infected larva, examination of *C. nigriceps* by using transmission electron microscopy showed that ascovirus virions and ascovirus vesicles adhered to the inner surface of the ovipositor. The ovipositor of *M. croceipes* was shorter than those of *C. nigriceps* or *C. sonorensis*, and this was correlated with a lower rate of ascovirus transmission by the former species. Observation of *C. nigriceps* populations in the field indicates this species survives even when ascovirus prevalence in *H. virescens* is high. Laboratory studies of this host-parasite-virus system showed *C. nigriceps* larvae survived infection of their host if parasitoid larvae were at least second instars at the time of infection. If an ascovirus infection in the first *H. virescens* host was no older 48 h, a *C. nigriceps* female sometimes did not transmit ascovirus to subsequent hosts. Exposure to environmental conditions of the field decreased the capacity of *C. nigriceps* to transmit ascovirus, and transmission also decreased over the longevity of female parasitoids.

KEY WORDS *Heliothis virescens*, ascovirus transmission, *Cardiochiles nigriceps*, *Campoletis sonorensis*, *Microplitis croceipes*

ASCOVIRUSES ARE A GROUP OF viruses that cause a chronic, ultimately fatal disease in larvae of a variety of Noctuidae species (Federici 1983, Hamm et al. 1986). In the southeastern United States, ascoviruses can be prevalent in *Heliothis virescens* (F.) and *Helicoverpa zea* (Boddie) larval populations in field crops, especially toward the end of the growing season (Adams et al. 1979, Hudson and Carner 1981, Carner and Hudson 1983). Stunting of infected larvae and production of virion-containing vesicles characterizes the disease caused by ascoviruses. As the infection progresses, the hemolymph of an infected larva becomes opaque white as vesicles released from infected tissues accumulate in the hemocoel. The virions of isolates of these viruses are large (400 nm in length),

allantoid in shape, and they have circular, double-stranded DNA genomes (Federici et al. 2000).

Ascoviruses are difficult to transmit *per os* (Federici 1983, Govindarajan and Federici 1990), but they are transmitted very effectively by the braconid parasitoid *Cotesia marginiventris* (Cresson) (Hamm et al. 1985). Ascoviruses also are readily transmitted by injection (Govindarajan and Federici 1990), suggesting that these viruses are vectored mechanically via the ovipositor of parasitoids during oviposition.

In Georgia, three parasitoids, *Cardiochiles nigriceps* Viereck, *Campoletis sonorensis* (Cameron), and *Microplitis croceipes* (Cresson), commonly attack *H. virescens*. *C. nigriceps* is a solitary endoparasitoid that can contribute substantially to the biological control of *H. virescens* on tobacco (*Nicotiana tabacum* L.) (Johnson and Manley 1983, Bidlack et al. 1991, Jackson et al. 1996) and cotton, *Gossypium hirsutum* L. (Lewis et al. 1972, Manley et al. 1991). The ichneumonid *C. sono-*

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rensis is an early season parasitoid of *H. virescens* in tobacco (Lingren et al. 1970), and the braconid *M. croceipes* has been reported to parasitize *H. virescens* in cotton (Lewis and Brazzel 1968). In 1998, in an experimental trap crop for *H. virescens* in cotton (Tillman 1999), 47.5% of the larvae trapped in tobacco developed an ascovirus infection, whereas a *C. nigriceps* third instar emerged from 43.6% of the larvae (P.G.T., unpublished data). In our study, we investigated transmission of ascovirus by *C. nigriceps*, *C. sonorensis*, and *M. croceipes* collected in the field or reared in the laboratory. Given the possibility for competitive exclusion of the parasitoid in the field, another aim was to determine the effects of ascovirus on developing parasitoids in hosts coinfecting with both agents.

Materials and Methods

Insects. Laboratory colonies of *H. virescens*, *C. nigriceps*, *C. sonorensis*, and *M. croceipes* were maintained at the Crop Protection and Management Research Laboratory (Tifton, GA). *H. virescens* were reared on pinto bean diet in 30-ml cups (Perkins et al. 1973). Unless otherwise stated, all insects were maintained in an environmental chamber at 27°C, 75% RH, and a photoperiod of 14:10 (L:D) h. Parasitoids were reared using procedures similar to those of Powell and Hartley (1987). Except where stated otherwise, second instars of *H. virescens* were used as hosts for *C. nigriceps*. Voucher specimens of all insects and the ascovirus are held in the USDA-ARS, Crop Protection and Management Research Laboratory.

Virus Maintenance and Identification. The *H. virescens* ascovirus isolate HV 99-14 used in the experimental tests originated from a *C. nigriceps* female collected from tobacco in Irwin County, Georgia, on 30 August 1999 and was maintained by serial injections of ascovirus virions into healthy *H. virescens* larvae. The isolate likely belongs to the HvAV-3a complex (Federici et al. 1990, 2000). The inoculum was obtained by cutting a proleg from an infected *H. virescens* larva and collecting the ascovirus-infected hemolymph that was released. Mechanical injections of ascovirus were accomplished by dipping the finely pointed tip of a cactus spine in the drop of hemolymph and then pricking a healthy larva with the ascovirus-coated spine (Hamm et al. 1985). All ascovirus-infected *H. virescens* larvae were injected with ascovirus virions in this manner and then held for 4 d by which time a high concentration of the ascovirus was reached in the hemolymph (E.L.S., unpublished data). Adult female and immature *C. nigriceps* and *H. virescens* larvae were prepared for light microscopy and transmission electron microscopy as described previously by Hamm et al. (1998). Immature stages of *C. nigriceps* include the egg, three larval stages, a prepupa, and a pupa (Lewis and Vinson 1968). Homogenized preparations of insects were examined for ascovirus by negative stain electron microscopy (Hamm et al. 1992).

Ascovirus Transmission. To examine ascovirus transmission by *C. nigriceps* females, each of five young

(2–3-d-old) laboratory-reared females of this parasitoid were allowed to parasitize five healthy laboratory-reared *H. virescens* larvae, followed by one ascovirus-infected *H. virescens* larva, and last another five healthy *H. virescens* larvae. *H. virescens* larvae were observed daily until either a parasitoid emerged or the hosts developed an ascovirus infection, as indicated by opaque white hemolymph. This test was repeated for four sets of females. At the end of one test, the ovipositor of a female was fixed in Trump's fixative, embedded, and sectioned for transmission electron microscopy.

To compare ascovirus transmission among three parasitoid species, *C. nigriceps*, *C. sonorensis*, and *M. croceipes*, an ascovirus-infected *H. virescens* larva was parasitized once by a female of each species. Then, each female was allowed to parasitize five healthy *H. virescens*. This was replicated for each parasitoid species 15 times by using a new ascovirus-infected larva for each of the replicates. For eight of the replicates, every third host was fixed for sectioning 7 d postparasitization to determine whether histopathology of the ascovirus differed among parasitoid species. The other replicates were held until either a parasitoid emerged or an ascovirus infection occurred. Ascovirus infection was determined by observing white color and opaque hemolymph for live hosts and by negative stain electron microscopy for dead hosts. External ovipositor lengths were measured using an ocular micrometer. All percentage data were converted by angular transformation. Percentage of ascovirus transmission and ovipositor length data were analyzed by PROC MIXED followed by least significant difference (LSD) separation of means (SAS Institute 1999).

To determine the presence of ascovirus on *C. nigriceps* in the tobacco trap crops, females were collected from the field in 1998 and 1999 and allowed to parasitize healthy *H. virescens* in the laboratory. On 4 August 1998, 29 and 19 *C. nigriceps* females were collected from tobacco in Colquitt County and Irwin County, Georgia, respectively. On 30 October 1998, 13 *C. nigriceps* females were collected from tobacco in Irwin County. The following year, 20 female *C. nigriceps* were collected on 30 August 1999 from tobacco in Irwin County. Except for 10 *C. nigriceps* collected in October 1998, each female was allowed to parasitize five healthy laboratory-reared *H. virescens*. All parasitized *H. virescens* larvae were held in an environmental chamber for 3 wk until either a *C. nigriceps* pupa or an ascovirus infection was verified by observing opaque hemolymph and/or detected by using negative stain electron microscopy. All 13 *C. nigriceps* females collected in October 1998 were homogenized and examined for ascovirus infection by negative stain electron microscopy.

To establish the occurrence of ascovirus-infected *H. virescens* larvae in the field, larvae of this pest were collected from tobacco in the trap crops. Stunted growth and the presence of opaque white discharge from the ventral eversible gland were the characteristics used to detect ascovirus-infected *H. virescens* larvae in the field. On 19 July and 15 September 1999,

10 and six presumptively ascovirus-infected *H. virescens* larvae, respectively, were collected in Irwin County from tobacco in which *C. nigriceps* females were observed searching for hosts. In the laboratory, the color and opacity of the hemolymph of all *H. virescens* larvae were determined and, in addition, the second set of *H. virescens* larvae collected was examined for ascovirus by negative stain electron microscopy.

Effect of Ascovirus on *C. nigriceps* Immature Survival. In the first experiment, *H. virescens* larvae first were parasitized by *C. nigriceps* and later injected with ascovirus to determine whether the age of *C. nigriceps* immatures at the time of host inoculation affected survival of this parasitoid. This test was conducted at a high temperature–relative humidity regime (27°C and 75% RH). Hosts were mechanically injected with ascovirus virions or dissected, for the controls, at 0 (1–2 h), 2 (48 h), 4 (96 h), 5 (120 h), 6 (144 h), 7 (168 h), and 8 (192 h) d after parasitization. Of the 250 first instars of *H. virescens* larvae parasitized by *C. nigriceps* females, 20 ascovirus-infected and 20 healthy larvae were used for each injection time. All *H. virescens* larvae were dissected using a Nikon SM2–2T dissecting microscope (Nikon Instruments, Inc., Melville, NY). Healthy *H. virescens* were dissected to determine the developmental stage of *C. nigriceps* on the day hosts were injected with ascovirus. Ascovirus-infected larvae were dissected 6–7 d postinjection to verify *C. nigriceps* progeny mortality and stage of development. This test was replicated three times. Fourteen of the female progeny that survived in ascovirus-infected hosts were allowed to parasitize 15 healthy *H. virescens* each to determine whether they could transmit ascovirus to their hosts. Also, nine *C. nigriceps* first instars were removed from *H. virescens* larvae inoculated with ascovirus 4 d postparasitization and fixed, embedded, and sectioned for transmission electron microscopy to determine whether they were infected with ascovirus. All percentage data were converted by angular transformation. Frequencies of occurrence of live *C. nigriceps* immatures in healthy *H. virescens* larvae and dead *C. nigriceps* immatures in ascovirus-infected hosts were analyzed by PROC MIXED followed by LSD separation of means (SAS Institute 1999) for each age group (postparasitization time) of *C. nigriceps* immatures examined. Frequencies of occurrence of live *C. nigriceps* second instars in healthy *H. virescens* larvae and frequencies of survival of *C. nigriceps* in ascovirus-infected hosts were analyzed by PROC MIXED followed by LSD separation of means (SAS Institute 1999) for each age group (postparasitization time) of *C. nigriceps* immatures examined.

In the second experiment, *H. virescens* larvae initially were injected with ascovirus and later parasitized by *C. nigriceps* to determine the effect of level of ascovirus infection in hosts on ascovirus transmission by *C. nigriceps* and survival of *C. nigriceps* immatures. Hosts (150) were mechanically injected with ascovirus virions 12, 24, 36, 48, 60, 72, 96, 168, 336, and 504 h before parasitization. At each time after injection of ascovirus, each of 13–19 females parasitized a single

healthy, uninfected *H. virescens* larva, followed by an ascovirus-infected *H. virescens* larva and then 10 healthy *H. virescens* larvae. Stage of *C. nigriceps* progeny development and viability and the presence or absence of ascovirus infection in *H. virescens* larvae were determined 6–7 d postparasitization. This test was replicated three times. The relationship between percentage of ascovirus transmission by a *C. nigriceps* female and time (hours) after injection of ascovirus in the first *H. virescens* host and was analyzed by PROC MIXED (SAS Institute 1999) with Satterthwaite option. The slope and intercept were tested at the center time (48 h) where minimum variance occurred (Draper and Smith 1981). Also, the relationship between mortality of *C. nigriceps* first instars and time after injection of ascovirus virions in *H. virescens* hosts was analyzed by PROC MIXED (SAS Institute 1999). Analysis for first instars was done only through 60 h postinjection because these instars were not present in hosts after this time.

Ascovirus Transmission over Lifetime of *C. nigriceps* Female. To determine the ability of *C. nigriceps* females to transmit ascovirus over their lifetimes, each of five newly emerged *C. nigriceps* females was allowed to oviposit once in an ascovirus-infected *H. virescens* larva and then allowed to parasitize 10 healthy *H. virescens* larvae every day until her death. All parasitized *H. virescens* larvae were held in an environmental chamber for 3 weeks until either a *C. nigriceps* pupa or an ascovirus infection was detected. Three of the females used in the test were fixed 10 d after parasitizing the ascovirus-infected *H. virescens* larva, embedded, sectioned, and examined by light microscopy to determine whether the females became infected with the ascovirus. The relationship between percentage of ascovirus transmission and longevity (days) of *C. nigriceps* females was analyzed by PROC MIXED (SAS Institute 1999).

Effect of Sunlight on Ascovirus Transmission. A test was conducted to determine the effect of exposure of ascovirus-contaminated *C. nigriceps* females to sunlight on percentage of ascovirus transmission by these wasps. Each of 45 *C. nigriceps* females (2–3 d old) was allowed to oviposit once in an ascovirus-infected *H. virescens* larva. Each female was placed in a cylindrical screen cage (8 cm in width by 8 cm in depth by 24 cm in length) and provided with honey and water. Fifteen of these females were placed in total darkness for 1, 3, and 6 d (five females per day), whereas 30 were exposed to sunlight for 1, 3, and 6 d (10 females per day). Females were exposed to sunlight from 10 a.m. to 4 p.m. by hanging cages outside. Females were held in the laboratory when not hung outside. At the end of the exposure time, each *C. nigriceps* female parasitized 10 healthy second instars of *H. virescens* in the laboratory. Ascovirus transmission by each female was recorded for each host. The procedure was repeated three times. The relationships between percentage ascovirus transmission and time *C. nigriceps* females were exposed to sunlight in the field or darkness in the laboratory were analyzed by PROC MIXED (SAS Institute 1999).

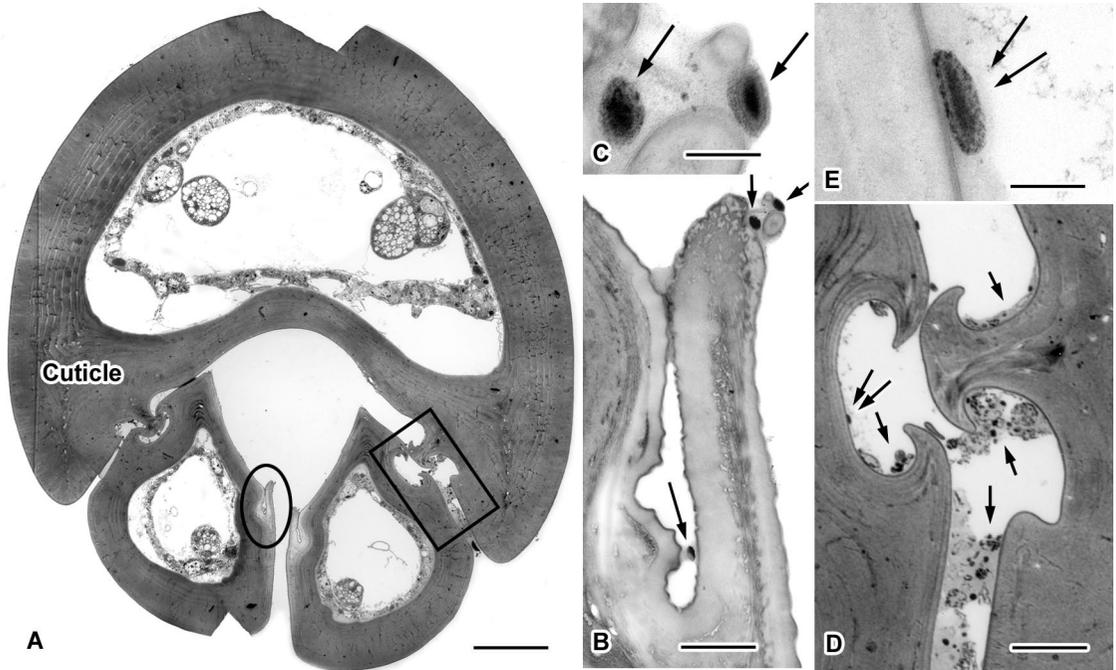


Fig. 1. Ovipositor of *C. nigriceps* immediately after oviposition in a *H. virescens* larva infected with ascovirus. (A) Composite photograph of a cross section of the ovipositor. (B) Enlarged view of the area within the oval outline. Arrows indicate single ascovirus virion adhering to the cuticle. (C) Enlarged view of B. (D) Enlarged view of the area within the rectangle. Single arrows indicate small accretions of cell debris from the parasitized larva that include ascovirus virions. The pair of double arrows indicates a single ascovirus virion. (E) Enlarged view of D. Magnification bars, 10 μm (A), 1 μm (B), 200 nm (C), 2.5 μm (D), and 200 nm (E).

Results

Ascovirus Transmission. After a single oviposition by laboratory-reared *C. nigriceps* in an ascovirus-infected *H. virescens* larva, 100% of the subsequent healthy *H. virescens* parasitized by this female parasitoid developed ascovirus infections. Using transmission electron microscopy to examine a *C. nigriceps* ovipositor after oviposition in an ascovirus-infected host revealed that ascovirus virions and small ascovirus vesicles adhered to the inner cuticular surfaces of ovipositors of females (Fig. 1). These results suggest that *C. nigriceps* ovipositors become contaminated with ascovirus virions during oviposition in infected hosts, and ascovirus virions are inserted into subsequent hosts when they detach from ovipositor surfaces.

Percentage of ascovirus transmission to healthy hosts was greater for *C. nigriceps* and *C. sonorensis* than for *M. croceipes* ($F = 4.34$; $df = 2, 40$; $P = 0.0196$) (Table 1). We were unable to obtain data from two of the *M. croceipes* females because they did not parasitize hosts. The ovipositor of *M. croceipes* was significantly shorter than the ovipositor of *C. nigriceps* and *C. sonorensis* ($F = 161.0$; $df = 2, 40$; $P = 0.0001$) (Table 1). Thus, ovipositor length can affect ascovirus transmission by parasitoids.

Regardless of the parasitoid species that transmitted ascovirus, tissues of host larvae were altered as expected for ascovirus-infected *H. virescens* larvae with

accumulation of large numbers of ascovirus-containing vesicles in the body cavity and heavily infected fat body with cells in all stages of infection (Fig. 2). There were no abnormal ascovirus infections or unusually low levels of infection. Tissues examined included hypodermis, muscle, fat body, tracheal epithelium, Malpighian tubules, midgut, hindgut, heart, paracardial cells, oenocytes, blood cells, testes, nerves, and ganglia.

Cross sections of mid-regions of ascovirus-contaminated ovipositors were similar for *M. croceipes* and *C. nigriceps*, with the exception that for *M. croceipes* a few ascovirus virions were present between the slightly shrunken mass of calyx fluid within the central lumen of the ovipositor and the surrounding cuticle. These ascovirus virions occurred along the margin of

Table 1. Transmission of *H. virescens* ascovirus isolate to new hosts and ovipositor length for three parasitoid species, *C. nigriceps*, *C. sonorensis*, and *M. croceipes*

Species	n	% Ascovirus transmission		Ovipositor length (mm)	
		Mean	SE	Mean	SE
<i>C. nigriceps</i>	15	94.7a	4.2	1.7a	0.04
<i>C. sonorensis</i>	15	97.8a	4.2	1.9a	0.07
<i>M. croceipes</i>	13	80.8b	4.5	0.9b	0.04

Means within a column followed by the same letter are not statistically different ($P < 0.05$; LSD).

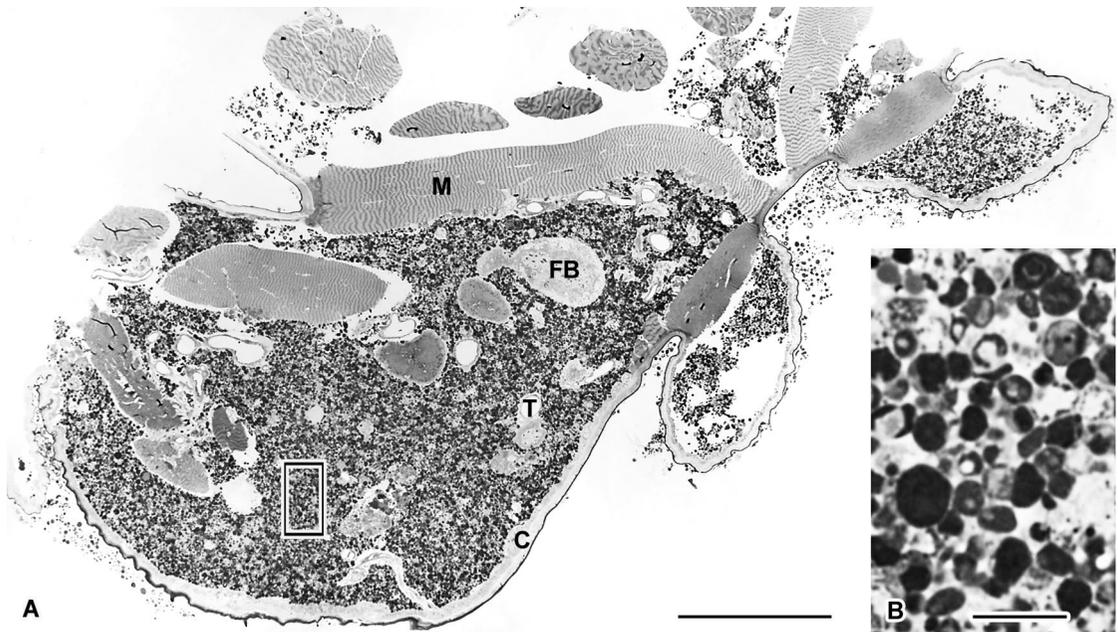


Fig. 2. (A) Cross section of a portion of a segment of a *H. virescens* larva 21 d after being parasitized by a *C. nigriceps* female that had previously oviposited in a host infected with ascovirus. The body cavity is filled with innumerable ascovirus vesicles. (B) Enlargement of the area within the rectangular outline in A. The densest structures are ascovirus occlusion bodies, some of which occupy the entire volume of their vesicle. M, muscle; FB, infected fat body; C, cuticle; T, trachea. Magnification bars, 200 μm (A) and 10 μm (B).

the plug of calyx fluid only. They were neither within the calyx fluid itself nor in contact with the adjacent cuticle. We concluded that the lower rate of ascovirus transmission for *M. croceipes* compared with *C. nigriceps* and *C. sonorensis* was related to the shorter ovipositor length of the former species.

C. nigriceps females collected from tobacco trap crops transmitted ascovirus to healthy *H. virescens* larvae in the laboratory. Of the *C. nigriceps* females collected in August 1998, 62 and 79% from Colquitt County and Irwin County, respectively, transmitted ascovirus to all of their laboratory hosts. In the 1999 field collections, 85% of the females collected from tobacco plots in Irwin County transmitted ascovirus to all their hosts. The remaining females did not transmit ascovirus to any of their hosts. The three females collected in October 1998 from Irwin County that were allowed to parasitize hosts transmitted the ascovirus to these hosts.

Negative stain electron microscopy did not detect ascovirus on any of 13 females collected in October 1998, including the three females that transmitted ascovirus in the laboratory. It seems that negative stain electron microscopy is not appropriate for detecting ascovirus on field-collected *C. nigriceps* females and suggests that ascovirus does not replicate within *C. nigriceps*. I. Newton (personal communication) has determined that the ascovirus did not replicate in *Microplitis demolitor* Wilkinson females. In Australia, polymerase chain reaction diagnostics were used to detect small amounts of ascovirus. The presence of ascovirus has been detected on the ichneumonids

Heteropelma scaposum (Morley) and *Netelia producta* (Brullé) collected from the field (I. Newton, personal communication).

Each of the 16 field-collected *H. virescens* larvae that were suspected to be infected by ascovirus had hemolymph characteristic of ascovirus infection, and six of these larvae were positive for ascovirus when examined by negative stain electron microscopy (Fig. 3). Thus, the ascovirus detection procedure used with *H. virescens* larvae in the field was not only as reliable as inspecting hemolymph and negative stain electron microscopy but also it was easier and less expensive than the laboratory procedures. Because *C. nigriceps* collected from the tobacco trap crops in 1999 transmitted ascovirus to healthy *H. virescens* larvae in the laboratory, and *H. virescens* larvae collected from these same trap crops in the same year were infected with ascovirus, we assume that *C. nigriceps* transmitted ascovirus to healthy *H. virescens* larvae in the field.

Effect of Ascovirus on *C. nigriceps* Immature Survival. In the first experiment, the frequency of occurrence of the immature stages that were killed in hosts was related to the timing of development of both the *C. nigriceps* immatures and the ascovirus infection. Nearly all *C. nigriceps* eggs died without hatching when parasitization and ascovirus injection occurred almost simultaneously (Table 2). However, 4.2% of the immatures that died in hosts injected with ascovirus immediately after parasitization were first instars, even though these parasitoids were eggs when ascovirus virions were introduced into their hosts. Parasitoid death did not occur immediately after inoculation

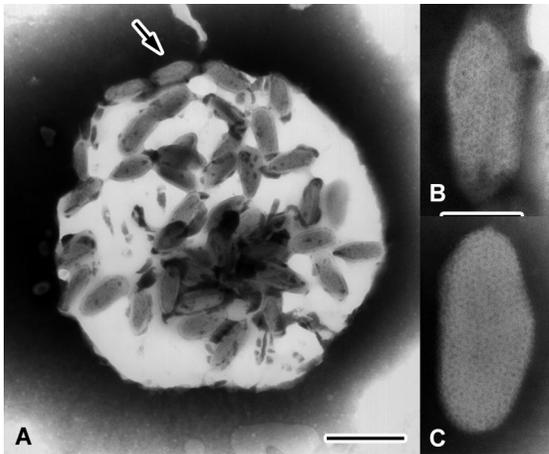


Fig. 3. Ascovirus occlusion body and individual virions from a negatively stained preparation of a *H. virescens* larva infected with ascovirus. (A) Ascovirus virions in process of envelopment and occlusion scattered over the surface of the occlusion body (arrow). (B) Magnified view of the virion indicated by the arrow in A. (C) Ascovirus virion not associated with an occlusion body. Magnification bars, 400 nm (A) and 100 nm (B and C).

of the host because the level of ascovirus infection develops over time (E.L.S., unpublished data). Thus, the *C. nigriceps* immature stage that died in an ascovirus-infected host possibly could be more mature than the stage present at the time of ascovirus inoculation. All first instars of *C. nigriceps* were killed when

Table 2. Frequency of occurrence of live *C. nigriceps* immatures in healthy *H. virescens* hosts and frequency of occurrence for each immature stage of *C. nigriceps* that eventually died in ascovirus-infected hosts for each immature parasitoid age

Day ^a	Stage	Frequency of live <i>C. nigriceps</i> immatures in healthy hosts			Frequency of dead <i>C. nigriceps</i> immatures in ascovirus-infected hosts		
		n	Mean	SE	n	Mean	SE
0	Egg	35	100.0a	0.02	107	95.8a	0.02
	1st Instar		0b			4.2b	
2	Egg	51	27.5b	0.02	121	0b	0.02
	1st Instar		72.5a			100.0a	
4	1st Instar	18	100.0a	0.03	53	100.0a	0.02
	2nd Instar		0b			94.6a	0.03
5	1st Instar	26	100.0a	0.03	28	5.4b	
	2nd Instar		0b			50.8a	0.02
	3rd Instar		0b			48.0a	
6	1st Instar	39	100.0a	0.02	41	1.2b	
	2nd Instar		0b			34.9b	0.02
	3rd Instar		0b			16.1c	
	Prepupa		0b			46.7a	
7	1st Instar	45	100.0a	0.02	36	2.3d	0.02
	2nd Instar		0b			2.3d	
	3rd Instar		0b			10.9b	
	Prepupa		0b			78.9a	
	Prepupa		0b			7.8b,c	
8	1st Instar	87	2.3b	0.02	68	2.3c	0.03
	2nd Instar		97.7a			10.9b	
	3rd Instar		0b			78.9a	
	Prepupa		0b			7.8b,c	

Means within a column followed by the same letter are not statistically different among *C. nigriceps* immature stages for a single parasitoid age (days) ($P < 0.05$; LSD).

^a Age of *C. nigriceps* immature when ascovirus was injected into host.

Table 3. Frequency of occurrence of live *C. nigriceps* second instars in healthy *H. virescens* hosts and frequency of survival of *C. nigriceps* in ascovirus-infected hosts at 6, 7, and 8 d postparasitization

Day ^a	% Occurrence of <i>C. nigriceps</i> second instars in healthy hosts			% Survival of <i>C. nigriceps</i> in ascovirus-infected hosts		
	n	Mean	SE	n	Mean	SE
6	39	0b	0.05	41	0b	0.02
7	45	0b	0.04	36	0b	0.08
8	87	97.7a	0.03	84	81.0a	0.07

Means within a column followed by the same letter are not statistically different among days hosts were injected with ascovirus ($P < 0.05$; LSD).

^a Age of *C. nigriceps* immature when ascovirus was injected into host.

hosts were injected 2 and 4 d after parasitization (Table 2). Mostly first instars died when ascovirus inoculation of the host occurred 5 d after parasitization. Mortality for first and second instars of *C. nigriceps* was approximately the same when these immatures were 6 d old at the time ascovirus was injected into the host. Mostly third instars of *C. nigriceps* died when these immatures were 7 and 8 d old at time of host inoculation.

Age of *C. nigriceps* immatures at the time of injection of ascovirus in hosts affected the survival of these parasitoids. Survival of *C. nigriceps* larvae was high in both healthy and ascovirus-infected hosts when these parasitoids were 8 d old at the time of injection of ascovirus, but there were no survivors in ascovirus-infected hosts when the parasitoids were 6 and 7 d old at the time of host inoculation (Table 3). Dissection of healthy hosts revealed that the transition from first to second instar for *C. nigriceps* occurred from 7- to 8-d-old immatures, corresponding to the age interval where *C. nigriceps* survival in ascovirus-infected hosts rose from 0 to 81% (Table 3). We conclude that *C. nigriceps* larvae can survive an ascovirus infection if parasite larvae are at least second instars at the time of host inoculation.

Ascovirus infections were not detected by transmission electron microscopy in tissues of dead first instars of *C. nigriceps* collected 4 d after parasitization in ascovirus-infected hosts. Ascovirus infections also did not develop in any of the healthy *H. virescens* larvae parasitized by female *C. nigriceps* that survived in ascovirus-infected hosts.

In the second experiment, the level of ascovirus infection in the first *H. virescens* host affected ascovirus transmission by a *C. nigriceps* female to subsequent hosts. Gradual increases in the ascovirus virions in host larvae from the initial infection of a limited number of host cells, to cell-to-cell spread within susceptible tissues, viral multiplication within a vastly increased number of infected cells, and release of increasing amounts of ascovirus virions and ascovirus vesicles into the hemolymph have been detected in host larvae (E.L.S., unpublished data). Therefore, we assumed that the level of ascovirus infection increased as the time after injection of ascovirus into the host in-

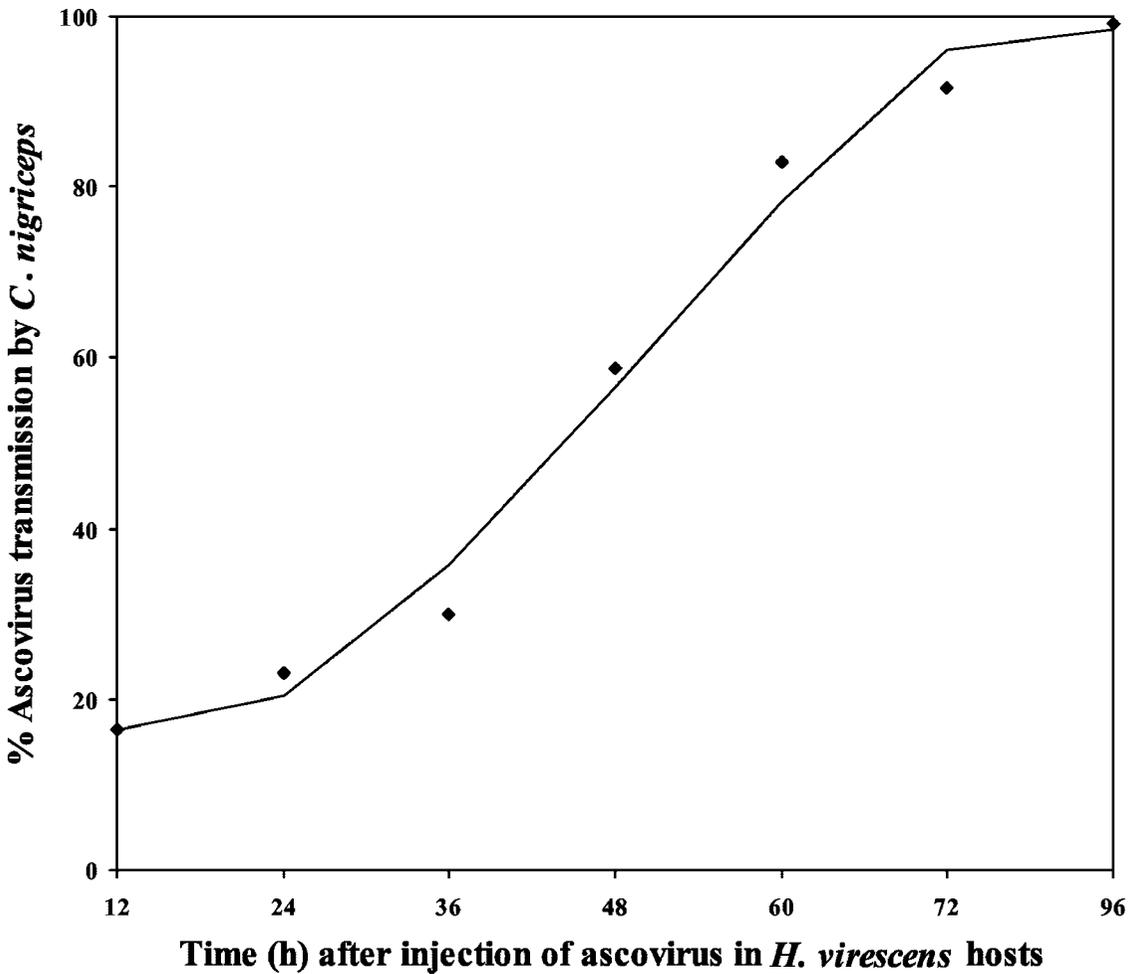


Fig. 4. Effect of time (hours) after injection of ascovirus in the first *H. virescens* host on percentage of ascovirus transmission by a *C. nigriceps* female in subsequent hosts; $y = 56.55 + 1.85x + 0.00292x^2 - 0.00049x^3$ (slope and intercept tested at center time of 48 h; Draper and Smith 1981).

increased. Ascovirus transmission by *C. nigriceps* increased cubically at the rate of 1.85%/h as the time (hours) after injection of ascovirus of the first host increased ($F = 12.06$; $df = 1, 3.25$; $P < 0.0355$) (Fig. 4). Ascovirus transmission was very low for the first 24 h after injection of ascovirus virions, increased from 24 to 72 h after injection of ascovirus, and leveled off after that time. Percentage of mortality of subsequent first instars followed a similar pattern, increasing from 12 to 60 h in a curvilinear manner ($F = 18.33$; $df = 1, 39$; $P < 0.0001$), presumably as ascovirus virion multiplication increased in the hosts (Fig. 5).

Ascovirus Transmission over Lifetime of *C. nigriceps* Female. *C. nigriceps* females were generally able to transmit ascovirus throughout their lifetimes, although ascovirus transmission decreased as the females aged (Fig. 6) ($F = 132.32$; $df = 2, 300$; $P < 0.0001$). These results also may have been influenced by the number of times a female inserted her ovipositor into a host, because a reduction of ascovirus virions on the ovipositor could occur after parasitizing

numerous hosts. We are currently conducting tests to study the effect of number of hosts parasitized on the ability of *C. nigriceps* to transmit ascovirus. There was no sign of ascovirus infection in the hypodermis, fat body, muscles, tracheal matrix, Malpighian tubules, heart, pericardium, ovarioles, calyx, nerves, oenocytes, midgut, or hindgut of any of the three wasps examined 10 d after exposure to an ascovirus-infected *H. virescens* larva. Thus, *C. nigriceps* females transmit ascovirus without becoming infected with the ascovirus.

Effect of Sunlight on Ascovirus Transmission. Throughout the period of exposure to sunlight in the field, percentage of ascovirus transmission by *C. nigriceps* decreased linearly at the rate of 12.05%/d ($F = 33.33$; $df = 1, 130$; $P < 0.0001$) (Fig. 7). For females remaining in darkness in the laboratory, percentage of ascovirus transmission by *C. nigriceps* decreased linearly only at the rate of 1.08%/d ($F = 33.33$; $df = 1, 130$; $P < 0.0001$) (Fig. 7). Exposure to field environmental conditions apparently reduced infectivity of ascovirus

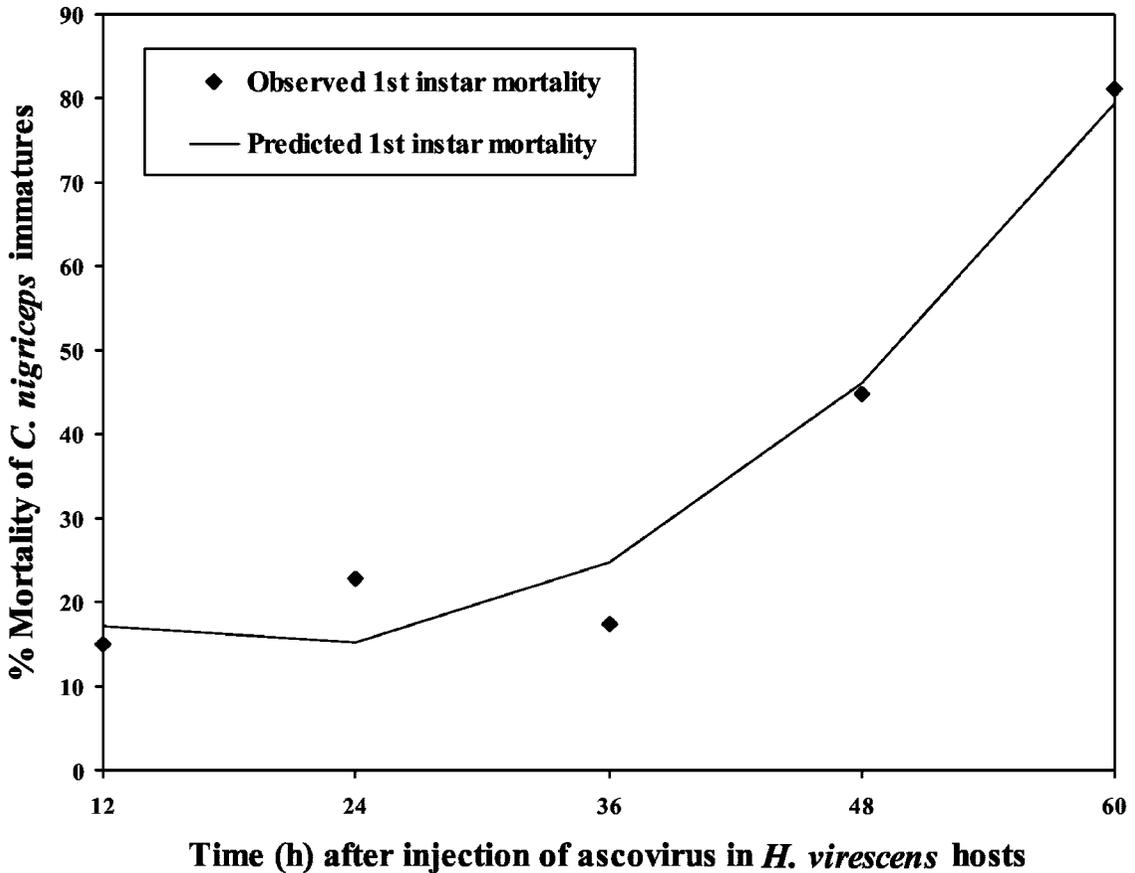


Fig. 5. Effect of time (hours) after injection of ascovirus in the first *H. virescens* host on percentage of mortality of *C. nigriceps* first instars in subsequent hosts; $y = 3.24x + 0.04x^2 + 79.26$.

virions on the ovipositors of *C. nigriceps* females so that by 6 d after exposure, only a small percentage of *H. virescens* developed an ascovirus infection after being parasitized by these females.

Discussion

Hamm et al. (1985) reported that the braconid *C. marginiventris* transmitted an ascovirus to *Spodoptera frugiperda* (J.E. Smith) larvae in the laboratory. In Australia, females of the braconid *M. demolitor* were vectors of an ascovirus of *Helicoverpa armigera* (Hübner) (I. Newton, personal communication). In our study, even though the shortness of the ovipositors of *M. croceipes* relative to those of *C. nigriceps* or *C. sonorensis* was correlated with a lower rate of ascovirus transmission by the former species, all three parasitoid species were vectors of ascovirus in *H. virescens*. Thus, ascovirus transmission via oviposition is not limited to a particular species or family of parasitoids. Ascovirus even can be transmitted mechanically by pricking healthy *H. virescens* larvae with an ascovirus-contaminated cactus spine (Hamm et al. 1985). Together, these data provide conclusive evidence that the ability of a parasitoid to transmit ascovirus is associated solely

with the mechanical ability of the ovipositor to penetrate into the host's body cavity to become contaminated with ascovirus virions and then later to inject ascovirus virions into another host along with the egg.

Ascoviruses have a very deleterious effect on larval parasitoids in hosts with this disease. Progeny of *M. demolitor* do not survive in ascovirus-infected *H. armigera* larvae (I. Newton, personal communication), and most of the progeny of *C. marginiventris* failed to complete development in ascovirus-infected hosts when inoculation occurred during and 4 d after parasitization, respectively (Hamm et al. 1985). However, ascovirus inoculation 5 d after parasitization resulted in only 30.0% mortality of *C. marginiventris* progeny. When host larvae were injected with ascovirus virions 5 d postparasitization, *C. marginiventris* immatures probably were second instars (Boling and Pitre 1970). Similarly, in our study *C. nigriceps* larvae were able to survive an ascovirus infection of their host if parasitoid larvae were at least second instars at the time of inoculation.

Preening by parasitoids is a behavior involving cleaning the mouth, wings, and legs. Female *C. nigriceps* have been observed in the field preening themselves after a host attack or exposure to sticky leaves,

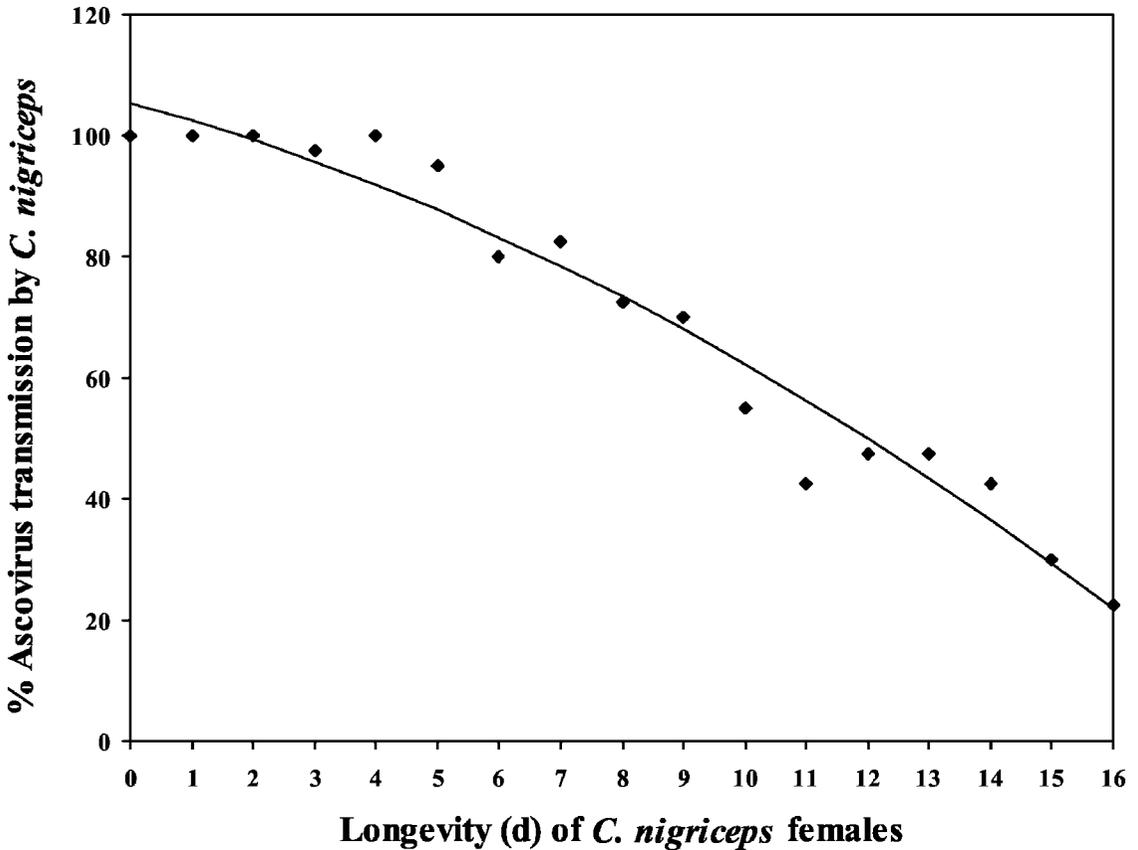


Fig. 6. Effect of *C. nigriceps* female longevity (days) on percentage of ascovirus transmission ($y = -2.79x - 0.15x^2 + 105.45$).

and the ovipositors of females are exposed to sunlight during this activity (Tillman and Mullinix 2003). Shapiro (1992) demonstrated that fluorescent brighteners could enhance viral infectivity for lepidopterous larvae by protecting the virus from UV irradiation (sunlight). Therefore, sunlight may have been responsible, in part, for reduced infectivity of ascovirus virions on the ovipositors of *C. nigriceps* females exposed to field environmental conditions. However, many of the ascovirus virions and small ascovirus vesicles adhere to the inner cuticular surfaces of ovipositors, which are protected from sunlight. Therefore, other environmental factors also are involved in the observed reduction in ascovirus infectivity. Research will be conducted to obtain a better understanding of this phenomenon.

Because ascovirus does not directly infect the *C. nigriceps* immature, why are the immatures slowly dying in infected hosts? Sometimes, the *C. nigriceps* stage that died in ascovirus hosts was older than the stage present when the host was inoculated. Also, a gradual increase in ascovirus transmission by *C. nigriceps* females occurred as the level of ascovirus infection increased, so the detrimental affect of the ascovirus infection was slow acting and possibly related to viral multiplication. The presence of ascovirus

virions within the hemolymph and/or ascovirus virion multiplication within host cells might alter, deplete, or divert normal host energy resources, effectively starving *C. nigriceps* larvae. Depletion of both glycogen and lipid has been observed in ascovirus-infected fat body cells (E.L.S., unpublished data). An ascovirus infection also might cause the release of specific compounds that are toxic to *C. nigriceps*. Kaya and Tanada (1972) demonstrated a toxic effect on the parasitoid *Glyptapanteles militaris* (Walsh) by a viral replication product of a granulosis virus. Hotchkiss and Kaya (1983) suggested a similar factor for the pathological response of *G. militaris* to a nuclear polyhedrosis virus infection.

The possibility for competitive exclusion of *C. nigriceps* exists in the field because immature parasitoids can die in ascovirus-infected *H. virescens* larvae. However, *C. nigriceps* populations have been observed to survive even when ascovirus prevalence in *H. virescens* is high. Laboratory studies of this host-parasite-virus system showed that there are several mechanisms for survival of the *C. nigriceps* population when these two biological control agents occur concurrently in the field. *C. nigriceps* larvae survived an ascovirus infection if the parasitoid larvae were at least second instars when the ascovirus virions were injected into the host.

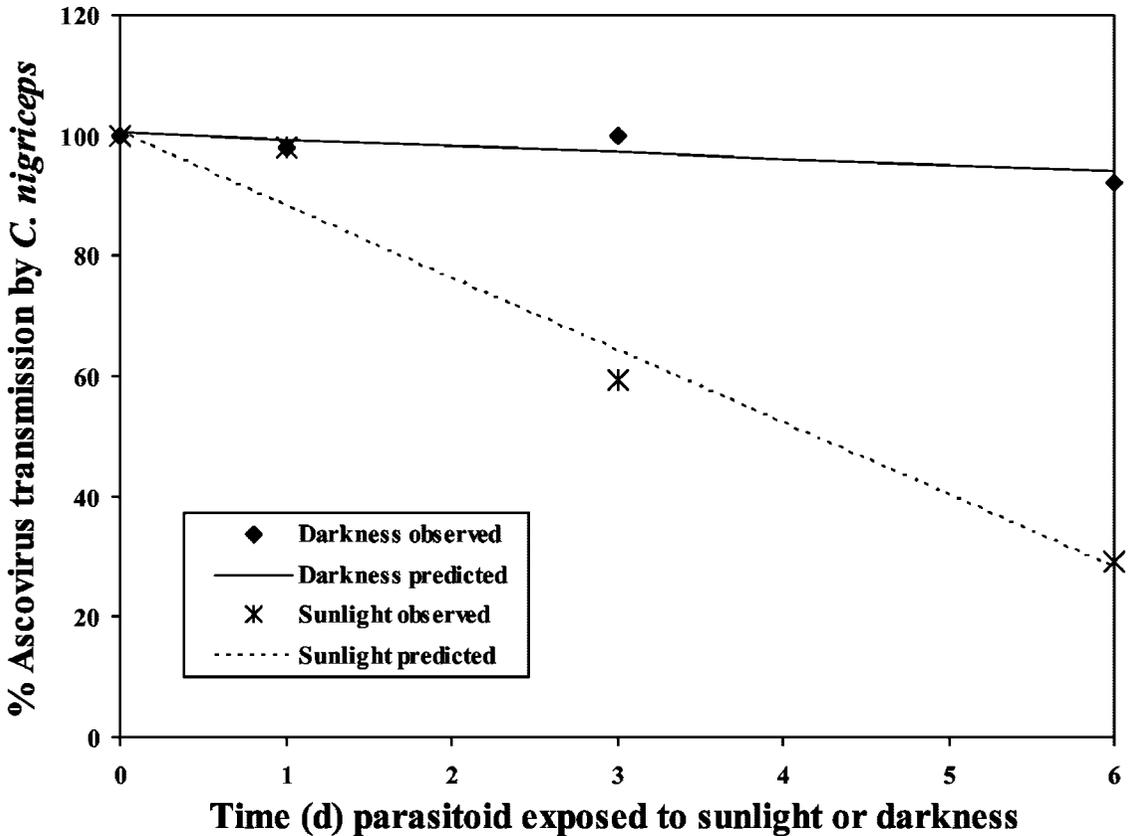


Fig. 7. Effect of number of days *C. nigriceps* females with ascovirus-contaminated ovipositors were exposed to sunlight in the field ($y = -12.04x + 100.42$) or darkness in the laboratory ($y = -1.08x + 100.42$) on percentage ascovirus transmission.

Furthermore, *C. nigriceps* females developing from larvae that survived ascovirus infections did not transmit the disease. Certain conditions limit the transmission of ascovirus by *C. nigriceps*. Female *C. nigriceps* sometimes were unable transmit ascovirus to subsequent hosts if the ascovirus infection in the first *H. virescens* host was <48 h old. Exposure to environmental conditions in the field decreased the capacity of *C. nigriceps* to transmit ascovirus. Also, ascovirus transmission decreased over the lifetime of *C. nigriceps* females, so potentially some of the progeny from an ascovirus-contaminated female could survive in the field. Still the likelihood exists that a female parasitoid could be recontaminated by the virus in the field. These mechanisms for the survival of *C. nigriceps*, however, presumably do operate to some degree during field outbreaks of ascovirus because mean percentage of occurrence of ascovirus in these pests did not exceed 50% for three growing seasons in tobacco trap crops (P.G.T., unpublished data).

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